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BOOK of ABSTRACTS

draft
A.01 LIFE SCIENCES - CLINICAL PROTEOMICS

1370 - EXOSOMES PURIFICATION FOR NEW BIOMARKERS DISCOVERY IN CANCER

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Keywords: exosomes, proteomics, nLC-MS/MS

Introduction:
Exosomes are cell-derived nanovesicles that are secreted from all types of cells and are present in all body fluids. Tumor derived exosomes contain molecules derived from the tumor, and thus represent an opportunity for health monitoring and diagnosis[1].
Here an exosome proteome purification procedure has been developed with the aim to purify exosomes from low sample amount and to study the correlation between exosome cargo and cancer progression.

Methods:
Exosomes were purified from 100 ul serum. Serum was previously depleted from bigger vesicles. Exosomes were then isolated by precipitation and the resulting pellet was ultra-filtrated on 3kDa spin filters, vesicles were lysed, and proteins concentrated. After protein digestion, peptides were analyzed by bottom-up-LC-MS/MS. To reduce the serum proteins level size exclusion chromatography(SEC) has been investigated as an additional purification step.

Results:
350 protein groups were identified, and which included 14 of the top 25 frequently identified exosomal proteins defined by the ExoCarta database. Functional enrichment analysis of the proteomics data was performed for cellular component geneontology. Gene ontology analysis performed by STRING reported the presence of exosomal proteins (194 proteins in GO:0070062, FDR: 1.87e-122), PANTHER reported vesicles as the main category present in the sample (135 proteins). Serum albumin, immunoglobulins and protein of the complement system were also identified in the EVs-UF-precipitated samples, indicating that the samples also contained soluble proteins as impurity. Serum exosomes purified by SEC reported lower level of albumin, immunoglobulins and complement system proteins showing the ability of the columns to further purify exosomes from contaminants.

Conclusions:
Our exosomes purification procedure will be applied to purify exosomes from cancer patient serum with the aim to study the role of these vesicles in inter-cellular communication and to study the correlation of exosome cargo and tumor progression. Further scaling down of the procedure will be evaluated so as to be able to analyze an even lower amount of starting sample.

Novel Aspect:
This procedure enable the in depth proteome analysis of vesicle cargo starting from very low amount of sample. Proteomics analysis of exosomes by bottom-up-LC-MS/MS have been overwhelmingly performed on exosomes purified by ultracentrifugation, but which requires a lot of sample volume available and thus is not feasible on the serum from individual mice (circa100ul available from a single mouse) for studying cancer mouse models [2]. The exosomes purification method reported here enables the analysis of exosomes from individual mice.

References:
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Development of a novel LC concept for clinical proteomics

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Mass spectrometry based proteomics and metabolomics are fast growing and powerful technologies, with the potential to revolutionize health care and precision medicine. However, separation technologies has so far limited throughput and robustness and thereby prevented omics technologies from being fully integrated and routinely used in a clinical setting. Here we describe a conceptually novel low-flow chromatography system that delivers the robustness and throughput required for clinical applications while maintaining the sensitivity of current nano-flow LC instrumentation. The new system uses four low-pressure pumps in parallel to elute samples from a disposable trap column while simultaneously creating an "offset" chromatographic gradient with embedded analytes.

Initially, two low-pressure pumps form a primary gradient that flows through a disposable trap column thereby eluting analytes of interest while two additional low-pressure pumps positioned right after the trap column are modifying the eluent to create an "offset" that increases the retention of the now embedded analytes at the separation column. The offset gradient with the embedded analytes are moved into a long, narrow holding loop that subsequently is switched in-line with a single high-pressure pump and a separation column. Thus elution from the disposable trap column and gradient formation become de-coupled from the high-pressure separation.

We have characterized the performance of the new system regarding cross contaminations (<0.05%, total TIC), retention time shifts and peak widths (<8.5 sec) in over 1,500 HeLa runs. The extremely short overhead time of approximately 3 min/gradient allowed us to measure 60 samples per day (21 min gradient, 3 min overhead time) compared to 40 samples with a standard nano-LC systems (22 min gradient, 15 min overhead time). To benchmark reproducibility, we measured 100 human plasma samples in 40 hours and each resulted in several hundred quantified plasma proteins, including more than 50 FDA-approved biomarkers. Assured of a high reproducibility, we then aimed to find significant biological information in the human plasma proteome. For this purpose, we analyzed a longitudinal weight loss study that included a total of 319 plasma samples, where we found strongest effects on proteins of the lipid metabolism and inflammation system.
Introduction
Matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) is a powerful analytical tool endowed with the capability to analyze biological samples ranging from biomolecules to micro-organisms, up to whole histological sections. Due to the complexity of such analytes, there is the need for ad-hoc sample pre-processing (e.g. washing, reduction/alkylation, enzymatic cleavage), which can be performed on-target, through the use of smart surfaces.

Methods
Cluster Beam Deposition [1,2] was adopted to deposit patterned functional films made of ultrafine TiO2 nanoparticles, either on polymeric, metallic and glass slides at room temperature. Peculiar nanoporosity and bio-affinity of this material play a synergic role in sample capturing. TiO2 super-hydrophilicity by UV irradiation [3] provides uniform spreading of liquid samples and optimal adhesion of histological sections onto the patterned functional areas.

Results
Hydrophobic barrier at the border of the super-hydrophilic functional areas acts as an effective droplet confinement structure, which allows easy and reliable on-target processing of liquid-phase samples. In comparison with standard in-vial approach, on-target processing of protein samples (including secondary cleaning and Trypsin digestion) avoids the loss of sample fractions, due to cleaning, as well as of peptide fractions due to their capture on vial plastic walls. Regarding histological samples, improved adhesion avoids detachment, tearing and shrinking, during tissue processing (e.g. dehydration, delipidation, fixation). Improved adhesion can also benefit pharmaco-kinetic studies, where any tissue treatment should a-priori be excluded [4]. Tissue stability permits matrix removal after MALDI Imaging, in order to carry out immunohistochemical analysis on the very same section. In general, nanoscale surface roughness offers distributed nucleation points favoring uniform and homogeneous crystallization of MALDI matrix.

Conclusions
Advanced surface engineering concepts have been adopted to develop innovative MALDI targets aiming to reliable on-target sample processing. Advantages were thoroughly demonstrated with protein samples, subjected to secondary cleaning and Trypsin digestion, and with histological samples subjected to harsh chemical treatments prior MALDI Imaging. Benefits are also expected in pathogen analysis as well as in robotic sample processing for clinical diagnostics.

Novel Aspect
Improved on-target sample processing in MALDI-MS through the use of nanoporous bio-affine films made of ultrafine TiO2 nanoparticles deposited on any kind of a substrate.

References
Proteome profiling of bronchioalveolar lavage fluid after exposure to magnetic iron and cobalt oxide nanoparticles

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Keywords: clinical proteomics, label-free, EvoSep, bronchioalveolar lavage, iron cobalt oxide nanoparticles

Introduction:
Magnetic nanoparticles are of great interest in biomedicine (e.g. magnetic resonance imaging, targeted tumor therapy), however, their biocompatibility is an ongoing concern and large-scale in vivo toxicity studies are mostly lacking. Here we examined the effects of magnetic iron and cobalt oxide nanoparticles on the lung in mice using label-free quantitative proteomics. The new EvoSep LC system, specifically developed for clinical proteomics, was evaluated.

Methods:
Toxicity of iron cobalt oxide nanoparticles was examined in vivo by intratracheal instillation in mice. Bronchioalveolar lavage fluid (BALF) samples were collected at day 1 and 3 post exposure. Nanoparticles with different iron cobalt composition were included (Fe Co: 1:0, 3:1, 1:1, 1:3, 0:1) testing two doses (54µg, 162µg). The BALF proteome of 168 mice was profiled by label-free LC-MS/MS (EvoSep coupled to Orbitrap Fusion Lumos, Thermo) using 21min gradients.

Results:
Performance of EvoSep was evaluated. Average Pearson correlation coefficient of 0.99 was determined among 16 technical replicates demonstrating very high reproducibility. To increase identification and quantification a mix of representative samples was separated by high pH fractionation and measured in parallel. Biological replicates (168 BALF samples) were run as single shot injections using 21min gradients. A total of 1400 proteins were quantified with average of 400 proteins per biological replicate (min: 269, max: 602). Statistical analysis was performed by empirical Bayes moderate T test incorporated in the Limma package. Differentially expressed proteins were clustered and enrichment analysis was performed. Highly enriched were proteins associated with extracellular vesicles, stress and immune response, as well as glutathione metabolism, response to metal ion and reactive oxygen species. To assist pathway identification in-depth BALF library from high pH fractionation was included in the bioinformatic analysis.

Conclusions:
We present here a proteomic toxicity screening testing simultaneously 6 different nanoparticle types (magnetic iron cobalt oxide nanoparticles with Fe Co ratio: 1:0, 3:1, 1:1, 1:3, 0:1; carbon black as control), after pulmonary inhalation at two time points and two concentrations. As demonstrated EvoSep combined with short LC gradients and high-resolution mass spectrometry enables high-throughput proteome profiling of clinical samples such as BALF or plasma.

Novel Aspect:
The EvoSep LC system designed for clinical proteomics allowing for high throughput and robust measurement was tested on real-life samples.
209 - A NOVEL MASS SPECTROMETRY BASED ASSAY FOR THE DIAGNOSIS AND TYPING OF SYSTEMIC AMYLOIDOSIS

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Keywords: systemic amyloidosis, SRM, diagnosis, fat tissue aspirates, clinical

Introduction:
In systemic amyloidoses (SA) different precursor proteins misfold, aggregate as amyloid fibrils and are deposited in various tissues. Correct identification of the precursor amyloid protein is key for successful clinical treatment. However, current diagnostic approaches are lacking. We developed an LC-MS method that assays in one analysis the major specific systemic amyloidosis proteins, their mutations and additional proteins as generic indicators of SA.

Methods:
We developed an SRM-MS assay targeting 13 major SA proteins and often-occurring mutations plus 20 additional proteins. To improve peptide detectability and allow for intra-sample and batch normalization, we spiked in heavy isotope labeled synthetic peptide analogs for all targeted peptides and iRT peptides. The final assay, containing 289 precursors and 1134 transitions, was condensed to a single, 35 minute LC-SRM-MS analysis method.

Results:
A 1-µg fraction of the trypsin digested tissue is enough to perform the developed SRM-assay. After target peptide and transition selection and optimization together with retention time scheduling, we could quantitatively measure more than 1000 transitions in a 35 minute LC gradient. Three sets of in total 226 samples consisting of control samples and patient samples with varying levels of the SA types ATTR, AA, AL-L and AL-K were measured with the SRM-assay. One set was used to create a prediction model based on amyloid protein intensities. The two other sets were used to test and validate the prediction model. The amyloidogenic precursor protein was correctly detected with >80% specificity and sensitivity. The correctness of diagnosis increased with increasing disease severity. Potentially present mutations could be detected simultaneously with the assay.

Conclusions:
An amyloid signature of 20 proteins was developed to diagnose the presence of amyloid fibrils in fat tissue aspirates from systemic amyloidosis patients regardless of type and to gauge SA severity. The high specificity and sensitivity of the assay combined with its relative straightforwardness make it an interesting alternative to the contemporary SA diagnosis pipeline.

Novel Aspect:
Single, 35-minute LC-SRM assay simultaneously diagnoses, types and grades 13 different SA types including their potential mutations.
1317 - ANALYSIS AND CHARACTERIZATION OF GASTROINTESTINAL METABOLISM OF MAJOR SOY ALLERGENS GLYCININ AND Β-CONGLYCININ IN AN IN-VITRO MODEL BY LC-MS/MS

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Keywords: food allergens, in-vitro digestion model, LC-MS/MS, label free quantification, proteomics

Introduction:
Soy is one of the eight major allergenic foods, that can cause severe reactions. But little is known so far about the fate of allergenic soy proteins during gastrointestinal digestion. Beside already published potential epitopic regions of different soy allergens with the ability to bind Immunoglobulin E and cause an allergic reaction, there is still no profound knowledge about kinetics of allergen degradation and characterization of degradation products [1].

Methods:
The three phases of human gastrointestinal digestion of dried soy beans were simulated with a standardized in-vitro digestion model [2]. While time dependent degradation of intact soy proteins was analyzed by SDS-PAGE, the identification of the highly complex set of degradation products was performed by micro-LC-MS/MS on a Q-TOF instrument in a software assisted proteomics approach. Degradation rates were estimated by label-free quantification for each phase.

Results:
For major soy allergens β-Conglycinin (Gly m 5) und Glycinin (Gly m 6) degradation was observed during gastrointestinal digestion. Gly m 5 β-subunit showed the highest stability during gastric digestion whereas α-subunits of Gly m 5 and Gly m 6 subunits were largely degraded during 30 minutes of gastric digestion. For both phases highly complex mixtures of low-molecular products (Gly m 5: 121 peptides, Gly m 6: 405 peptides) could be identified. Gastrointestinal digestion seems to follow a multi-step mechanism with repetitive patterns regarding time dependent formation and degradation kinetics of peptides with different length. Investigation of digestion kinetics by label-free quantification verifies that some proteins are degraded into larger peptides at an early digestion state, whereas others are degraded as late as in the end of gastric phase. Regions in the amino acid sequence overlapping with previously identified epitopes could be determined, suggesting that these peptides can trigger allergic reactions after intestinal absorption.

Conclusions:
By Simulation of gastrointestinal digestion of soy in an in-vitro model time dependent degradation of major soy allergens β-Conglycinin und Glycinin could be determined as well as differences in formation kinetics of degradation products. Additionally, degradation products of both allergens with intact epitopic regions could be identified, keeping the ability of IgE-binding to potentially cause an allergic reaction after absorption by the intestinal mucosa.

Novel Aspect:
Time dependent degradation of allergens and formation of potential allergenic products could be observed by simulated gastrointestinal digestion in an in-vitro model.

References:

1291 - PROTEOMIC PROFILING OF EXTRACELLULAR VESICLES DERIVED FROM MESENCHYMAL Stromal CELLS

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Keywords: Mesenchymal stromal cells, Extracellular vesicles, Shotgun Proteomics

Introduction:
Mesenchymal stromal cells (MSCs) are adult multipotent stem cells used for regenerative medicine and immunomodulation [1]. Extracellular vesicles (EVs) released by MSCs act as their paracrine effectors by delivering transcription factors, oncogenes, mRNA, microRNA, and proteins to target cells [2]. In this study, we have investigated the immunomodulatory capabilities of EVs derived from primed-MSCs (pEVs) compared to control (cEVs), through a SWATH-MS approach.

Methods:
Shotgun mass spectrometry (MS) analysis has been performed on pEVs and cEVs in order to characterize and compare their proteomic profile. Proteins differentially expressed were evaluated in silico using different bioinformatic prediction tools. Multivariate pattern recognition methods like Principal Component Analysis (PCA) and Partial Least Squares Discriminant Analysis (PLS-DA) were also employed. Selected proteins and pathways were validated using Western Blot and flow cytometry analysis respectively.

Results:
Shotgun proteomic analysis revealed in pEVs, a strong modulation of proteins involved in many processes related to immune response. The results obtained through SWATH-MS analysis, were validated through western blotting, by selection of four candidate proteins according to their immunomodulatory potential. Proteins differentially expressed in p-EVs compared to c-EVs were mapped to terms in the GO database. Biological process classification showed enriched terms categories such as “leukocyte migration”, and “leukocyte cell-cell adhesion” suggesting a possible involvement of pEVs in modulating immune effector cells activity. Enrichment analysis of pEVs downregulated proteins revealed that the top four identified pathways are: “PI3K-AKT signalling pathway”, “regulation of actin cytoskeleton”, “focal adhesion” and “leukocyte trans-endothelial migration”. The expression and phosphorylation profile of different components of PI3K-AKT signalling pathway was evaluated by flow cytometry in activated B lymphocytes. Our results showed that, the treatment with pEVs, induced a significant down regulation of the pathway compared to cEVs.

Conclusions:
The molecular characterization at protein level of pEVs derived from MSCs, and the consequent functional validation of candidate pathways in activated B lymphocytes, will suggest novel therapeutic perspectives for the treatment of inflammatory and autoimmune diseases.

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Concurrent Quantification of Synaptotagmin-1 and SNAP-25 in Clinically Relevant Volumes of CSF by Immunoaffinity LC-MS/MS

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Keywords: PRM-MS, immunoprecipitation, cerebrospinal fluid, synaptic proteins

Introduction:
Reduction of synaptic activity is an early event in several neurodegenerative diseases. In two earlier studies we have used affinity purification combined with PRM-MS to demonstrate increased CSF levels of the synaptic proteins synaptotagmin-1 and SNAP-25 in patients with Alzheimer’s disease compared with controls (1, 2). In this study the strategy was further developed to concurrently measure levels of both proteins in individual patient CSF samples.

Methods:
The immunoprecipitation was performed on a KingFisher™ Flex System which uses magnetic rods to move particles through the various binding, mixing, washing and elution phases in a 96 well plate format. HR-PRM analyses were performed on a Q Exactive™ Hybrid Quadrupole-Orbitrap™ MS coupled to an Ultimate 3000 standard liquid chromatography system. The instrument was set to acquire scheduled pairs of PRM scans in profile mode.

Results:
In this study, we developed and validated a high-throughput assay for concurrent quantification of synaptotagmin-1 and SNAP-25 in clinically relevant volumes of CSF. The assay was utilized to compare the levels of synaptotagmin-1 and SNAP-25 in a clinical cohort with CSF samples from patients diagnosed with early AD, prodromal AD, mild cognitive impairment, late AD, mixed AD, and non-demented controls. The increased CSF levels of both synaptotagmin-1 and SNAP-25 in AD patients compared to controls are in agreement with the results from two previous studies were CSF synaptotagmin-1 and SNAP-25 were quantified separately.

Conclusions
We have shown that automated immunoprecipitation combined with PRM-MS is robust and sensitive enough to concurrently measure two synaptic proteins in CSF from individual patients.

Novel Aspect:
This platform will make it feasible to perform larger and diagnostic diversified studies as well as adding other biomarkers mirroring other pathologies.

References
414 - SINGLE SHOT DIA PROFILING OF >1500 PLASMA PROTEOMES OF THE WEIGHT LOSS AND MAINTENANCE STUDY DIOGENES

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Keywords:

Introduction:
Comprehensive, robust, high-throughput analysis of the plasma proteome has the potential to enable analysis of the health state. Up to now, analysis of the plasma proteome was achieved using delicate nano-flow setups. To reduce this limitation, we established a robust capillary-flow LC-MS-DIA setup capable of measuring 32 plasma proteomes a day. Using this setup, we acquired the samples of a large-scale weight loss and maintenance plasma sample study(DiOGenes).

Methods:
Plasma samples were randomized and prepared with an optimized in-solution digestion protocol and spiked with Biognosys' PlasmaDeepDive. The samples were acquired on a Thermo Scientific Orbitrap Fusion Lumos connected to a Waters M-class UPLC and a 300μm*150cm Waters CSH 1.7μm column. DDA runs were performed using HPRP fractionation. DIA was done in a 45 min injection-to-injection mode. Analysis of DIA runs was performed using Spectronaut Pulsar.

Results:
After testing several combinations, we obtained optimized conditions using a 300 μm inner diameter column. This setup has the advantage of offering increased flow rates (5 μl/min) resulting in small injection-to-injection overhead times (i.e., 5 min). A gradient of 40 min was determined as an optimal gradient length, resulting in 90% of the maximally achieved identifications with longer gradients. Further characterization of the system showed precise quantification of 47 FDA approved plasma biomarkers (CVs below 20%).

We successfully used this capillary LS-MS DIA setup to acquire 1,508 (plus 66 control pools) samples of the DiOGenes project. We identified in average 450 proteins per samples and achieved a dataset completeness of 77% at the protein level. Proteins of the inflammation system were the most variable and proteins involved in blood coagulation were the least variable protein groups. Unsupervised clustering showed that the weight loss was most severe intervention accompanied with significant protein changes in plasma.

Conclusions
Differential abundance analysis identified proteins to be significantly differential between all of the time points revealing biology. Non-enzymatic addition of glucose to proteins (i.e., glycation) was detected and could be followed over the time course of the study. The PlasmaDeepDive kit could be used to perform extrapolation to all identified proteins enabled label-free absolute quantification of 490 additional proteins in of 1,472 plasma samples.

Novel Aspect:
High-throughput capillary-flow DIA of 1,574 human plasma proteomes with label-free absolute quantification and unbiased determination of glycation sites.
853 - IDENTIFICATION OF COMPLETE POOL OF BIO ACTIVE PEPTIDES IN MOUSE BRAIN STRIATUM INCLUDING SEPS, A NOVEL CLASS OF NON-CLASSICAL BIO ACTIVE PEPTIDES

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Keywords: short Open Reading Frame (sORF), Mass spectrometry, Bio active peptides, Peptidomics, sORF encoded polypeptides (SEPs).

Introduction: Bio active peptides are involved in the regulation of major physiological processes that occur in an organism [1]. Classical bio active peptides are cleaved from their precursor and are directed towards the secretory pathway [2]. Recently, another novel class of bioactive peptides often termed as SEPs (sORF encoding polypeptides) alias micro peptides alias non-classical bio active peptides were discovered [3]. These are called non-classical because they are translated directly from small ORFs [3]. Therefore, they lack a signaling sequence and are released into the cytoplasm, not directed through the secretory pathway [3]. Despite their unconventional expression, some of these peptides are found to play critical functional roles [4-10]. The first SEP was discovered in c.elegans in 1996 [11]. From then, many sORFs were discovered in plants such as Polaris gene (Arabidopsis thaliana), ROTUNDIFOLIA4, Brick, Zm401 and Zm908p1 [12-16]. Recently, with the advent of ribosome profiling (an RNA sequencing technique), millions of sORFs potentially translated to peptides were discovered in Saccharomyces cerevisiae, Drosophila melanogaster, Mus musculus, Danio rerio and Homo sapiens by various research groups [17-18]. Although ribosome profiling experiments predicted SEPs that are being translated, proving their actual bio chemical existence is quite challenging. In 2012, with the aid of mass spectrometry 90 SEPs were identified in a human cell line [19]. This was followed by the identification of 237 SEPs in humans in 2017 [20]. Since their discovery, millions of SEPs were predicted through ribosome profiling data but only few (hundreds) SEPs were biochemically identified. Four challenges hamper these identifications. The peptides are thought to be in Low abundance (1) – In general natural peptides are less abundant than cytosolic proteins; 2) Narrow mass range – The mass range of SEPs is between 0 - 10 KDa which is often missed in the regular proteomic analysis, therefore they are absent in most proteomic data repositories. In addition they often lack of cleavage site for the proteases; 3) Difficulties in data analysis – Search engines are typically not good to identify long peptides with multiple charges; 4) False positives – (During the database search) The databases that are typically used are concatenated ones that contains both known large proteins and predicted SEPs. Due to (up to) a 100 fold larger number of the predicted SEPs (millions) in the database when compared to known uniprot entries, false discoveries are likely to aggregate more in the SEP database. In present study, we performed an extensive analysis on mouse brain striatum sample with an intention to identify SEPs. In order to do so, we developed a protocol to overcome the challenges that arise during the process of identifying these peptides with a high confidence.

Methods:

The mouse brain peptides were extracted using a mixture of methanol:water:acetic acid (90:9:1). The peptides were fractionated into 20 fractions in reverse phase liquid chromatography. All the fractions are analyzed in nanoLC QXactive plus orbitrap mass spectrometer, 60 min linear gradient for each fraction. Two replicates for each fraction were run.

Peptide identification: A concatenated database was used: “mouse uniprot reviewed proteome downloaded on 2017-03-01 + predicted SEPs from mouse ribosome profiling data (sorfs.org) + contaminant database [2017-03-01]”. The database searching is performed in three methods: PEAKS (version 7.0, Bioinformatics Solutions Inc.) was used as the first strategy for the database search. Variable modifications used in the settings are: oxidation of methionine, C-terminal amidation of peptides, N-terminal acetylation of proteins, pyro-glu formation from glutamine and glutamic acid at N-terminus, acetylation of lysine, phosphorylation of tyrosine, threonine and serine.

The peptides are searched with Mascot (Version 2.1, Matrix science Pvt Ltd) and Sequest HT (University of Washington, USA) with no enzyme selected. The spectra from precursors with mass range from 500 to 10000 Daltons were selected for the identification process. The database search was conducted on deconvoluted data that was exported from PEAKS. The precursor mass tolerance was set to 10 ppm and the fragment ion mass tolerance was set to 0.02 Daltons. Only high confident peptides (1% False Discovery Rate) were chosen in this analysis.
Dynamic modifications including N-terminal acetylation of protein, N-terminal pyro-glutamic acid formation from glutamine, C-terminal amidation of peptide and oxidation of methionine were set as variable modifications. A global false discovery rate (FDR) for peptide and protein identification was set to 1%. The third approach is similar to the second approach but without using a deconvolution step.

Peptide validation: After identifying peptides with high confidence. The resulting peptides were validated through four steps.

Manual verification of the fragmentation spectrum for the ion series match.

BLASTp analysis for the novelty of the identified SEPs. In this section, the fragment that is matched with the in-silico generated ion series was only searched through BLASTp but not the whole identified sequence.

MS2PIP based validation: MS2PIP is a tool that generates predicted ion peak signal intensities based on artificial neural network. The experimentally identified fragmentation spectra was be matched to the MS2PIP generated fragment intensities [21].

The sORF attributes such as ORF score, FLOSS score, Ribosome occupancy, conservation, and in frame coverage of the identified SEPs will be checked [18,22].

Only those peptides passing all validation steps, are considered confident. Finally experimental fragmentation spectra of the identified peptides were compared to that obtained from a synthetic equivalent.

Results:
In total 5650 unique peptides were identified combining both the replicates. Using charge state deconvolution of peptides, we observed 36% increase in unique peptide identifications and we identified longer peptides ranging from 0-85 AA long. Classical bio active peptides: 926 peptides originating from 37 neuropeptide precursors were identified. Non classical bio active peptides: 12 peptides originating from 4 SEPs were identified. Prior to the validation steps, 53 putative SEPs were identified with 1% FDR (this number can be comparable to the previous studies identifying 90 SEPs). Out of the 53, 49 identifications passed through the first filter (manual sequence verification). Out of 49, only 11 identifications passed through the second filter (BLASTp based verification). Out of 11, 6 identifications survived the third filter (MS2PIP based validation). Out of 6, 4 SEPs passed through the fourth filter (sORF attributes cross check).

Conclusions:
A protocol to identify a complete set of natural bioactive peptides is created. Using this approach we identified 926 peptides originating from 37 neuropeptide precursors. To our knowledge, using peptidomics approach, this is the highest number of peptides derived from neuropeptide precursors identified in mouse brain in any study so far. Using a four step validation approach we effectively reduce the chances for eliminated false positive identifications and confidently identified 4 SEPs, out of which 3 SEPs are novel.

References:
Novel aspect:

First, identification of 926 peptides originating from 37 neuropeptide precursors through peptidomics approach in mouse brain striatum is the highest number of unique peptide identifications reported till date.

Second, the concept of SEPs is relatively novel which originated in 1996. There are studies proving the functional importance of the SEPs. These SEPs can be a heavy interest for the pharmaceutical companies. Most importantly, we observed false positive identifications in the data where few studies claim to identify SEPs. In this study we proposed a method to eliminate the false positives and result with confidently identified SEPs. In our study we confidently identified 4 SEPs, out of which 3 SEPs are novel and not reported previously.
Introduction:
Accurate differential diagnosis of hypertensive disorders is important during pregnancy, because it affects the outcome for the mother and fetus. The high potential of urine peptidomics has been demonstrated for clinical purposes. Several MS based approaches have been successfully applied for urine peptidome analysis and potential biomarker search. Herein we apply LC MS to identify a peptide panel for differentiation of hypertensive disorders during pregnancy.

Methods:
A case-control study was designed for 64 women from 4 groups: preeclampsia (PE), superimposed PE, chronic hypertension, and healthy pregnant individuals. The urine peptidome fraction was separated using ultracentrifugation, purified by gel-filtration, and analyzed with nano-HPLC coupled to a high resolution 7T LTQ-FT Ultra (Thermo) mass-spectrometer and semiquantitative label free analysis was performed.

Results:
Analysis of the LC-MSMS data from different groups of patients showed that 36 peptides were common for them all. 22 belonged to alpha-1-chain of collagen I, 9 were from alpha-1-chain of collagen III, 2 from alpha-2-chain of collagen I, 1 from alpha-1/2-chain of collagen I, 1 from alpha-1-chain of collagen I/XVIII and one from uromodulin. Patients with hypertensive disorders had 34 common peptides: 12 from alpha-1-chain of collagen I, 10 from fibrinogen alpha-chain, 8 from alpha-1-chain of collagen III, and 4 per other types of collagen. The most important is a group of 16 peptides common for PE and superimposed PE cases. It consists of peptides from alpha-1-chain of collagen I, alpha-2-HS-glycoprotein, apolipoprotein A-I – one per each and the rest 13 belong to alpha-1-antitripsin. Fibrinogen peptides in the urine point to inflammation processes and those of alpha-1-antitripsin – to changes in metabolic ways of protein degradation.

Conclusions
Fragments of alpha-1-antitrypsin confirmed their importance as markers of pre-eclampsia suggested earlier. Statistical data analysis by non-parametric Kruskal-Wallis and Mann-Whitney tests showed the presence of 12 peptides differentiating at least one pair of groups.

Novel Aspect:
Comparative analysis revealed 12 peptides, which could be used as a diagnostic panel for confident discrimination of various hypertensive disorders.
PROTEOMIC CHARACTERIZATION OF HUMAN PLATELET LYSALE BY LABEL-FREE NLC-MS/MS: TOWARDS THE EFFECTORS OF ITS BENEFICIAL EFFECT IN REGENERATIVE MEDICINE

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Keywords: Regenerative medicine, Proteomics, platelet lysate, scaffold, MALDI imaging

Introduction:
Hydrogel-forming polymeric scaffolds (HyPS) provides a favourable 3D biomimetic and biodegradable environment for Human Mesenchymal Stromal Cells (hMSCs) [1]. Recently, in HyPS engineered with hMSCs, human platelet lysate (hPL) has been shown as highly effective to stimulate the hMSCs cell-growth and osteo-chondral differentiation [2-4]. The proteins responsible for its beneficial effect have been not yet clarified and will be herein investigated by MS.

Methods:
Quali-quantitative evaluation of hPL (n=4) compared to PlateletsPoor Plasma (PPP)(n=4) was achieved by label-free shotgun proteomics. IgG and albumin depleted and not depleted samples were trypsinized [5] and analysed by nLC-UHRTOF. Data was submitted to PEAKS studio and Mascot. Proteomic images of chondrogenic differentiated hMSCs from bone marrow (BM) and adipose tissue (AT), grown in HyPS for 21 days with hPL, were obtained by RapifleX MALDI TissueTyperTM [6].

Results:
About 450 and 570 protein IDs were identified in not depleted and depleted hPL and PPP, respectively. 59 of them resulted specific of hPL and not present in PPP independently from depletion, representing a possible panel of factors involved in cell stimulating effect of this supplement. Relative quantification highlighted 7 and 43 proteins significantly altered in their abundances comparing hPL vs PPP in not depleted and in depleted samples (fold change>1.5; p<0.05; ≥2 unique peptides). 3 of these differences were in common, including a critical proliferation regulator. The functions and networks of the proteins of interest were investigated and compared. In situ proteome evaluation of AT-hMSCs and BM-hMSCs grown in HyPS in vitro models for 21 days in presence of hPL and chondrogenic medium was also provided. Histology evaluation was also performed with H&E. The molecular images of trypsinized samples were co-registered with the stained counterparties and co-localization of specific peptide signals with differentiated cells were enlightened.

Conclusions:
Shotgun analysis and label-free quantification of hPL and PPP allowed to ‘proteomically’ characterize the supplement and to highlight possible candidates responsible for the beneficial effect onto the proliferation and differentiation of in vitro models. MS imaging analysis of seeded scaffold permitted also to recognize proteomic profiles specific of chondrogenic differentiated cells for in situ monitoring cell expansion and maturation.

Novel Aspect:
Proteomic characterization of hPL and MS imaging analysis give important information to clarify and monitor the hMSCs chondrogenic differentiation in vitro HyPS3D model.

References:
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F. Re et al., Abstract number: B338, 44th Annual meeting of the European Society for Blood and Marrow Transplantation, 18-21 March (2018), Lisbon.

Fundings:
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Keywords: Elderly yoga, Healthy plasma, Comparative glycoproteomics, Lectin affinity chromatography

Introduction:
As the elderly population grows, yoga gets more popular as one of the exercises to improve the quality of life and health of the elderly. Glycoproteins in human plasma are known to be associated with multiple diseases, cell differentiation, aging, and etc. This study intends to examine the efficacy of yoga by identifying and comparing the glycoproteins in human plasma from elderly yoga groups before and after participation in a fall-prevention yoga program.

Methods:
Human plasma were collected from both Advanced level group and Beginner level group before and after participation in a fall-prevention yoga program. The plasma were pooled and affinity-selected using self-packed LEL columns. The captured glycoproteins from each plasma were then trypsin digested and deglycosylated with PNGase F. After that, proteins were identified with nLC-MS/MS and database searches in both groups.

Results:
Plasma glycoproteins were identified from both Beginner level group and Advanced level group before and after 4-month of yoga practice. The average age and sex ratio were equally matched in each group for the same experimental conditions. The results show that the number of plasma glycoproteins in Advanced level group is less than that of Beginner’s group, demonstrating an inverse correlation between number of plasma glycoproteins and yoga experience.

Conclusions
As the result of 4-Month yoga Program, the numbers of glycoproteins in the blood plasma tend to decrease for yoga participants. The longer the participants have enrolled in the program, the greater the change in the number of detected glycoproteins for the same 4-Month of yoga Program. Glycoprotein compositions of healthy elderly people have been obtained as reference data for future related studies.

Novel Aspect:
This is a comparative plasma glycoproteomics between elderly healthy yoga groups to see the efficacy of yoga programs with lectin affinity selection.

References
VALIDATION OF A LC-MS ASSAY FOR ASSESSMENT OF ALPHA-DEFENSIN IN SYNOVIAL FLUID

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Keywords: Synovial fluid, periprosthetic joints infections, alpha defensin, liquid chromatography-mass spectrometry

Introduction:
Synovial fluid alpha defensin is regarded as a potential biomarker for rapid identification of periprosthetic joints infections (PJI). The availability of a method not prone to interferences, with high sensitivity and specificity, could be of great help to clinicians, to make treatment decisions. LC-MS technology is widely used to measure new biomarkers, due to high selectivity and high sensitivity. We developed and validated a LC-MS method to perform synovial fluid alfa-defensin quantification.

Methods:
Synovial fluids (SF) samples were sample left over obtained from Patients referring to a large Italian private hospital. A pre-analytical step was performed based on chemical protocol to obtain a peptide from tryptic digestion of protein. The quantification of peptide are carried out by blank synovial fluid (spike solution) and simulant of synovial fluid (simulant [1]) in range from 0.1 to 100 µg/ml. To develop the LC-MS method, two different platforms (LC-QTOF and LC-MS/MS) were used.

Results.
The isolation and identification of the marker peptide from alpha defensin was achieved by LC-QTOF analysis by Auto-MS acquisition of data, after tryptic digestion of synovial fluid sample, positive for the presence of alpha defensin (Positive in according to MSIS criteria). The raw data were elaborated with Spectrum Mill Software, to verify the uniqueness of marker peptide of alpha defensin: the peptide LWAFCC is identify as a specific marker for the presence of alpha defensin. To evaluate the method performance, LOD and LOQ are calculated with 60 replicates of negative samples, in accordance to CLSI EP17-A2:20012 [2]: the best results are 0.09 µg/ml for LOD and 0.29 µg/ml for LOD performed by nagative matix spike and LC-QTOF analysis. For December 2016 to November 2017, 119 synovial fluid samples are tested (from 119 patients, 92 aseptic and 27 positive by MSIS criteria): the results are cut-off value of 1.0 µg/ml, AUC 0.992 (95% C.I. 0.98-1.0), sensitivity 100%, specificity 96.3%, PPV 90% and NPV 100%.

Conclusions:
Presently, Alfa defensin measure in SF is performed either by laboratory based alpha- defensing immunoassay or by Synovasure “quick test” (lateral flow test kit). Both methods claim high degree of accuracy in the diagnosis of PJI [3]. LC-MS offers the potential to be an alternative to immunoassays and offers the highest sensitivity and precision for the identification and detection of analytes, although it still requires high skilled professionals and may need a long TAT.

Novel Aspect.
The results of the LC-MS assay validation show a robust method for qualitative and quantitative research of alpha defensin in synovial fluid.

References.
Introduction:

One of the prominent obstacles in hepatocellular carcinoma (HCC) is the diagnosis in advanced stages when patients have several liver dysfunctions, limiting treatment options and life expectancy. A specific molecular signature is “a must” to be established to complex diseases, as HCC, to better monitor the development and progression of those pathological stages [1,2]. Cancer multiomics approach could reveal global signature under different biological scenarios, especially for redundant alterations as those seen in HCC [3].

Methods:

Nine patients with HCC, submitted to orthotopic liver transplant, and nine non-pathological patients (donors, negative control) were evaluated. Label-free LC-MS/MS-based proteomics (350 to 1,800 m/z, positive mode) and MALDI-TOF/MS-based lipidomics (600 to 1,200 m/z, positive mode) analysis were performed associated with statistical analysis (Progenesis QI®) and chemometric approach (MetaboAnalyst®) to compare plasma global protein and lipid profiles. The present study was approved by the Research Ethics Committee of the Federal University of Ceara (COMEPE 026/98).

Results:

The results displayed two distinct proteomic and lipidomic profiles within pathological conditions versus control. The criteria for protein classification for biomarkers prospecting study used the highest fold change variation (≥ 5), the highest VIP score (1.9 – 1.4) after PLS-DA (PLS-DA imp, p < 0.05) performance and GO annotation analysis. In total, seventeen proteins were highly indicated for future validation. Cofilin-1 and Ig heavy chain variable region highly contribute to pathological phenotype when compared to control group under PLS-DA analysis. In patients with HCC, the major variations on fold change were detected at up-regulated Immunoglobulin kappa variable 4-1 (fold change 1,716.93) and down-regulated Procollagen C-endopeptidase enhancer 1 (fold change 221.49) proteins. The criteria for lipid identification by important features of relative concentrations used the highest VIP score (2.5 – 1.0) after PLS-DA analysis. The most abundant lipid in control patients is phosphatidylcholine (PC), with four ions contribution under PLS-DA analysis. In contrast, in HCC patients there was a shift on lipid profile, with phosphatidylethanolamine (PE) contributing as equally as PC, under PLS-DA analysis. Glycerophospholipids highly contribute to pathological phenotype when compared to control group.

Conclusions:

In this research, variations on fold change and VIP score display aberrant expression and contribution of several proteins, allowing the screening of 17 biomarkers candidates for future validation. Alteration in ratio between PC and PE revealed glycerophospholipid imbalance as potential diagnostic biomarkers pathway in a non-invasive method for HCC, as well for other liver diseases. Lipidomics and proteomics analysis, assessed individually or together, revealed possible candidates for diagnostic markers in a non-invasive method for HCC, as well for other liver diseases.

Novel Aspect:

Applying mass spectrometry to clinical questions under a translational-multiomic-approach provides deeper understanding on advanced HCC and possible biomarkers candidates [3,4].

References:
Introduction:
Sepsis causes one death every 5.2 seconds worldwide [1]. To achieve rapid patient care, prognostic markers are needed. Predictive value of S100A8/9 complex was recently highlighted with an ELISA [2]. However, antibody affinity to particular isoform is questionable. Our study has assessed the prognostic value of plasmatic S100A8 and S100A9 by combining intact protein mass spectrometry for isoform characterization and bottom-up quantification of total isoforms.

Methods:
Proteoform characterization includes immunoprecipitation using specific antibodies coated on magnetic beads, acidic elution and intact protein analysis on a Q-Exactive instrument operated in full MS mode. Bottom-up assay: the antibody-free assay consists in a simple pellet and tryptic digestion protocol using 10µL of plasma. The Q-Exactive was operated in the PRM mode and four specific peptides were monitored with labeled peptides for quantification.

Results:
Intact protein analyses following immunoprecipitation were used to characterize the circulating proteoforms of S100A8 and S100A9. In plasma of septic patients, truncated, acetylated and oxidized isoforms were detected. Peptides common to all detected proteoforms were selected to quantify the total forms. A simple antibody-free sample preparation was developed for targeted analysis of S100A8 and S100A9 signature peptides. Application to clinical cohort analysis requires robust and quantitative assays. Validation in plasma of septic patients demonstrated good reproducibility, linearity and peptide stability. A cohort of 45 plasma of septic patients was analyzed with the present assay, in parallel to an ELISA [3]. Remarkably, we found a significant difference in S100A8 and S100A9 concentration between survivor and non-survivor patients (p= 0.009). Additionally, excellent correlations between MS analysis and ELISA were observed (r=0.97), thus demonstrated similar performance for both assays.

Conclusions:
Intact mass spectrometry allowed identification of S100A8 and S100A9 isoforms in patients with sepsis and all isoforms were then quantified by a simple antibody-free bottom-up assay. The present method demonstrated high reproducibility and accuracy, in agreement with the latest bioanalytical recommendations [4]. It should help harmonization of S100A8 and S100A9 quantification across laboratories, with the objective of reaching a better sepsis prognosis.

Novel Aspect:
Novel validated LC/MS assay for S100A8 and S100A9 quantification in a cohort of patients with sepsis, provide new significant variables for prognosis in sepsis.

References:
Introduction:
Nowadays infertility affects a significant number of couples and for many of them ART procedures provide a unique opportunity of achieving pregnancy. The major factor limiting the success of IVF procedures is embryo implantation. Studies showed that PIF peptide can be responsible for tolerances of the immune system of the mother [1]. The aim of the project is to understand the protein interactions of the PIF peptide in human serum to understand molecular mechanisms responsible for implantation.

Methods:
For binding proteins from human serum to PIF peptide we used affinity chromatography. PIF peptide was immobilized on solid support and then human serum was applied to microcolumn with immobilized peptide. The eluted proteins were subjected to bottom up analysis by liquid chromatography tandem mass spectrometry. Fragmentation spectra obtained in connection with available bases of amino acid sequences enabled to create a list of proteins that bind to PIF peptide.

Results:
The PIF peptide consisting of the 15 aminoacids residues (H-MVRIKPGSANKPSDD-OH) was chemically synthesized with the variants containing cysteinpentaglycine. These variants enable to prepare affinity column for binding proteins. The bottom up analysis with trypsin as a digestion enzyme enable to identify more proteins in a sample. Fragmentation spectra obtained from LC-MS/MS analysis in connection with SwissProt databes enabled to identify several proteins which bind to PIF peptide. These proteins have potential impact on trophoblast invasion, eventually affecting placental development in the peri-implantation period.

Conclusions:
Performed methods of affinity chromatography and LC-MS/MS technique are good tools for analysis of PIF interactome in human serum and other fluids. Presented method reveals that PIF peptide interacts with proteins which are responsible for implantation of the embryo and immune tolerance of the mother. However, holistic approach should be performed to understand whole mechanism of the implantation.

Novel Aspect:
The information obtained in this project can be used to develop diagnostic and therapeutic methods for monitoring the development of pregnancy and maintaining endangered pregnancies.

References:
**Introduction**

Fibromyalgia (FM) (prevalence of 2-4%) is a multifaceted chronic pain condition. The pathophysiological mechanisms behind FM involve both central and peripheral sensitization. Validated objective markers for involved pathophysiological processes in FM are crucial for diagnosis, but are today unfortunately lacking. The aim of this study were to investigate the interstitium muscle proteome in FM and healthy subjects to identify activated molecular pathways before and after progressive resistance exercise.

**Methods**

30 FM patients and 30 healthy subjects were included. Microdialysis (MD) was performed in the vastus lateralis muscle using commercial catheter (100 kDa). Dialysate samples were collected every 20 minutes during a four hours session at a flow rate of 5 μL/min. The proteome was analyzed by nLC-MS/MS on LTQ Orbitrap Velos Pro (Thermo). Multivariate statistical analysis and bioinformatics were used to compare and describe the proteome profile between the FM and controls.

**Results**

The proteome fingerprinting analysis of the interstitial muscle identified proteins involved in inflammatory responses, stress response, immune system processes, muscle contraction and nociception. The insertion of MD catheter causes an acute tissue trauma and nociception. The equilibration period for the muscle was two hours. To investigate if the myalgic muscle reacts differently to the insertion of MD catheter compared to healthy muscle, dialysate samples from the trauma period (20-120 minutes) were analyzed. There was a significantly altered protein pattern in FM during the acute tissue trauma compared to the healthy controls. Important proteins whose levels were lower in FM during the tissue trauma were proteins involved in muscle contraction. The expression levels of proteins involved in inflammation, gluconeogenesis and stress responses were higher in FM compared to healthy subjects. Statistical analysis on the effect of the exercise regime on the proteome profile are ongoing.

**Conclusions**

This study shows that the nociceptive reaction of the muscle due to the MD catheter insertion in FM is different compared to healthy pain free subjects. The findings in this study together with the findings in plasma from the same cohort contribute to increased knowledge about peripheral and systemic molecular mechanisms involved in the nociceptive mechanisms in FM. Taken together our results can contribute to a better understanding of how to improve outcomes of treatments.

**Novel Aspect**

MD-LC-MS/MS is a promising tool to provide new insights into the interstitial proteome of muscle and to finding altered molecular pathways in fibromyalgia.
Keywords: top-down proteomics, bottom-up proteomics, snake venom

Introduction:
Venoms produced by viper snakes contain medically significant toxins that play a key role in the pathophysiology of snakebites disease. The most widely used techniques for venom analysis is LC-MS/MS-based bottom-up (BU) proteomics[1]. On the contrary, intact protein measurement with top-down (TD) MS strategies, offers the richest data, covering the almost complete protein sequence and the ability to identify proteoforms and to localize modulations [2-3].

Methods:
Snake specimens were captured and milked in their localities around three different geographical regions of Iran. Size-exclusion chromatography (Bio-Rad NGCQuest Plus) was used for protein fractionation prior to LC-MS/MS. Mixed and fractionated venom samples were subjected to TD and BU proteomics analysis using Ultimate 3000 RP-UHPLC coupled to a Fourier transform orbital trapping mass spectrometry (Q Exactive HF-X, Thermo Scientific GmbH, Bremen, Germany).

Results:
The venom toxin composition of three geographically different saw-scaled viper samples were primarily measured as a venom mix through both, direct TD and BU strategies. The results of these approaches provide an initial overview of the venom toxin family on the protein level. The venom mix measurement illustrated that the venom proteomes were dominated by PLA2 and SVMP. Considering the proteome complexity of snake venoms, pre-fractionation is an essential part of venomics prior to MS analysis. We therefore de-complexed the venom of three populations by size-exclusion chromatography into 6 fractions for each sample. The fractions were then applied to LC-MS/MS in reduced and non-reduced form (TD) and also digested in solution (BU) for proteomics measurements. The data indicated sequence similarities to more than 300 proteins, belonging to 20 protein and peptide families.

Conclusions:
Accurate understanding of venom proteome contents can greatly help to improve the treatment of this neglected tropical disease and also drug discovery. Venom research is constantly developing, based on advanced analytical technology. Implementation of multi-dimensional separation techniques and TD proteomics using high-performance mass spectrometry accelerates the identification and characterization of venom components.

Novel Aspect:
First comprehensive qualitative and quantitative proteomics study of saw-scaled viper (Echis carinatus sochureki) venom from three different geographical regions of Iran.

References:
Introduction:
Amyotrophic Lateral Sclerosis (ALS) is a progressive neurodegenerative disease that affects motor neurons in the brain and the spinal cord. The progressive motor neuron degeneration eventually leads to death usually within 5 years of diagnosis[1]. ALS can be often mistaken for primary lateral sclerosis (PLS), which progresses more slowly[2]. In addition, comorbidities in ALS patients further complicates diagnosis, treatment and proper care[3, 4].

Methods:
In an effort to investigate protein biomarkers for better diagnosis and disease progression, serum samples from a longitudinal cohort of 48 subjects, including ALS, re-classified from PLS to ALS, and age- and sex-matched healthy controls, were investigated. Highly abundant serum proteins were depleted and subjected to LC-MS/MS analysis. For label-free protein quantification and data analysis, MaxQuant, Perseus and R software were employed.

Results:
Thirty-nine differentially expressed proteins between ALS patients (including subjects re-classified from PLS to ALS) compared to healthy controls were identified. These proteins include complement factor H, complement C2 and apolipoprotein A1, suggesting abnormality or impairment in the regulation of complement cascade and cholesterol metabolism pathways. These findings were in agreement with the previous published studies[5, 6]. Only six proteins were common between the ALS and reclassified ALS when they were compared to the healthy control. This could be due to human heterogeneity or may actually representing different molecular mechanisms affecting disease progression, which warrant further investigation. Analysis of the longitudinal patient samples revealed a number of proteins potentially involved in disease progression. The role of candidate proteins in ALS pathophysiology and progression will be further evaluated.

Conclusions:
Our study revealed the presence of a distinct set of proteins in the serum of ALS patients that can potentially be used for tracking disease progression and diagnosis. A better understanding of the pathological features characterizing ALS will shed light onto the common and unique cellular events shared among disorders of similar etiology. This will ultimately help to develop effective diagnostic tools and will potentially identify novel treatment strategies.

Novel Aspect:
Identification of diagnostic protein markers for ALS disease stratification from disorders of similar etiology and for tracking ALS disease progression.

References
INTRODUCTION:
Metastasis formation is the major cause for cancer-related deaths and the underlying mechanisms remain poorly understood. In this study we describe spontaneous metastasis xenograft mouse models of human neuroblastoma used for unbiased identification of metastasis-related proteins by applying an infrared laser (IR) for sampling primary tumor (PT) and metastatic tissues, followed by mass spectrometric proteome analysis.

METHODS:
IR aerosol samples were obtained from ovarian and liver metastases, which were indicated by bioluminescence imaging (BLI), and matched subcutaneous primary tumors. Tissue ablation products were tryptically digested using a filter aided sample preparation protocol [1].

RESULTS:
Corresponding histology proved the human origin of metastatic lesions. Ovarian metastases were commonly larger than liver metastases indicating differential outgrowth capacities. Among ~1,700 proteins identified at each of the three sites, 89 proteins were differentially regulated in ovarian metastases while 290 proteins were regulated in liver metastases. There was an overlap of 26 and 10 proteins up- and downregulated at both metastatic sites, respectively, most of which were so far not related to metastasis such as LYPLA2, ACTL8, EIF4B, LGALS7, GFAP, and ELAVL4. Moreover, we established in vitro sublines from PT and metastases and demonstrate differences in cellular protrusions, migratory/invasive potential and glycosylation.

CONCLUSIONS:
Our study demonstrates that BLI-guided collection in the LAN-1 neuroblastoma model is suitable to identify novel candidate proteins that contribute to spontaneous metastasis formation in vivo. The number of regulated proteins (showing differential expression levels in PT vs. metastatic sites) decreases in advanced metastases. The derived sublines maintain pro-metastatic features in vitro making them highly attractive tools for future functional studies.

NOVEL ASPECT:
Summarized, this study identified potential novel putative drivers of metastasis formation and provides novel sublines of metastatic neuroblastoma.

REFERENCES

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1275 - BLOOD PLASMA PROTEOME AND PEPTIDOME COMPARATIVE SCREENING FOR POTENTIAL BIOMARKERS OF ALZHEIMER’S DISEASE

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Keywords: Amyloid-beta, Alzheimer’s disease, blood, proteomics, peptidomics

Introduction:
Alzheimer’s disease is a fatal neurodegenerative disorder of the elderly, characterized by slow onset of memory loss, impaired consciousness and other brain functions, which are not detected until severe and possibly irreversible signs appear. Study of proteome changes during all stages of pathology – normal aging, mild cognitive impairment and dementia – may allow to obtain understanding on the mechanisms of disease onset or approaches to monitor progression.

Methods:
Comparative analysis of blood plasma proteins, peptides and their post-translational modifications was carried out for three groups of patients at different stages of the disorder. Proteome and peptidome fractions were extracted by a combined set of preparative procedures and analyzed by HPLC MS/MS analysis on an Agilent 1100 HPLC system coupled to a 7T FT ICR LTQ FT Ultra mass-spectrometer in DDA mode.

Results:
Label-free analysis of obtained data allowed to show potential protein markers specific for different levels of cognitive disorders. Over 300 proteins were identified in total. Among the common 76 coreproteins, 15 proteins were shown to reliably change in the MCI group in comparison with the controls. Of them 8 proteins were associated with various neurological diseases. A similar approach was used to find the significantly changing proteins for the AD group. A significantly less pronounced difference was observed between the AD and MCI conditions. In addition, a search for significant differences in the post-translational modifications of plasma proteins was carried out with a special attention to oxidative modifications known to be one of the early and valid tissue damage indicators, especially, for brain tissues. Significant changes in levels of PTMs such as deamidation of glutamine residues, oxidation of methionine and N-terminal residues and glutathione binding were observed.

Conclusions:
The found difference in the proteomes between groups of healthy individuals and patients with MCI and a less significant variation between mild and heavy disease states allow to hope for a possibility to develop methods of diagnosis at the earlier and potentially more treatable stages of the disease progression.

Novel Aspect:
The obtained protein panel can be potentially used for validation of biomarkers for screening of significant cognitive function changes and AD onset in particular.
1168 - IMMUNOASSAYS IN MULTIPLEX FOR BIOMARKER DISCOVERY AND VALIDATION

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Keywords: Biomarker, Immunoassays, Safety biomarkers, Signaling

Introduction:
Array-based assay systems allow the analysis of hundreds of molecular parameters in a single experiment. Within the last decade multiplexed protein analytical assays achieved robust analytical performance and enable to screen for a multitude of parameters using minimal amounts of sample material. There are a variety of technologies and methods available to address assay requirements in terms of covering analyte concentration, sample variety and sample origin.

Methods:
Two assay formats addressing unmet needs in biomarker discovery developed at NMI will be presented. Our DigiWest assay format transfers the western blot to a bead-based microarray platform extending traditional western blotting to enable the rapid quantification of hundreds of specific proteins and protein modifications in complex samples (1). The second assay format combines sensitive and selective immunoprecipitation coupled with mass spectrometric readout. The use of peptide group-specific antibodies called MS-based immunoassays employing such terminal specific antibodies are established for several applications, including a number of cross-species drug induced organ injury assays that represent truly translational biomarker assays (2).

Results:
The DigiWest high-throughput western blot approach allowed us to identify alterations in cellular signal transduction that occur during the development of resistance to the kinase inhibitor Lapatinib. A total of 185 western blot equivalents were performed. Differential phosphorylation was observed for a variety of proteins; a substantial increase in phosphorylated Aurora A, p27 and IRAK4 was detected; for other proteins a decrease in phosphorylation was observed (e.g. MEK1, Src, Erk1/2, different sites in EGFR). (1) For our immunoaffinity mass spectrometry assays the quantification of drug induced kidney biomarker candidates in canine and monkey urine samples will be presented. The expression profiles of osteopontin, retinol binding protein 4, clusterin and KIM-1 in urine samples correlates with histopathology and conventional clinical pathology parameters when compared to tissue samples from the same animal. The biomarker assays are more sensitive than blood urea nitrogen (BUN) and serum creatinine (sCr) to detect early drug induced kidney injury. (4)

Conclusions:
Biomarker discovery and subsequent validation require robust technologies and assays combined with an excellent study design to include appropriate sample sets. This is a prerequisite for any biomarker qualification process. Our new assay formats enable the generation of such relevant data sets and can be implemented into a generic qualification strategy. Such new biomarkers will allow early identification, assessment and management of drug-induced injuries throughout R&D as well as biomarkers relevant for precision medicine.

References
Introduction:
Acute myocardial infarction (AMI) is a chronic disease and major cause of mortality worldwide, together with cancer. AMI is currently diagnosed based on an electrocardiogram and elevated levels of protein biomarkers such as troponin I, troponin T, myoglobin, creatine kinase, and CK-MB [1]. Once a heart attack occurs, surgery should be done within a short period of time, and the prognosis is very poor. If a novel biomarker in early stage is discovered, then it will help to reduce the burden of life for heart disease patients. We used a mixed non-targeted and targeted approach to discover novel biomarker candidates for AMI from the comparison of healthy and patient serum using quantitative analysis.

Methods:
Serum samples of healthy controls (n = 22) and AMI patients (n = 14) were pretreated using an albumin and immunoglobulin G depletion kit before trypsin digestion, and desalting. UHPLC-LTQ-Orbitrap analysis was achieved by data dependent analysis followed by a Mascot search. Comparative and shot gun proteomics developed were modified for discover biomarker candidates [2, 3]. Multi reaction monitoring by UPLC-LTQ-Orbitrap and ELISA were used for the quantitative analysis of biomarker candidates from serum samples for validation.

Results:
Thirty four peptide biomarker candidates were screened. Multi reaction monitoring showed that alpha-1-acid glycoprotein 1 level was significantly decreased (P < 0.05) and those of antithrombin III, clusterin, obscurin, and titin were significantly increased (P < 0.001) in AMI patient samples compared to levels in healthy controls. Four significantly decreases peptides, which were unidentified in Mascot Search, were also discovered (P < 0.05) as AMI diagnostic biomarker candidates. We quantified the biomarker candidates further using commercial ELISA kits. ANOVA showed that obscurin and titin concentrations were significantly elevated in AMI patients compared to levels in healthy controls, together with that of troponin I. Obscurin and titin showed the highest decision performance, with sensitivities of 86% and 85%, specificities of 100% and 94%, and diagnostic criteria of 11.4 ng/ml and 36.1 ng/ml, respectively.

Conclusions
A modified comparative proteomics approach provided excellent results in the search for novel biomarker candidates of AMI. A combination of obscurin and titin could be used for the diagnosis of AMI with high diagnostic performance.

Novel Aspect:
Combination of new technology, abundant protein depletion; UHPLC-LTQ-Orbitrap; data dependent analysis; Mascot search, was applied to discover AMI biomarker candidates by comparative proteomics. Obscurin and titin are novel biomarker candidates for AMI patient in early stage.

References
Introduction:
Rheumatoid factor (RF) is auto-antibody that is involved in autoimmune such as rheumatoid arthritis. RF is diagnostic biomarker of rheumatoid arthritis. However, single RF factor is not sufficient to reflect all of the state of rheumatoid arthritis. To increase efficacy of diagnosis of rheumatoid arthritis, our aim is to discovery RF-correlated proteins that can be used complementarily with RF.

Methods:
For proteomics approach, Serums were classified into RF-low (RF<18 IU/ml), RF-intermediate (18<RF<54 IU/ml), and RF-high (RF>54 IU/ml). Nano-liquid chromatography-tandem mass spectrometry is used to identify and quantify global proteins in RF-low and RF-high subjects. Candidate biomarker proteins were validated using Enzyme-linked immunosorbent assay (ELISA) in RF-low, RF-intermediate and RF-high.

Results:
Among identified proteins by nano-liquid chromatography-tandem mass spectrometry, 4 proteins, including cholinesterase, histidine-rich glycoprotein (HRG), lipopolysaccharide-binding protein (LBP) and C-reactive protein, were more than 2-fold differentially expressed with statistically significance (p<0.05). Of these 4 proteins, the ELISA results of HRG and LBP was consistent with the results of nano-liquid chromatography-tandem mass spectrometry. Areas under the ROC curve of HRG and LBP were 0.861 and 0.828, respectively. The correlation between RF and HRG was statistically significant (p =0.003), and that with LBP was also statistically significant (p = 0.044).

Conclusions:
As a results, HRG and LBP are altered in RF-high serum, which is suggested that HRG and LBP can be complementarily used with RF. In validation discovery set, the efficacy of HRG and LBP proved in patients with rheumatoid arthritis by ELISA.

Novel Aspect:
We propose that HRG and LBP can be useful screening markers for rheumatoid arthritis with RF.

References:
NOVEL BIOMARKER DISCOVERY IN EXTRACELLULAR VESICLES FROM PLASMA FOR COLORECTAL CANCER

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Keywords: extracellular vesicles, plasma, biomarker, colorectal cancer

Introduction:
Targeted proteomics using SRM has been proved to be highly sensitive and quantitatively accurate. However, it is expensive and time-consuming to perform large-scale SRM analysis. We introduce a systematic and scalable SRM workflow for discovery and validation of novel biomarkers in plasma extracellular vesicles (EVs) targeting more than 400 proteins effectively.

Methods:
We developed a three step strategy for selecting biomarker candidates (BCs). Step 1: Primary BCs were selected from three sources; experimental data, literature data and database. Step 2: Targeted proteomics was performed for selecting secondary BCs from primary BCs. Isolated plasma EVs obtained from patients with colorectal cancer (n=59) and healthy volunteers (n=59) were analyzed in Step 1/2. Step 3: We evaluated the performance of secondary BCs in another sample set.

Results:
Step 1; 1,300 proteins were identified from plasma EVs as experimental data. 900 and 5400 proteins were extracted from PubMed literature search and ExoCarta database respectively. From these sources, 450 proteins were selected as primary BCs. Step 2; More than 1,000 stable isotope-labeled peptides were synthesized by our original peptide synthesis method, MS-based Quantification By isotope-labeled Cell-free products (MS-QBiC) [1]. We performed SRM analysis by using synthetic peptides to confirm their retention time and select top 5 highest intensity transitions. Next, spike-in plasma EVs isolated from pooled plasma were analyzed to select measurable peptides. Optimized SRM analysis was performed for selecting secondary BCs in individual plasma EVs. Step 2/3 is now still in the investigation stage.

Conclusions
Although the selection of final BCs is still ongoing, Step 1/2 are supported by each unique logical method to choose candidates which is the best for purpose. Our three step strategy would be innovative and practical approach for novel biomarker discovery.

Novel Aspect:
We proved the feasibility of targeted proteomics using more than 1,000 stable isotope-labeled synthetic peptides for clinical samples.

References
Keywords: cervicovaginal fluid; preterm birth; quantitative proteomics; biomarkers

Introduction
Preterm birth (PTB) before 37 weeks of pregnancy is one of major causes of perinatal mortality and neonatal morbidity. Despite of previous studies in developing biomarker(s) of PTB [1-3], unfortunately, the valuable protein maker for the early detection of PTB has not been unveiled. In this study, we performed quantitative profiling of cervicovaginal fluid (CVF) proteome for discovery of PTB-specific marker(s) via online 2D-nLC-ESI-MS/MS with isobaric labeling.

Methods
Comparative proteomic analysis of CVF proteome from PTB and control (its normal counterpart) was carried out using online two dimensional nanoflow liquid chromatography-electrospray ionization-tandem mass spectrometry (2D-nLC-ESI-MS/MS) with Isobaric tags for relative and absolute quantitation (iTRAQ)-based labeling.

Results
From 2D-nLC-ESI-MS/MS experiments, a total of 1346 proteins were identified in both CVF proteome from PTB and control and resulted in identifying 30/72 (up-/down-regulated by 1.5-fold change) proteins that were differently expressed in PTB compared to control. Bases on Gene Ontology (GO) enrichment analysis, it revealed that these significantly changed proteins were functionally involved in structural constituent of epidermis and cytoskeleton, serine-type endopeptidase inhibitor activity and oxygen carrier activity. We further evaluated the PTB biomarker candidates in five PTB individuals and seven control individuals, resulting in 55 proteins showed statistically significant difference between PTB and control. Among these, two proteins (transaldolase and junction plakoglobin) have been previously proposed as candidate PTB biomarkers [3] and other proteins are novel PTB biomarker candidates.

Conclusions
In summary, we analyzed the proteomic difference of human CVF between PTB and control group, which allowed the identification of novel CVF protein biomarkers for diagnosis of PTB. Further investigations will focus on the assessment of these biomarkers (specificity and selectivity) in a large cohort of subjects.

Novel Aspect
This study provided novel CVF protein biomarkers that have the potential to be applied in clinical diagnosis of PTB employing quantitative proteomics.

References

For information please contact: scientific@imsc2018.it
468 - ANTIBODY-FREE LC-MS METHODS FOR LOW TO SUB NG/ML QUANTIFICATION OF THE SOLUBLE RECEPTOR OF ADVANCED GLYcation END-PRODUCTS IN SERUM

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Keywords: Biomarker, Immunoaffinity enrichment, Ion-exchange, LC-MS, Quantification

Introduction:
The soluble Receptor of Advanced Glycation End-products (sRAGE) is a decoy receptor moderating pro-inflammatory signals in the lungs. For COPD, sRAGE is considered to be a promising biomarker candidate based on data obtained using antibody-based assays. Here we describe two antibody-free approaches for LC-MS-based sRAGE quantification, one using affimers and one using strong cation exchange (SCX).

Methods:
A UPLC-MRM/MS method using affimers, a novel class of affinity ligands, and a method employing SCX solid-phase extraction (SPE) were developed to enable sRAGE quantification in the range of 0.2-10 ng/mL using 50 µL of serum. Both methods were validated according to EMA and FDA guidelines and were compared with a previously developed antibody-based LC-MS method for sRAGE.[1]

Results:
Efforts to establish an affimer-based sRAGE method led to an easily automatable workflow, which is capable of analyzing hundreds of samples per week thus having considerable potential for clinical applications. SCX-based sample cleanup at pH 10 enabled sRAGE enrichment with good precision owing to the protein’s bipolar charge distribution, despite of having an average pl of 7.8. Compared to the antibody-based assay, our affimer method showed good correlation (R-squared of 0.9) and a bias around -25%, whereas the SCX assay showed moderate correlation (R-squared of 0.5) and reported on average 20% higher results. Plausible explanations for the observed differences include different subsets of sRAGE proteoforms being quantified and varying impacts of sRAGE ligands on the enrichment of this protein.

Conclusions:
We developed and validated two antibody-free LC-MS methods for sRAGE quantification. Levels reported by these assays are different from each other as well as from those reported by antibody-based assays. Thereby, these results indicate that sRAGE assays should be critically evaluated and adequately characterized in order for this protein to become useful as a biomarker.

Novel Aspect:
We present the unique applications of affimers, a novel class of affinity ligands, and strong cation exchange for low abundant protein enrichment.

References
PROTEOME ANALYSIS OF HUMAN NEUROMELANIN GRANULES AND NEURONS IN THE CONTEXT OF DEMENTIA WITH LEWY BODIES

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Keywords: Dementia with Lewy bodies (DLB), Neuromelanin (NM) granules, laser microdissection, LC-MS/MS, proteomic study

Introduction:
Neuromelanin is a dark insoluble pigment, which forms so called NM granules with lipids and proteins in the substantia nigra. It is widely debated if those granules are neuroprotective or neurodegenerative. For example, there is a loss of dopaminergic neurons containing NM during the course of DLB. A proteome analysis of NM granules and neurons could allow deeper insight into the function of these granules in the pathogenesis of neurological disorders like DLB.

Methods:
NM granules were enriched from fresh frozen DLB and control tissue by laser microdissection. A tryptic digestion was performed afterwards. The samples were analyzed by liquid chromatography tandem mass spectrometry (LC-MS/MS). MaxQuant was used for the evaluation of the MS/MS spectra. A data synchronization was conducted with Gene Ontology for further analysis.

Results:
Mass spectrometric analysis and subsequent evaluation of the data in this pilot study of four DLB and five control samples led to the identification of around 660 proteins. A significant differential study were performed to give a statement about over- and underrepresented proteins. 116 significantly (t-test <0.05) differential proteins were identified: 72 proteins overrepresented and 44 proteins underrepresented in NM granules from DLB cases. Analysis revealed that lysosomal associated proteins are significantly overrepresented and mitochondrial associated proteins are underrepresented in NM granules of DLB compared to healthy control cases. To affirm these results and increase the statistical significance, the study will be extended by 18 DLB and 20 control cases. Additionally, a simultaneously enrichment and comparison of NM granules and neurons will be performed. This could give deeper insight in the function of NM granules in the pathogenesis of DLB.

Conclusions:
The results of this pilot study support the hypothesis of the NM granules formation as described in the literature that NM granules are probably lysosome related organelle species. NM forms in the cytoplasm at physiological temperature and pH as a side product of dopamine synthesis and is incorporated by endosomes or lysosomes. Furthermore, 536 proteins were identified in all cases, which can be considered as biochemical essential composition of the NM granules.

Novel Aspect:
For the first time a protein biochemical characterization of NM granules in the context of DLB was performed.

References
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PROTEOMIC PROFILING OF MELANOMA CELL LINES AND TISSUE USING DATA INDEPENDENT ACQUISITION MASS SPECTROMETRY TO PREDICT TREATMENT RESPONSE AND PATIENT SURVIVAL

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Keywords: Melanoma, SWATH-MS, Targeted Therapy, Inhibitor Resistance, Survival Prediction

Introduction:
Melanoma is the most frequent cause of skin cancer-related deaths and is the most common lethal malignancy in young people (<40yrs). Inhibition of crucial survival pathways, such as the MAPK pathway, has become a routine in melanoma, however, response rates vary and responders cannot be reliably predicted by genotype alone. We conducted mass spectrometric screening to detect cellular processes that might predict MEK inhibition (MEKi) response.

Methods:
Ten subcutaneous lymph node metastatic melanoma derived cell lines with known MAPK status and 32 fresh frozen NRASmut or BRAFmut regional lymph node metastatic melanoma specimens were obtained. Lysed cells and tissues were digested and analyzed by Data-Independent Acquisition (SWATH-MS) using a TripleTOF 5600/6600 mass spectrometer. Cell line viability over ten days in presence of 2µM of the MEK inhibitor Selumetinib was determined by a PrestoBlue assay.

Results:
Across all cell lines, about 2500 proteins were quantifiable. Principal component analysis segregated melanomas based on in vitro MEKi sensitivity, whereas genotype alone did not. In total, 63 proteins were highly correlative with MEKi response. Survival analysis demonstrated significantly better survival (p=0.01) for patients with MEKi sensitive (>7.5 years) versus patients with MEKi resistant cell lines (<1.7 years). About 2000 proteins were quantifiable in the tissue cohort. The MEKi proteome phenotype was also a dominant signal in tissues, however, neither this phenotype nor genotype correlated directly with survival. Dividing patients into active and low or inactive melanogenic tumors and combining it with expression level of two plasma membrane proteins, allowed for accurate segregation between patients with good and poor post-biopsy survival. The three marker penal was further validated on a data set retrieved from a publically accessible data base containing RNA sequencing information of 460 melanoma specimen.

Conclusions:
Mass spectrometric profiling of patient derived melanoma cell lines using SWATH-MS led to the identification of predictive markers for MEKi therapy response. In a validation cohort using 32 melanoma tissues, SWATH-MS identified the MEKi response as a major driver of patient segregation. Additionally, a three protein marker panel was identified in the tissue cohort predictive for patient post-biopsy survival independent of genotype and MEKi response phenotype.

Novel Aspect:
SWATH-MS profiling demonstrated MEKi phenotypes in cell lines and fresh frozen metastatic melanoma and identified a marker panel predicting patient post-biopsy survival.
Introduction:
Aquaculture is one the most rapidly growing food sectors globally [1] Still, high economic losses occur due to infections, for example by the gram negative bacterium Aeromonas salmonicida as this organism causes a septicemic disease called furunculosis in freshwater fish [2]. In this study we utilized mass spectrometry based proteomics to study the physiology of this pathogen with special interest in the outer membrane vesicles that may interact with the host.

Methods:
The A. salmonicida strain JF2267 was grown in lysogeny broth liquid medium. The bacteria were harvested in the logarithmic and stationary growth phase. Cytoplasmic, inner membrane, outer membrane and extracellular proteins as well as proteins of outer membrane vesicles were enriched by subcellular fractionation and subsequently analyzed in a mass spectrometer. The resulting data were processed with the MaxQuant [3] and Perseus [4] software.

Results:
Overall, app. 50 % of the proteins predicted for this A. salmonicida strain were identified, indicating a high proteome coverage. For validation of the applied fractionation protocol the predicted cellular location (according to PSORTb [5]) and the subcellular fraction in which the proteins were identified, were compared. This comparison showed a clear enrichment of the proteins according to their prediction. Further, clear alteration between the logarithmic and stationary phase could be observed on proteome level. The changes in the fraction of the outer membrane vesicles are particularly interesting, as more than twice as many proteins were identified in the vesicles of the stationary phase compared to the logarithmic phase.

Conclusions:
In summary, in this study a very good proteome coverage could be achieved by application of a subcellular fractionation for A. salmonicida. Moreover, as expected, significant alterations were observed in the comparison of the proteome of the logarithmic and stationary phase. Interestingly, the most striking changes were detected in the vesicle fraction. Therefore, in future experiments, we will have a deeper look into the physiological role of the vesicles.

Novel Aspect:
For the first time the protein composition of the outer membrane vesicles of Aeromonas salmonicida was determined.

References
INTRODUCTION

Proteomics aims to complete profiling of the protein contents and their modifications in cells, tissues, and biofluids and to quantitatively determine changes in their abundances. This information reveals cellular processes and signaling pathways and serves to identify candidate protein biomarkers and/or therapeutic targets. Therefore, analysis should be comprehensive and efficient.

METHODS

A novel online two-dimensional reversephase/ reverse-phase liquid chromatography separation platform, using six switching valves based on a newly developed online non-contiguous fractionating and concatenating device (NCFC device).

RESULTS

In bottom-up proteomics analyses of a complex proteome, this system provided significantly improved exploitation of the separation space of the two RPs, resulting in a considerable increase in the numbers of peptides identified compared to a conventional contiguous 2D-RP/RPLC method.

CONCLUSIONS

The fully automated online 2D-NCFC-RP/RPLC system bypassed a number of labor-intensive manual processes (offline fractionations, pooling, clean-up, drying/reconstitution, and autosampler injection) required with previously described offline 2D-NCFC-RP/RPLC method, offering minimal sample loss and highly reproducible 2D-RP/RPLC experiment.

NOVEL ASPECT

The developed system was the first online 2D RP/RPLC system that NCFC technology was applied. This system allowed comprehensive proteome profiling using small amount of sample without loss of sample and their information.

REFERENCES


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Introduction
Infliximab is a chimeric IgG1 kappa monoclonal antibody, used in the treatment of inflammatory diseases targeting tumor necrosis factor-alpha (TNF). However, studies have identified a substantial proportion of patients (between 30-40%) who fail to respond to anti-TNF therapy. In this study, we describe the use of nSMOL (nano-surface and molecular-orientation limited) proteolysis to selectively target the Fab CDR region of the mAb and LC-MS/MS detection.

Methods
Plasma samples (5 μL) were initially captured using FG beads and digested with trypsin to selectively target the Fab CDR region of the mAb, followed by LC-MS/MS detection using reversed phase UHPLC separation (Nexera LC, Shimadzu Corporation using a Shimpack GISS 1.9µm 50x2.1mm C18).

Results
A novel method was developed that performed selective proteolysis of the Fab region of mABs using nano-surface and molecular-orientation limited (nSMOL) proteolysis (Iwamoto et al. 2014). The approach decreases sample complexity through mAb protein enrichment from plasma and uses proteotypic peptide sequence specificity for quantitative LC-MS/MS analysis. By restricting digestion, tryptic fragments are limited to the Fab region, allowing a selective quantification of target mAb peptides with higher efficiency and selectivity in combination with MRM measurement. Skyline software (MacCoss Lab, University of Washington) was used to perform in-silico protein digestion and predict candidate peptides and MRM transitions. SINSATHYAESVK, YASESMSGIPSR and DILLTQSPAILSVSPGER were selected based on response, linearity and accuracy.

Conclusions
Quantitation of the mAb infliximab in plasma was implemented using standard sample preparation and generic peptide chromatographic conditions. Method development using Shimpack GISS column achieved a cycle time of 8 minutes per sample. nSMOL technology in combination with LC-MS/MS can offer a rapid alternative to mAb quantitation by ELISA. Inclusion of an internal standard in nSMOL& LC-MS/MS increases accuracy and robustness not available in ELISA type assays.

Novel Aspect
Using a Fab CDR restricted trypsin digestion method, infliximab was quantified in plasma by LC-MS/MS resulting in an alternative technique compared to ELISA.

References
TOP DOWN MASS SPECTROMETRY WITH MULTIPLE MS/MS STRATEGIES TO IDENTIFY AGE-RELATED PROTEOFORM CHANGES IN TEAR FLUID.

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Keywords: biofluids, precision-medicine, top-down proteomics, UVPD, Orbitrap

Introduction:
Tear fluid is of key importance to maintain the health of the front of the eye and provide clear vision. In addition, it represents a promising body fluid that can help in the diagnosis and prognosis of various eye diseases. Here we explore the use of top-down proteomics to map the proteoform diversity in the tear fluid. The proposed work flow includes an array of top-down fragmentation techniques on a chromatographic time scale that allows for identification of over 50 proteoforms simultaneously in less than 1 hour. We then applied this workflow to a cohort of sixteen individuals with ages ranging from 18 up to 70 years old to identify molecular changes due to age

Methods:
Tear samples were purchased from Lee Biosolutions. Extracted proteins were directly transferred to an injection vial and analyzed by LC-MS using an Orbitrap Fusion Tribrid Instrument modified with UVPD. MS/MS acquisition was performed using ETD, EThC, HCD and UVPD fragmentations at a resolution of 120,000 at m/z 200. Data analysis was performed using Thermo Scientific Deconvolution 4.0 and Prosight PD nodes in the Proteome Discoverer 2.2 software. Label free analysis was done using Biopharma Finder 3.0. Statistical analysis to identify differentially expressed or processed proteoforms was done using R.

Results:
Tear fluid functions as lubrication of the eye surface, contains a cocktail of antimicrobials to prevent bacterial infections, washes away foreign matter that is deposited in the eye during our daytime activities, and keeps the eye surface clean and smooth. Tears have the potential to be a source of biomarkers for diagnosing eye-related diseases. First, it is a fluid that can easily be obtained in a non-invasive manner. Second, it is localized to the organ of interest to the ophthalmologist (the eye) and not prone to the same dilution problems associated with biomarker fluids like plasma. Third, tear fluid composition changes with the health of the eye (as well as with age).
In this work, we have coupled high sensitivity top-down mass spectrometry to enable the characterization of tear proteoforms in less than 1 h with an elegant combination of bioinformatics algorithms. Our approach consists of a simplified two-step workflow. First, samples are then analyzed over a 30 min LC gradient by MS and MS/MS using various fragmentation mechanisms. And then, MSMS are searched against a protein database. Label free quantitation is perform by aligning the raw files in the chromatographic dimension and extracting the areas under the curve for each of the identified molecular species. Intensities were then normalized and a t-test was performed to identify differential events. PCA analysis shows that samples within the age groups are more similar. And among the significant proteins in the dataset, lacritin is the one that changes most with age. This protein is involve in the promotion of basal tearing and which low levels are associated with dry eye syndrome. Overall, this strategy offers a powerful option for discovery and characterization of potential tear biomarkers that could be used for the screening of diseases, both eye related or other.
From our analyses, we have proven that tear fluid could be of great interest for understanding eye related diseases and stratifying individuals. In addition, this work also proves that top-down proteomics in clinical research presents a great opportunity to inform about the actual changes that are happening, because it enables to correlate exact proteoforms to a given phenotype. And opens the door for the development of proteoform specific assays in the clinical setting.

Novel Aspect:
Tear proteoform biomarker discovery in hours using label free proteomics and an Orbitrap Fusion Lumos.
**674 - ADVANCES IN CLINICAL PROTEOMICS FOR ANALYSIS OF THYROID FINE NEEDLE ASPIRATION BIOPSIES: EVALUATING PROTEOMIC STABILITY IN PRESERVATIVE SOLUTIONS.**

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**Keywords:** thyroid lesions; Fine needle aspiration biopsies, proteomics, cytological sample stability, mass spectra similarity index.

Introduction: Thyroid nodule lesions is one of the diseases diagnosed using liquid-based cytology [1]. Such samples are collected via fine-needle aspiration biopsy (FNAB) and deposited into preservative solutions in order to maintain their morphological integrity. However, the proteomic stability of these preserved samples is yet to be investigated and must be ascertained in order for them to be reliably employed in proteomic studies aimed at biomarker discovery.

Methods: Thyroid FNABs were collected from 14 patients (San Gerardo Hospital, Monza, Italy) and transferred into CytoLytsolution, centrifuged and re-suspended in PreservCyt solution. Cytospin spots have been positioned onto ITO-conductive slides and MALDI-MSI intact proteins analysis was performed using an ultrafleXtreme MALDI-TOF/TOF (Bruker Daltonik). Spectra pre-processing and data analysis were performed using the open-source R software v.3.4.3.

Results: Each FNAB was split into several samples in order to investigate the experimental repeatability (intra-day and inter-day) of the proteomics analysis and the cytological samples stability after 7, 14 days and 2 months in PreservCyt and after 7 days in CytoLyt at 4°C. All samples were compared with the one prepared at t0. Mass spectra similarity was evaluated by using two score systems. The score S3, derived from a previous study [2], was the sum of three components (fit, retrofit and spearman’s correlation). The second score system (S4) ranges 0–4 and includes a fourth feature that measure the overlap, which takes into account the whole shape of the two spectra. Intra-day and inter-day CV were very low within ranges of 8.64%-12.03% for S3 and of 7.37%-10.43% for S4, respectively. The results suggest no substantial deviations from t0 when the cytological samples were stored in PreservCyt until 14 days and in CytoLyt until 7days. However longer storage time (2months) in PreservCyt does not preserve the specimens, and the spectra overlap with t0 was only 50%.

Conclusions: This study represents a step forward towards the implementation of MALDI-MSI, combined with a trustworthy and robust sample preparation methodology, into the cytopathology routine, integrating the morphology with the proteomics data to improve the diagnosis. Moreover, this protocol allows simple sample collection and shipment to be used not only for the proteomic MALDI-MSI analysis of thyroid FNABs but also for other biological liquid based specimens.

Novel Aspect: A new similarity score was introduced to equally take into account the number of signals (fit and retrofit) and their intensities (spearman’s correlation and spectra overlap).

References

FUNDING: This work was funded thanks to AIRC (AssociazionItaliana per la RicercasulCancro) MFAG GRANT 2016-ID. 18445.
Introduction: VEGFA as biomarker for blood vessel formation and tumor progression
Tumor progression is often accompanied by ‘aberrant angiogenesis’, a process of blood vessel formation with a special permeable structure. Such structures lead to an adequate supply of the tumor with oxygen and nutrients [1]. Vascular endothelial growth factor A (VEGFA) is an angiogenesis stimulating factor and can therefore serve as a prognostic biomarker for several types of cancer [2, 3].

Methods: VEGFA enrichment and analysis by LC-HR/MS
Human plasma samples were mixed with isotopically labelled VEGFA protein as internal standard. Unlabeled, recombinant VEGFA was used to create a calibration curve. Samples were denatured and digested before VEGFA peptides were enriched by an immunocapture step using the KingFisherTM Flex platform. After further SPE clean up, samples were analyzed in the fullscan mode of a Q ExactiveTM HF system connected to a nanoLC system (all Thermo Fisher Scientific).

Results: Quantification of endogenous VEGFA levels from human plasma with high precision
We were able to set up and validate a robust LC-MS method for the quantification of VEGFA as a biomarker from human plasma down to 0.7 ng eq/mL (~ 1.8 fmol absolute per sample) with a CV < 10%. Since the method is based on an immunoprecipitation approach using a polyclonal antibody as capturing agent, antibody lot-to-lot variance was examined as well and found to be negligible.

Conclusions: Approach can be applied to patient samples
The newly developed LC-MS approach can be used to analyze endogenous VEGFA levels and drug treatment profiles of patient samples. As the method is semi-automated, up to 96 samples can be processed in one run.

Novel Aspect: Development of a LC-MS method for VEGFA
To our knowledge it was the first time that VEGFA was quantified from a complex plasma sample by an LC-MS assay.

References:
765 - CHARACTERIZATION OF THE MODIFICATION IN MITOCHONDRIAL PROTEOME DUE TO EIF6 DEPLETION BY SWATH-MS ANALYSIS AND MULTIVARIATE STATISTICAL TOOLS

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Keywords: eIF6; mitochondrial proteome; shotgun proteomics; multivariate statistical analysis; biomarker identification

Introduction:
Eukaryotic Initiation Factor 6 (eIF6) binds 60S ribosomal subunits, has with an anti-association property, and rate limiting for tumour onset and progression. eIF6 haploinsufficient cells are normal, but not efficiently transformed in vitro. Some lines of evidence have shown that alterations in mitochondria (the main compartments of energy production) contribute to the development of metabolic syndrome.

Methods:
The mitochondrial proteome of AML-12 (non-tumourigenic murine liver hepatocytes) cell line, with eIF6 down-regulated by shRNA, and of liver tissues from wild type and +/- mice for eIF6 has been analysed by uHPLC-QTOF-MS/MS exploiting the SWATH-MS acquisition, which is a high throughput label-free method for protein quantitation combining shotgun proteomics with the quantitative accuracy and reproducibility of selected reaction monitoring (SRM).

Results:
The data collected were further processed by multivariate statistical tools to identify the effects of eIF6 depletion. Partial least squares-discriminant analysis (PLS-DA) coupled to a variable selection procedure in cross-validation was applied to this purpose, in order to identify the most relevant panel of markers with the best predictive ability. The analysis allowed the identification of panels of markers with a very good predictive ability. Bioinformatic tools applied to the identified markers allowed the identification of deranged pathways.

Conclusions
We found that depletion of eIF6 by shRNA induces profound and varied impact on mitochondrial proteome, impairing the energy production, steering the metabolism toward the up-regulation of aerobic glycolysis and the inhibition of oxidative phosphorylation.

Novel Aspect:
We identified interesting deranged pathways in the mitochondrial proteome after eIF6 depletion.
800 - IDENTIFICATION OF PROTEOMIC AND LIPIDOMIC MARKERS IN INFLAMMATORY BOWEL DISEASES BY MS-BASED TECHNIQUES AND MULTIVARIATE STATISTICAL ANALYSIS

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Keywords: Inflammatory bowel diseases; proteomics; lipidomics; multivariate statistical analysis; biomarkers identification

Introduction:
The causes of Inflammatory Bowel Diseases (IBDs) are still little known, the prevailing hypothesis being an immunologic reaction towards antigens. The diagnosis of IBDs is usually carried out by quite invasive instrumental techniques; some plasma biomarkers have been proposed, but much can be done to clarify the etiology of the pathology and identify reliable biomarkers with predictive ability.

Methods:
Plasma proteins and fatty acids from controls and patients affected by Chron’s disease (CD) and Ulcerative Colitis (UC) were analysed using mass spectrometry-based techniques. Low-abundant plasma proteins were quantified after depletion of the 14 most abundant and tryptic digestion, followed by uHPLC-QTOF-MS/MS. Several fatty acids were simultaneously analysed by Gas Chromatography Mass Spectrometry (GC-MS), after derivatization.

Results:

uHPLC-QTOF-MS/MS was applied exploiting the SWATH-MS acquisition, a high throughput label-free method for protein quantitation combining shotgun proteomics with the quantitative accuracy and reproducibility of selected reaction monitoring (SRM). The data collected were further processed by multivariate statistical tools to identify markers of CD and UC but also identify markers able to discriminate UC vs CD samples. Partial least squares-discriminant analysis (PLS-DA) coupled to a variable selection procedure in cross-validation was applied to this purpose, in order to identify the most relevant panel of markers with the best predictive ability. Proteomic and lipidomic data were also integrated to provide new combined biomarkers including both proteomic and lipidomic markers. The analysis allowed the identification of panels of markers with a very good predictive ability and pointed out the importance of including fatty acids.

Conclusions

The combination of proteomic and lipidomic data allowed to obtain panels of biomarkers with improved predictive ability, pointing out the importance of fatty acids, combined with proteins, in the identification of IBDs. Bioinformatic tools applied to the panels of identified markers allowed the identification of deranged pathways (up and/or down regulated).

Novel Aspect:
The combination of proteomic and lipidomic data provided panels of markers with improved predictive ability with respect to the only proteomic analysis.
Introduction:
Spinal cord injury (SCI) belongs to currently incurable disorders of the CNS and is accompanied by permanent health consequences—disability. In order to mimic a SCI, a balloon-compressive technique was used at thoracic Th8-9 spinal level in adult rat.

Methods:
4D MALDI Imaging, lipidomics, Saptiotemporal tissue microproteomics were undertaken combined with confocal imaging. Exsosomes from Stem Cells, functionalized biomaterial, Rho A inhibitor have been tested in pre-clinical way to develop a smart biomaterial.

Results:
We determined the spatio-temporal events occurring in acute phase after SCI. Caudal segment has clearly been detected as the therapeutic target [1,2]. We then assessed in a rat SCI model the in vivo impact of a sustained RhoA inhibitor administered in situ via functionalized-alginate scaffold [3,4]. In order to decipher the underlying molecular mechanisms involved in such a process, an in vitro neuroproteomic-systems biology platform was developed in which the pan-proteomic profile of the dorsal root ganglia (DRG) cell line ND7/23 DRG was assessed in a large array of culture conditions using RhoAi and/or conditioned media obtained from SCI ex-vivo derived spinal cord slices. A fine mapping of the spatio-temporal molecular events of the RhoAi treatment in SCI was performed. The data obtained allow a better understanding of regeneration induced above and below the lesion site.

Conclusions
Results notably showed a time-dependent alteration of the transcription factors profile along with the synthesis of growth cone-related factors (receptors, ligands, and signaling pathways) in RhoAi treated DRG cells and involvement of IgG by binding to their receptors on the DRG cells

Novel Aspect:
We established a novel origin of IgG, their role in neurites outgrowth modulation, and developed a smart biomaterial for treating SCI.

References:
LABEL-FREE PROTEOMIC ANALYSIS OF URINARY EXOSOMES FROM PATIENTS WITH CC-RENAL CELL CARCINOMA: STAGE-SPECIFIC DIFFERENTIAL PROTEIN PROFILE

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Keywords: ccRCC, Urinary Exosomes, Proteomics, Label-free, Biomarkers

Introduction:
RCC represents 2-3% of all cancers and its incidence is increasing [1]. Clear cell RCC (cc-RCC) is the most frequent type. Although ccRCC are diagnosed at early stages, their aggressiveness and clinical outcomes result heterogeneous within each staging group; thus, novel predictors are needed. A non-invasive test using urine will have a significant impact on patient management. Urinary Exosomes (UE) are proposed as a useful biomarkers source for ccRCC.

Methods:
The aim of this study is to highlight a characteristic UE protein profile of RCC progression. UE pools obtained from patients with cc-RCC classified as pT1 (n=6) and pT3 (n=6), and from age- and sex-matched healthy subjects (n=6) were isolated by ultracentrifugation. The corresponding proteome of each sample was digested using the FASP-based method and analysed by label-free nLC-ESI MS/MS.

Results:
The UE proteome of patients with ccRCC (low- and high-stage) was investigated by mass spectrometric approach following correlation of the data with clinical features (TNM) [2]. The MS analysis allowed the identification and quantification of about 500 proteins. Considering only the protein identification, the data show that the UE protein composition remained rather constant in the three groups (pT1, pT3 and CTRLs). However, by evaluating the protein abundance, 230 had a statistically significant variation. The pathway network elaboration revealed that many typical urinary system proteins changed their levels in UE. Furthermore, the results shown that some proteins involved in signaling pathways change their abundance, increasing disease stage. This finding suggest a possible relationship of these quantitative variations with the process of tumour dedifferentiation and spread. Finally, a panel of proteins were selected in order to validate their differential content between ccRCC and Ctrl UE, through immunoblotting.

Conclusions:
UE comparative proteomics could represent a promising starting point for the identification of ccRCC biomarkers for prognostic purposes. The data obtained may be a useful finding for the future investigation of the molecular mechanism of ccRCC progression. More important, with the prospective validation in a larger cohort, information derived from this study may lead to the development of ccRCC candidate biomarkers.

Novel Aspect:
In this work, for the first time, the proteomic characterization of UE in patients affected by ccRCC at different stages is approached.

References

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534 - MAXIMIZED THROUGHPUT AND ANALYTICAL DEPTH FOR SHOTGUN PROTEOMICS USING PASEF ON A TIMS EQUIPPED QTOF AND A NOVEL LC SYSTEM

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Keywords:timsTOF Pro, PASEF, Evosep One High throughput, Clinical Research

Introduction:
The achievable depth in high throughput proteomics analyses is most often limited by the sequencing speed of mass spectrometers, while the nano-LC turnover rate limits the sample throughput. Here we combine a trapped ion mobility spectrometry (TIMS) QTOF capable of Parallel Accumulation Serial Fragmentation (PASEF) acquisition to a front end allowing efficient use of short gradients, the Evosep One to achieve, high depth at high throughput analysis.

Methods:
50ng of a peptide digest of a Hela cell line were separated on an Evosep One (Evosep) and analyzed on a timsTOF Pro instrument (Bruker Daltonics). PASEF acquisition allowed for very high sequencing speed at high sensitivity. HeLa digests were separated on very short gradients of 11.5 and 5.6 min corresponding to 100 and 200 samples/day, respectively. Data were analyzed using PEAKS studio (Bioinformatics Solution Inc.) and MaxQuant (Max-Planck-Institute of Biochemistry).

Results:
The use of short nano-LC gradients reinforces the need of a fast MS instrument to guarantee reasonable analytical depth. TIMS provides a further dimension of separation in addition to the retention time and m/z ratio that increases peak capacity, and it releases the ions from the TIMS cell as concentrated ion packets. By applying the PASEF scan mode on those packets we obtain almost 100% duty cycle, increased sequencing speed (> 100Hz) and high sensitivity leading to a deep coverage of the proteome over 4 orders of magnitude dynamic range. To test if we can profit from the high speed and sensitivity of PASEF we set up very short gradients on the Evosep system with a total runtime of 14.0 min (11.5 min gradient) and 7.1min (5.6 min gradient), resulting in 100 samples/day and 200 samples/day, respectively. Evotips were loaded with only 50 ng of HeLa trypic digest and analyzed. Using the 200 sample per day turnover we found more than 1200 protein families and 8000 peptide matches.

Conclusions
Our results demonstrate that PASEF on the timsTOF Pro coupled to the Evosep One enables fast and robust analysis of large sample numbers with potential applications for high throughput screening and clinical research.

Novel Aspect:
High Depth, High Throughput Label-free proteomics for clinical research.
Targeted quantitation of proteins for discriminating obese from normal-weight adolescents by liquid chromatography-mass spectrometry

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Keywords: protein quantification, stable isotope-labeled (SIL) peptide, LC-MS/MS, protein biomarker, multiple reaction monitoring (MRM)

Introduction:
Targeted quantitation of proteins based on isotope-labeled synthetic peptides with multiple reaction monitoring (MRM) LC-MS/MS has been reported as one of the important techniques. The aim of the present study was to select unique peptides of target proteins and to determine the concentration of the target peptides with LC-MS/MS in human plasma to compare between obese and non-obese subjects.

Methods:
The process began with a list of target proteins that identified by Tandem mass tag (TMT) strategy in the discovery phase. The next step was to select tryptic peptides and transitions of peptides after collision energy optimization using isotope-labeled peptides with Skyline software. Then, peptides were to analyze the samples by MRM LC-MS/MS method and to integrate and to evaluate the data.

Results:
Using detectability test of the endogenous peptides, we selected 100 representative target peptides from the 39 proteins. Stable isotope labeled internal standard peptides for 39 proteins have been synthesized. The concentration of the internal standards for each peptide was spiked to the sample was obtained by level of the unlabeled peptides in the plasma and randomized samples (n=60) were analyzed in triplicate by MRM LC-MS/MS. Relative quantitation of the peptides was carried out by monitoring the ratio of heavy-to-light peptide. Intra-day and inter-day precision of stable isotope-labeled peptides showed less than 15.62%. P value and AUC of the peptides were calculated with Medcal software and AUC of 12 peptides (9 proteins) that represented below 0.05 is showed below 0.0417.

Conclusions
100 Target peptides for the 39 proteins that distinguishing between obese and non-obese subjects were quantified and assessed by MRM LC-MS/MS in human plasma. This method is sensitive, robust and fit for purpose to verification of peptides. Finally, 60 human samples with obese or non-obese were successfully analyzed using our MRM method.

Novel Aspect:
Fully integrated MRM LC-MS/MS quantitation approach of targeted proteins to compare obese and non-obese subjects.

References
85 - DEVELOPMENT OF A SANDWICH ELISA FOR THE THROMBIN LIGHT CHAIN IDENTIFIED BY SERUM PROTEOME ANALYSIS

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Keywords: pancreatic cancer, biomarkers, serum, TMT, early detection

Introduction:
Pancreatic ductal adenocarcinoma (PDAC) accounts for 85–90% of all pancreatic tumours. The median survival of all PDAC patients is less than 6 months, and the 5-year-survival rate is 3–5%[1]. One of the most crucial reasons for the poor prognosis is the lack of early diagnostic markers for PDAC. To overcome this and improve the outcomes of patients with PDAC, there is an urgent need to identify highly sensitive and specific markers for early detection.

In the present study, we employed the TMT method to generate comparative protein profiles of sera samples obtained from pre- and postoperative PDAC patients. Furthermore, we compared the serum levels of candidate proteins to evaluate the ability to discriminate between PDAC and healthy controls. Serum CA19-9 cannot discriminate the PDAC from other diseases such as pancreatitis and major gastroenterological cancers including biliary tract cancer (BTC).

Methods:
Using tandem mass tag labelling and LC-MS/MS, we performed comparative analyses of pre- and postoperative sera from PDAC patients to identify specific serum biomarkers for PDAC. In validation studies, we evaluated the discriminatory power of candidate proteins.

Results:
Among the 302 proteins analysed, 20 were identified as potential biomarkers, with C4b-binding protein α-chain (C4BPA) and polymeric immunoglobulin receptor (PIGR) being selected for further analysis. The sera levels of C4BPA and PIGR were significantly higher in the preoperative PDAC patients than in the postoperative ones (P<0.008, P<0.036, respectively). In addition, serum C4BPA levels, but not PIGR, in patients with PDAC were significantly higher than those in healthy controls as well as in patients with pancreatitis and other malignancies including biliary tract cancers (BTC) (P<0.001). The respective area under the receiver operator characteristics (ROC) curve (AUC) was 0.860 for C4BPA, 0.846 for CA19-9 and 0.930 for the combination of C4BPA and CA19-9 in PDAC vs non-cancer individuals. The respective AUC was 0.912 for C4BPA, 0.737 for CA19-9 in Stages I and II of PDAC, 0.854 for C4BPA and 0.264 for CA19-9 in PDAC vs BTC.

Conclusions:
We have demonstrated that C4BPA is a novel serum biomarker for detecting early stage PDAC, as well as for distinguishing PDAC from other gastroenterological cancers. Further analysis in large cohort studies will warrant C4BPA as a promising biomarker of PDAC in clinical use.

Novel Aspect:
C4BPA is a novel serum biomarker for detecting early stage PDAC.

References:

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USE OF THE MALDI BIOTYPER SYSTEM WITH MALDI-TOF MS FOR RAPID IDENTIFICATION OF MICROORGANISMS CAUSING BACTERIAL URINARY TRACT INFECTION FROM URINE SAMPLES

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Keywords: direct identification, LUTD, MALDI-TOF MS, rapid BACpro, urine

Introduction:
With the increasing number of cats maintained as pets, the opportunities to treat cats with lower urinary tract disease have recently increased in the clinical veterinary field. Several studies have reported urinary tract infection of felines with LUTD accounting for 2–10% of cases [1]. We performed direct identification of bacteria in urine using pretreatment kits for the direct application of positive blood culture bottles to MALDI-TOF MS, aiming to improve the low identification rates of E. faecalis and 2-morphology colony types.

Methods:
Urine samples collected from 50 cats with bacterial cystitis brought to Maeda Veterinary Hospital between August 10, 2015 and March 31, 2017 were used in the study. Sample preparation of the urine was performed using the MALDI Sepsityper kit and rapid BACpro. To identify the isolates, MALDI-TOF MS was performed on the AutoFlex TOF/TOF mass spectrometer.

Results:
MALDI-TOF MS seems to require high bacterial counts to be able to provide reliable scores. Experimental inocula with at least 1.0 × 10^5 CFU/mL were required to obtain reliable scores for E. coli by MALDI Sepsityper Kit and rapid BACpro, and the required inocula were even higher for E. faecalis by MALDI Sepsityper Kit (5.0 × 10^6 CFU/mL) and BACpro (1.0 × 10^5 CFU/mL).

Of the 50 urine specimens, the growth of colonies was observed in 37 specimens: 29 specimens with single colony morphology, 8 specimens with two colony morphology. Thirteen specimens did not grow in culture, and MALDI-TOF MS did not identify any significant protein profile in any of these cases.

Single colony morphology correctly identified 25 isolates (86.2%) using the MALDI Sepsityper Kit and 25 isolates (86.2%) with the rapid BACpro. In 4 cases in which identification was not possible, the bacterial count was 1.0 × 10^5 CFU/mL or lower, which was below the detection limit of sensitivity. In the specimens with two-colony morphology, the MALDI Sepsityper Kit identified only one species (Citrobacter freundii, Enterobacter cloacae, Klebsiella pneumoniae). The rapid BACpro was capable of identifying 2 species in 7 urine specimens. It was not able to identify any bacterium in one case, in which E. faecalis and E. cloacae were present, and the E. cloacae count was 1.0 × 10^5 CFU/mL, which was below the detection sensitivity.

Conclusions:
The MALDI-TOF MS method employing the rapid BACpro for pretreatment is a quick and reliable method for the identification of bacteria from infected urines, whose shortened analysis time enables an earlier and more accurate selection of antibiotics for feline LUTD treatment.

Novel Aspect:
The identification of bacteria by the MALDI-TOF MS method using the rapid BACpro for pretreatment is possible when the bacterial level is 1.0 × 10^5 CFU/mL or higher.

References:

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INTRODUCTION

Adipose tissue inflammation is a well-observed consequence of obesity and a prelude of the metabolic syndrome. It may result from infiltrating immuno-inflammatory cells in adipose tissue, which disturbs metabolic homeostasis and initiates low-grade systemic inflammation. Obesity-induced lipid spillover in adipocytes provokes an aberrant adipokine secretory profile, resulting in the release of increased pro-inflammatory mediators such as cytokines and signaling lipid derivatives. Arachidonic acid is the main source of lipid derivatives (i.e. prostaglandins, thromboxanes, leukotrienes, resolvins, protectins and epoxyeicosatrienoic acids), that mediate inflammation. Different enzymatic conversions downstream of arachidonic acid, i.e. cyclooxygenase (COX), lipoxygenases (LOX) and cytochrome P450 epoxygenases (P450), yield specific lipid derivatives. The correlation between adipose tissue levels of these lipid derivatives and the metabolic healthy or unhealthy phenotype of the obese subjects is not clear [1]. Moreover, it is unclear to what extent circulating lipids reflect the inflammatory state of the diseases adipose tissue [2]. We hypothesize that we can determine the diagnostic value of these molecular lipids as biomarker for adipose tissue inflammation through the identification of aberrant local lipid expressions in diseased adipose tissue and subsequently connecting these to circulating lipid levels. Therefore, this project aims to analyze circulating pro-inflammatory lipids and oxylipins that reflect adipose tissue inflammation. The topological distribution of the molecular lipid profile in adipose tissue will be analyzed with mass spectrometry imaging (MSI), whereas circulating lipids in matched patient plasma samples will be analyzed with LC-multiple reaction monitoring-(MRM) MS.

METHODS

Matched adipose tissue and plasma samples of 10 lean- and 10 obese metabolic healthy subjects were included into this study. For MSI experiments, fresh frozen adipose tissue biopsies were cryosectioned at 10 µm thickness slides. Each cryosectioned biopsy was split into three different slides randomly to minimize batch bias effects. A primary MALDI-MSI analysis on human adipose tissue was performed on a RapifleX MALDI Tissuetyper system (Bruker Daltonik GmbH, Bremen, Germany) in both positive and negative ionization mode. Representative tissue sections with aberrant lipid expression profiles from the primary screening were subsequently analyzed with high mass resolution imaging on an Orbitrap Elite mass spectrometer (Thermo Fisher Scientific, Bremen, Germany). Data analysis was performed as described previously [3].

A UPLC-ESI-MRM targeted mass spectrometry assay for oxylipins was developed on a Xevo TQ-S triple quadrupole, in negative ESI mode, coupled to an ACQUITY UPLC system using a Waters ACQUITY HSS PFP, 2.1x100 mm, 1.8 µm as analytical column (Waters Corporation, Milford, Massachusetts, USA). COX metabolites were obtained from Cayman Chemicals (Ann Arbor, Michigan, USA) to assess the method’s performance, sensitivity and specificity by plasma spike-in assays with mixed mode solid-phase extraction (SPE) using Oasis MAX µElution plates (Waters Corporation, Milford, Massachusetts, USA). Heavy labeled COX metabolites were used as reference standards in patient samples. Sample preparation and SPE conditions were performed as described previously [4]. Data analysis was performed with TargetLynx V.4.1 (Waters Corporation, Milford, Massachusetts, USA).
Results
Lean and obese subjects were well defined based on anthropometric parameters (e.g. BMI and total body fat mass), biochemical analysis (e.g. plasma glucose, insulin and C-reactive protein) and markers of adipose tissue inflammation (e.g. IL-6, IL-8, number of macrophages and crown-like structures). Topological distribution of the COX-, LOX- and P450 metabolites in adipose tissue is being determined. Subsequently, aberrant lipid profiles in adipose tissue will be analyzed with existing metadata of individual subjects to infer the biological relevance of these metabolites. This holistic approach will indicate those relevant oxylipin profiles that are related to adipose tissue inflammation, which are then selected for targeted oxylipin MRM approach in circulating plasma samples.

MRM methods using specific transitions were developed for analysis of fourteen COX metabolites individually (i.e. 6 keto-Prostaglandin F1α, Thromboxane B2, 11β-Prostaglandin F2α, Prostaglandin F2α, 11β-13,14-dihydro-15-keto PGF2α, Prostaglandin E2, Prostaglandin D2, 13,14-dihydro-15-keto Prostaglandin E2, 13,14-dihydro-15-keto Prostaglandin D2, 15-keto Prostaglandin E2, Prostaglandin J2, 15-deoxy-Δ12,14-PGD2, 15-deoxy-Δ12,14-Prostaglandin J2 and Arachidonic Acid). MRM methods show good linearity across at least two magnitudes of standard (R2 >0.97). Purification by SPE was further optimized to extract spiked COX metabolites standards (unlabeled and heavy labeled) in human plasma.

We currently perform assay validation of the MRM assay prior to analysis of patient samples. This pilot study of 10 lean and 10 obese subjects will be evaluated first prior to analysis of all lean (n=35) and obese (n=37) subjects and type 2 diabetic (T2D) patients (n=33). The T2D patients will be included as the metabolic unhealthy subjects.

Conclusions
This approach will allow to identify aberrant lipid expressions in diseased tissue and its connection to circulating lipid levels, which indicates the diagnostic value of these molecular lipid profiles as biomarker for adipose tissue inflammation.

Novel Aspect
The unique availability of matched patients samples (viz. adipose tissue biopsies and plasma) enables biomarker research for adipose tissue inflammation. This holistic approach that combines complementary mass spectrometry approaches will strengthen personalized biomarker research. This is the first study that combines MALDI-MSI with LC-MRM-MS to analyze molecular characteristics and location of lipid profiles in the adipose tissue and how this relates to the circulating lipid profiles per individual.

References
639 - LC-MS/MS-BASED CHARACTERIZATION OF GlioBLASTOMA CELL RESPONSES TO TYPE I INTERFERONS FOR ESTABLISHING DEFECTS IN ANTIVIRAL MECHANISMS

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Keywords: glioblastoma, interferon signaling, oncolytic viruses, LC-MS/MS-based proteomics, label free quantitation

Introduction:
Therapy of glioblastoma multiforme (GBM) is challenging due to specific property of glioblastoma stem cells (GSC) to penetrate far from the tumor localization, thus, avoiding therapeutic actions. However, experimental therapy using oncolytic viruses have demonstrated ability to kill GSC and cause stable remissions. In this work, we studied specific defects in GBM antiviral mechanisms, enabling success of virus therapy.

Methods:
8 GBM cell cultures treated with interferon α,β were analyzed using Orbitrap Fusion Lumos mass spectrometer coupled with UltiMate 3000 nanoflow LC (Thermo Scientific). Protein identification, post-search validation and label free quantification were performed using X!Tandem [1] and MPscore [2], respectively. Statistical analyses of differential protein regulation followed by GO analysis were implemented using python-based libraries and GOrilla [3].

Results:
Tests for GBM cells sensitivity to vesicular stomatitis virus Indiana strain (VSV) have revealed 5 GBM cultures developing resistance to virus infection due to pre-treatment with interferon α or β (IFNα, IFNβ). Other GBM cultures have either developed resistance after pre-treatment with IFNα or kept sensitivity to the virus regardless of IFNα and IFNβ treatment. Based on these results, 5 GBM cultures were combined into the group with intact type I IFN signaling, while the other 3 cultures were defined as cultures with broken (at least partially) type I IFN signaling. Proteomics-based analysis of untreated and IFN treated GBM cells followed by analyses of differentially regulated protein products of IFN stimulated genes (ISGs) and biological process enrichments have revealed that GBM cells with intact IFN signaling respond to IFNα and IFNβ treatment by enhanced production of ISGs proteins with antiviral properties. The number of ISGs proteins is considerably less in the group with broken type I IFN signaling.

Conclusions
Results of proteomic analysis of GBM cell responses to IFNα and IFNβ treatment supported the results of VSV sensitivity tests. In our data, a molecular signature of significantly upregulated IFIT1/2/3, OAS1/2/3/L, MX1/2 proteins was a marker of developed resistance to VSV infection. We assume inactivation of STAT1 and STAT2, the key components of IFN signaling; can be responsible for the broken antiviral defense in GBM cells.

Novel Aspect:
The interactions between GBM cells, GSC and oncolytic viruses as well as the role of IFN signaling for virus therapy are the studies of primary interest, promising novel and effective therapy against glioblastoma.

References:

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1202 - EPITOPE IDENTIFICATION OF GLYCATED HSA AND THE EXTRACELLULAR REGION OF HUMAN RAGE BY SPR AND AFFINITY-MS SPECTROMETRY

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Keywords: VC1-RAGE, glycated human serum albumin, affinity-mass spectrometry, epitope identification, surface plasmon resonance

Introduction
In the pathogenesis of diabetic complications, the interaction between the receptor for advanced glycation end products (RAGE) and its ligands AGEs is evident: however AGE structural properties and the interaction mode is still unknown [1]. Aimed at clarifying AGE structural requirements, the study intended to characterize the binding of VC1 (RAGE ectodomain) and commercial glycated human serum albumin (HSAgly) to gain new insights on AGE-RAGE interaction.

Methods
HSAgly and HSA were covalently immobilized onto carboxymethyl-dextran surface and the binding with recombinant VC1 was investigated by means of surface plasmon resonance (SPR) [2]. Then, an affinity column was prepared by immobilizing VC1 on CNBr activated sepharose beads for VC1–HSAgly interaction studies by MALDI-ToF mass spectrometry (MS). Epitope extraction MS [3] on HSAgly tryptic digestion was performed to identify HSAgly peptides involved in the binding.

Results:
Two surfaces of HSAgly and HSA were prepared via amine coupling chemistry and a SPR-based assay was used to verify the selectivity of the binding between VC1 and HSAgly in comparison with the unglycated isoform. VC1-HSAgly affinity analyses were carried out and a 1:1 binding model was used to extrapolate the equilibrium dissociation constant of the complex (KD). The binding constant (KD = 4.9 M) showed a moderate affinity of VC1 for HSAgly and allowed to make some consideration on the strength of such interaction. Furthermore, a VC1-based affinity column was prepared and VC1-HSAgly interaction was confirmed by MALDI-ToF MS. HSAgly was tryptic digested and analyzed by ESI-Q-ToF and MALDI-ToF MS in order to characterize the glycation sites on the protein scaffold. Epitopes extraction method allowed the isolation and the characterization of HSAgly peptides involved in the interaction. The analysis of the model structure showed that they belong to discontinuous epitope included in domain I and sub-domain IIA of the protein.

Conclusions
The results of this study enabled the assessment of VC1-HSAgly interaction by affinity-MS. VC1 showed to well discriminate between glycated and unglycated HSA confirming that HSAgly acts as VC1 binder. The determination of the affinity binding constant and the structural identification of epitope involved in such interaction provided a reliable characterization of commercial HSAgly as marker for AGE-RAGE interaction studies.

Novel Aspect
The affinity-MS approach for identification of epitopes and affinity quantification of the complex provided key information on the glycation sites involved in RAGE binding.

References
THE MOLECULAR PATTERN OF PLASMA PROTEINS AND THEIR CORRELATION TO PAIN INTENSITY AND SENSITIVITY IN WOMEN WITH FIBROMYALGIA – A MULTI-CENTER STUDY

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Keywords: Proteomics, chronic pain, plasma, multivariate statistics, fibromyalgia

Introduction:
The complete knowledge of the molecular mechanisms behind fibromyalgia (FM) is still lacking and few studies have analyzed the peripheral molecular pattern in women with FM. The primary aim of this study was to investigate and compare the plasma proteome profile between women with FM and healthy controls (CON). Secondly, to investigate the correlation between plasma protein patterns and pain intensity (Visual analogue scale, VAS) and pressure pain thresholds (PPT).

Methods:
Clinical variables and background data were collected and PPTs were recorded using an electronic algometer. Plasma proteome profile from women with FM (n=30) and CON (n=32) was analyzed by 2-DE combined with MS. The 2-DE results were analyzed with Principal Component Analysis (PCA) and Orthogonal Partial Least Squares – Discriminant Analysis (OPLS-DA) models. Correlation between plasma proteins and pain intensity, pain sensitivity was analyzed in FM using OPLS.

Results:
As expected significant differences were found between FM and CON in the clinical variables Fibromyalgia impact questionnaire (FIQ), VAS, PPT, hospital and anxiety depression scale (HADS) and pain catastrophizing scale (PCS). The OPLS-DA model, identified 27 plasma proteins as being important for the group separation between FM and CON. In the OPLS model of pain intensity, 12 proteins were multivariately correlated with pain intensity in the FM group; eight of 12 proteins were associated with higher pain intensity score (VAS>50 mm). In the OPLS model of pain sensitivity in FM, 11 proteins multivariately correlated with PPT; three out of 11 proteins were associated with very low pain sensitivity score (<200 kPa).

Conclusions:
In this cohort prominent differences exist in plasma protein patterns between FM and CON. Several plasma proteins correlated with pain intensity and sensitivity and these proteins needs to be further identified to confirm their role in FM. Combining clinical and proteomic data with multivariate statistics, potential biomarkers could be identified that can lead to improved clinical assessments, treatments and eventually new pharmacological therapies for FM.

Novel Aspect:
Plasma proteomic generate large data set, which in combination with multivariate statistics, is an important tool for identifying potential biomarkers associated with FM.
133 - THERAPEUTIC PROTEINS IN SPORTS DRUG TESTING: DETECTION OF DIFFERENT ACTRII INHIBITORS IN SERUM BY MEANS OF (IMMUNO-)AFFINITY PURIFICATION, TRYPTIC DIGESTION, AND LC-HRMS

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Keywords: Doping, ActRII-Fc fusion proteins, Therapeutic antibodies, Affinity purification, LC-HRMS

Introduction:
Therapeutic proteins represent an emerging class of pharmaceuticals comprising several drug candidates with potential performance-enhancing properties. Especially inhibitors of the ActRII signaling pathways possess the potential for being misused as doping agent in sports as they were found to have positive effects on muscle mass, bone formation, and red blood cell production[1, 2].

Methods:
Ammonium sulfate precipitation, (immuno-)affinity purification, tryptic digestion, and LC-HRMS were employed to develop targeted detection assays for the therapeutic antibody Bimagrumab and the ActRII-Fc fusion proteins Sotatercept (ActRIIA-Fc) and Luspatercept (modified ActRIIB-Fc) in doping control serum samples.

Results:
Three complementary assays for the detection of different ActRII inhibitors were successfully developed by using different proteomic techniques [3, 4]. The methods were comprehensively characterized and found to be fit for purpose for doping control routine analysis. In addition to LC-HRMS, ion mobility was employed to facilitate the unambiguous identification of the target peptides. For Bimagrumab, clinical samples were analyzed to demonstrate the applicability of the approach to authentic serum specimens.

Conclusions:
Mass spectrometric assays for the detection of emerging protein drugs are of great value for both the pharmaceutical industry and preventive doping research. The presented assays will expand the range of available tests and can readily be modified to include other therapeutic fusion proteins and antibody-based drugs.

Novel Aspect:
Although no ActRII inhibitors have obtained clinical approval yet, LC-HRMS detection methods for three candidates were proactively developed.

References:
A NOVEL HIGH-THROUGHPUT ANTIBODY-FREE LC-MS/MS ASSAY TO QUANTITATE CHROMOGGRANIN-A IN SERUM

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Keywords: Tumor biomarker; diagnostics; clinical mass spectrometry; proteomics

Introduction:
Chromogranin-A (CgA) is a protein produced in the secretory granules of neuroendocrine tissue, with increased plasma levels being indicative of neuroendocrine tumors (NET)[1]. Currently, CgA is measured using various immunoassays [2]. However, limitations arising from non-specific binding and a reduced dynamic range pose hurdles in implementing such tests in clinical laboratories. Here we describe a novel, antibody-free LC-MS/MS assay to quantitate CgA in serum.

Methods:
Exploiting the acidic properties of CgA, 100 μL of serum is extracted using an anion exchange solid-phase extraction plate followed by addition of internal standard (IS). The extracted sample is concentrated, enzymatically digested, and a unique peptide to CgA is chromatographically resolved and analyzed by SRM on a Sciex 6500+ QTrap mass spectrometer. The ratio of the analyte peak area to the isotopically labeled IS peak area is used to achieve quantitation.

Results:
Chromogranin-A showed linearity across a wide range (50-50,000 ng/mL, R2=0.99), as well as inter- and intra-assay reproducibility (CV 5.2-15.7%). Analytical sensitivity and limit of quantitation were determined to be 35.5 and 50 ng/mL, respectively. This method has no cross reactivity with interfering substances, and shows sample stability up to 5 days at room temperature, up to 14 days at 4C, and up to 31 days at -20C. Samples from 308 patients were analyzed to compare CgA serum values measured by the Cisbio CGA-ELISA-US immunoassay with values from our LC-MS/MS assay. After natural logarithm-transformation of the measurements, a normal distribution was seen in the 308 patients. A paired t-test was performed on these data and the concordance between the assays was measured by the Pearson correlation coefficient and Passing & Bablok curve fitting (R2=0.76; -0.25 + 1.03x).

Conclusions:
We have developed and validated a fully automated LC-MS/MS assay for quantitating chromogranin-A out of serum. Unlike immunoassays, our workflow is not dependent on capture and detection antibodies, and therefore structural changes in CgA due to denaturation, aggregation, or post-translational processing will have no impact on detection.

Novel Aspect:
This is a first-of-its-kind LC-MS/MS assay to detect and quantitate chromogranin-A out of serum.

References
A.02 LIFE SCIENCES - QUANTITATIVE PROTEOMICS

516 - OPTIMIZATION OF BIOTIN ENRICHMENT PROTOCOL AND ITS APPLICATION FOR THE ANALYSIS OF ORGANELLE-ENRICHED PROTEINS BY LC-MS/MS

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Keywords: BioID, Affinity Enrichment, Organelle, MS

Introduction:
The strong interaction of biotin and avidin is widely used for the enrichment of proteins from complex mixtures. The recovery of biotinylated proteins and the discrimination between unspecific binding and specifically enriched proteins remain major challenges. We compared different strategies for the purification of biotin-enriched proteins followed by MS analysis and applied them to the analysis of organelle proteomes.

Methods:
Equal amounts of heavy control and light stably BioID [1] transfected SILAC cells were combined, lysed and processed with 4 methods (three replicates): 1,2: Binding to beads followed by digestion and off-gel fractionation using 2 sets of washing buffers [2]; 3. Digestion prior to biotin capture [3]; and 4. Elution from the beads and in-gel digestion [4]. Organelle proteomes were enriched using different BioID constructs. Samples were analyzed using LC-MS/MS.

Results:
Using unbiotinylated 3plex SILAC labeled HEK cells (in two replicates), a significance threshold for biotinylation (95% of proteins) was calculated. Peptides with experiment/control ratios above this threshold were considered to be specifically enriched and therefore biotinylated. On bead digestion followed by offgel fractionation resulted in identification of 3165 and 3006 proteins on average for 2 different sets of washing buffers (82.3% and 81.6% biotinylated). Incubation of peptides with beads yielded on average in 173 proteins identifications (64% biotinylated) while elution of biotinylated proteins followed by in gel digestion resulted on average in 711 proteins (81.3% biotinylation). Using method 1, cells transient transfected with 4 different BioID constructs (three replicates) located at the cytoplasm, endoplasmic reticulum, nucleus, and mitochondria were analyzed, resulting in distinct subcellular proteomes.

Conclusions:
Except digestion of proteins prior to on bead biotin capture, all approaches resulted in similar enrichment efficiencies for biotinylated proteins. On bead digestion resulted in the highest number of identifications being in our hands the best method for the analysis of affinity captured biotinylated proteins. It was possible to identify subcellular proteomes using different BioID constructs.

Novel Aspect:
Comparison of methods for avidin affinity capture of biotinylated proteins for MS analysis and analysis organelle proteomes using BioID constructs.

References:
Keywords: protein correlation profiling; Trypanosoma brucei; cross-linking; size exclusion chromatography; cryomilling

Introduction
Trypanosoma brucei is a protozoan parasite and the causative agent of sleeping sickness in humans. Approximately 9100 protein coding genes have been identified by whole genome sequencing[1], yet 4900 of these lack classifiable homology to known proteins in other organisms. To further ascribe functional classification to these “hypothetical” proteins [2,3], we performed a protein correlation profiling (PCP) based on a cross-linking mass spectrometry approach.

Methods
Cells were cryomilled and the lysate powder was reacted with the cross-linking reagent DTSSP. After, the cross-linked protein samples were submitted to size exclusion chromatography (SEC) followed by their fractionation. Each fraction was submitted to nanoLC-MSMS analyses and their PCP analysis was performed based on label free quantification (LFQ) intensities processed by MaxQuant.

Results
PCP identifies cofractionating proteins to enable a global characterization of functionally related proteins via “guilt by association”, as many intracellular biological processes are dependent on the stable physical association between two or more proteins. We utilised cryomilling to maintain in vivo protein associations, whilst using chemical crosslinking to stabilise such interactions during further sample processing.

In order to optimise DTSSP mediated crosslinking conditions, α-tubulin was monitored as a reference protein by Western blotting with anti-α tubulin. The established reaction condition of 4 mM DTSSP for 30 mins was sufficient to induce crosslinking. As a next step, the SEC was performed, and the eluted protein complexes were collected in 12 fractions. The PCP results revealed protein complexes that were not seen in the previous study carried out using native conditions [4]. Moreover, we have found hypothetical proteins associated with annotated proteins, that might help to accredit their shared function.

Conclusions
We established a crosslinking approach with DTSSP on cryomilled Trypanosoma brucei cell lysates, followed by their proteins separation on SEC and fractionation. Using a small number of fractions, PCP preliminary data have shown the capability to reveal not only protein complexes that were not seen in the previous study, but also the protein associations between annotated and hypothetical proteins.

Novel Aspect
The novel aspect of this approach relies on the combination of cryomilled cell lysates, the covalent protein association using crosslinking, SEC and PCP.

References
Alsford, S. et al. Genome Res. 21, 915-924 (2011)
Introduction:
Sample multiplexing is an important tool for improving the throughput of MS analysis. Herein, we introduce the concept of Isoelectric Point Coding (pl-Coding) for an additional x2 or x3 level-multiplexing power of isobaric labeled samples. An in-solution isoelectric focusing device for fast pl-Coding is described, especially suitable for combining biological-replicates into a single sample. The utilization of pl-Coding for LFQ and TMT samples is demonstrated.

Methods:
Steps for pl-Code multiplexing:
1. Separation. Isolation of non-overlapping pl windows from each sample; Sample 1: peptides with pl < 5.0, Sample 2: peptides pl between 5.2-7.5, and Sample 3: peptides with pl > 7.8.
2. Pooling of all isolated pl ranges into one sample, followed by its LC MS/MS analysis.
3. Signal deconvolution: theoretical pl calculation of every peptide & assignment according to: pl < 5 to Sample 1, pl between 5.2 and 7.5 to Sample 2, pl > 7.8 to Sample 3.

Results:
In isobaric labelling, the sample number information is encoded into a chemical tag, which is later decoded by MS [1]. Similarly, the isoelectric point value can be used as an information tag, since its value can be easily calculated, and because each protein produces tryptic peptides having pl values from acidic to neutral to basic, with empty valleys between them. In fact, it has been shown full human proteome coverage using peptides with pl ranges between 2.5 - 4.7 [2].

pl-Coding was developed as followed:
In-silico modelling. Several isoelectric focusing ranges were studied for optimum multiplexing. The results showed that two to three pl-channels provide at least 3 peptides for each protein.
pl-Coding. An isoelectric focusing device was developed for optimum collection of 3 pl areas in a way compatible with automation.
Validation. The results showed that pl-coding could be used for x2 or x3 multiplexing for the analysis of thousands of proteins in a highly parallelized manner for iTRAQ/TMT-based or label-free quantification.

Conclusions:
The pl-Coding works with LFQ and TMT-reagents to double or triple their throughput. In LFQ, pl-coding is especially suitable for combining 3 biological replicates into a single sample (using 3 pl-Code channels). In the case of TMT-labeled samples, pl-Coding allows up to 20-30 sample multiplexing. In-silico modelling suggest that pl-Coding could be applied to different organisms. To our knowledge this is the highest level of multiplexing described in the literature.

Novel Aspect:
pl-Coding works with LFQ and TMT-labeling to double or triple their throughput. In TMT-labeled samples, it allows up to 20-30 sample multiplexing.

References:
Keywords: Differential Proteomic Analysis of Actinic Keratosis, Bowen’s Disease and Cutaneous Squamous Cell Carcinoma by Sequential Window Acquisition of All Theoretical Mass Spectra (SWATH-MS)

Introduction:
Actinic keratosis (AK), Bowen’s disease (BD) and cutaneous squamous cell carcinoma (cSCC) are heterogeneous skin tumours with high prevalence rates globally. This study reports comprehensive protein biomarkers with discriminatory capabilities towards these lesions.

Methods:
Proteolytically digested peptides from formalin-fixed and paraffin embedded (FFPE) samples of normal skin, AK, BD, well differentiated cSCC (n=14), moderately differentiated cSCC (n=15) and poorly differentiated cSCC (n=15) were analysed in TripleTOF® 6600 using sequential window acquisition of all theoretical mass spectra (SWATH-MS) workflow. Peptide peaks were extracted using Skyline followed by their differential abundance analysis and bioinformatics analysis.

Results:
We reliably identified and quantified 3574 proteins across 93 samples. Differential abundance analysis revealed that 15, 26 and 403 proteins were exclusively changed in cSCC, BD and AK samples compared to the normal skin, respectively. Also, 16 and 225 proteins were differentially abundant exclusively in BD and cSCC compared to AK samples, respectively. Ten proteins were differentially abundant in cSCC compared to BD samples. In addition, a total of 118 proteins were commonly changed in moderately-differentiated and poorly-differentiated cSCC samples compared to well-differentiated cSCC subtype while 55 and 809 proteins were differentially abundant exclusively in moderately and poorly differentiated cSCCs compared to well-differentiated cSCC samples, respectively. Majority of these proteins were involved in pathways associated with cell proliferation, apoptosis and DNA damage response which are crucial in carcinogenesis.

Conclusions:
To our knowledge, this is the most comprehensive and complete SWATH-MS proteomic study in cSCC and its premalignant lesions. The identification of differentially abundant proteins and potentially important molecular pathways in this study form a rich resource for biomarker discovery in the future.

Novel Aspect:
For the first time we successfully applied SWATH proteomics on FFPE skin samples. Our methodology opens invaluable opportunities to explore proteome of other skin conditions.
Keywords: tape stripping, mass-spectrometry based proteomics, actinic keratosis, noninvasive skin biopsy

Introduction:
Actinic keratoses (AK) are frequent premalignant tumors that can be clinically difficult to differentiate from skin cancer. To definitely confirm or rule out the diagnosis, a biopsy is needed, but this is an invasive procedure that leads to increased morbidity. An easy, quick and reliable non-invasive alternative to biopsy is needed to definitively confirm the clinical diagnoses. In this paper, we evaluate Tape Stripping (TS) of stratum corneum (SC) for biomarker analysis of AK.

Methods:
Lesional and non-lesional human SC samples were obtained by application of stripping tapes on the skin of five AK patients. Following sample preparation, protein digests were analyzed by liquid chromatography tandem mass-spectrometry (LC-MS/MS). Bioinformatics analysis were performed using Funrich, Ingenuity Pathway Analysis (IPA) and Oncomine bioinformatics and analytical tools.

Results:
Of the total 613 unique proteins identified, 477 overlapped with proteins identified in our proteomic analysis of formalin-fixed and paraffin-embedded (FFPE) AK samples. Additionally, 32 proteins were significantly increased, and four proteins decreased in AK samples compared to the normal skin (p<0.05). In line with proteomic analysis of FFPE samples, IPA and Funrich analysis showed that differentially abundant proteins in the TS AK samples are implicated in PI3K/AKT and EGF signaling pathways. These findings were confirmed at the transcript level.

Conclusions:
Tape stripped AK sample is suitable for biomarker analysis using mass spectrometry.

Novel Aspect:
The application of this technique further could revolutionize management of keratinocytic skin tumors by reducing the need for traditional invasive biopsy.
INTRODUCTION:
Protein degradation plays important roles in biological processes and is tightly regulated. Further, targeted proteolysis is an emerging research tool and therapeutic strategy. However, proteome-wide technologies to investigate the causes and consequences of protein degradation in biological systems are lacking.

METHODS:
mPDP enables the sensitive and comprehensive relative quantification of cellular mature and nascent protein pools across a range of different treatment conditions in biological duplicates and in a single mass spectrometric experiment by combining dynamic SILAC with chemical labeling using neutron-encoded tandem mass tags (TMT).

RESULTS:
We developed ‘multiplexed proteome dynamics profiling’, mPDP [1], a mass spectrometry-based approach combining dynamic-SILAC labelling with isobaric mass tagging for multiplexed analysis of protein degradation and synthesis. When applied combination with other quantitative proteomics approaches such as chemoproteomics and thermal protein profiling, mPDP provides unique insights in drug mechanism-of-action. The method was applied in three proof-of-concept studies, and uncovered different responses induced by the bromodomain inhibitor JQ1 versus a JQ1-proteolysis targeting chimera. Further, we elucidated distinct modes-of-action of estrogen receptor modulators, and we comprehensively classified HSP90 clients based on their requirement for HSP90 constitutively or during synthesis demonstrating that constitutive HSP90 clients have lower thermal stability than non-clients, higher affinity for the chaperone, vary between cell types and change upon external stimuli.

CONCLUSIONS:
We have established multiplexed proteome dynamics profiling a powerful means to elucidate the mechanism of action of bioactive molecules, thus complementing current proteome-wide target identification strategies. mPDP identifies targets of protein degraders and more generally enables the discovery of regulatory degradation mechanisms in biological systems.

novel Aspect:
PROTAC target identification and detection of regulated degradation events.

References
Savitski MM et al., Cell 173, 260-274(2018)
Keywords: organellar proteomics, subcellular fractionation, relative quantification, hierarchical clustering, protein complexes

Introduction:
In previous studies we have presented comprehensive interactome analysis using multi-epitope AP-MS ([1], [2]) and stoichiometric complexome analysis by csBN-MS ([3]) for membrane proteins. Notwithstanding, these techniques do not provide information on biogenesis of assemblies, subcellular distribution or spatiotemporal dynamics. We, therefore, took on extending/refining organellar proteomics workflows ([4], [5], [6], [7]).

Methods:
Primary epithelial cells (RPTEC) were used as a model for endosomal recycling. After isotonic homogenization, hypotonic lysis and disruptive sonication multidimensional protein fractionation was applied. Fractions were analyzed by nano-LC-MS/MS. After label-free quantification, Euclidian-distance based hierarchical clustering and dimensionality reduction by t-distributed stochastic neighbor embedding (t-SNE) data were visualized in two dimensions.

Results:
Key elements of the approach are subcellular fractionation (which is inherently limited), comprehensive and quantitative MS profiling, and unsupervised cluster analysis. High reproducibility and accuracy are required for optimal results. The higher the number of non-redundant fractions, the higher the precision of quantification. Nano-LC-MS/MS analysis of 80 trypsin-digested RPTEC samples (40 sub-fractions, each divided into an upper and a lower molecular weight range sample by SDS-PAGE) with an LTQ Orbitrap XL instrument yielded more than 3700 quantitative protein distribution profiles. Hierarchical clustering revealed more than 20 distinct entities representing classical cellular organelles or organelle substructures, populations of trafficking vesicles, multiprotein machineries and cytoskeletal networks as shown by successful mapping of 1074 prequalified marker proteins and validated functional annotations from UniProtKB/Swiss-Prot.

Conclusions:
Organellar proteomics is a powerful method for comprehensive, robust and quantitative assessment of the subcellular localization of proteins with a broad range of applications: Identification and verification of subcellular markers, classification of interactomes, unbiased definition of subcellular proteomes, analysis of spatial dynamics of proteins and localization determinants, etc.

Novel Aspect:
With multidimensional fractionation and advanced quantification, cluster analysis and visualization tools protein clusters were resolved with unprecedented resolution.

References:
ABUNDANT PROTEIN DEPLETION OF HUMAN PLASMA SAMPLES – SAMPLE PREPARATION APPROACHES FOR QUANTITATIVE COMPARISON STUDIES

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Keywords: plasma, depletion, TMT, sample preparation

Introduction:
Depletion of abundant plasma proteins is required to identify and measure changes in prognostic or diagnostic proteins [1]. We have developed new top2 and top12 abundant protein depletion resins and evaluated them for depletion specificity, efficiency, and reproducibility. We also combined our abundant protein depletion resins with a streamlined Tandem Mass Tag (TMT) sample preparation procedure.

Methods:
Commercially-obtained pooled human plasma samples were used to assess depletion resins by SDS gel, ELSIA, and LC-MS. HighpHreversed-phase fractionation was performed on TMT-labeled samples before LC-MS analysis. All samples were analyzed on Thermo Scientific Orbitrap Fusion Tribrid mass spectrometer and processed using Thermo Scientific Proteome Discoverer 2.2 software [2].

Results:
After optimization of the antibody ligand conjugation chemistries and resin blending protocols, we achieved >97-99% depletion efficiency of twelve high abundance protein targets in human plasma samples. Abundant protein depletion from plasma enabled detection of 34% more peptides and 25% more proteins compared to undepleted samples. Assessment of depletion capacity of the new resins showed high reproducibility for samples ranging from 10-100ul. These depletion resins were also used in combination with a new sample preparation workflow for TMT-reagent labeling which minimizes sample handling and the number of steps. Using this workflow, we identified and quantified nearly 1100 proteins in normal and lung cancer patient plasma samples.

Conclusions:
Abundant protein depletion from plasma enables detection of more peptides and proteins for better detection and quantitation of relevant biomarkers. Combining abundant protein depletion with a streamlined TMT reagent sample preparation workflow and high pH reversed phase fractionation resulted in quantification of nearly 1100 proteins in normal and lung cancer patient plasma samples.

Novel Aspect:
Development and comprehensive analysis of new top2 and top12 depletion resins for abundant human plasma proteins for quantitative comparison studies.

References


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Introduction: (Limit of 400 characters)
No consensus have been made whether label or label free proteomics quantitative methods are better for the quantitative analysis of complex mixtures. We have analyzed human reconstructed skin samples of 4 donors at different time using 10-plex TMT labeling, label free quantitation, DIA (Data Independent Acquisition) and SRM for a comprehensive analysis of the phenomena of skin reconstruction and comparison of proteomics quantitative techniques and softwares.

Methods: (Limit of 400 characters)
Epidermal skin reconstruction of 4 donors at 10times (0 to 28 days) were used for the comparison. Peptides were labeled with 10-plex TMT and injected on the orbitrap fusion. The label-free experiment was injected on the orbitrap fusion and analysed by MaxQuant and Proteome Discoverer 2.2.60 selected proteins were targeted by SRM on the 6500 QTRAP and analyzed by Skyline. DIA experiments were performed on the orbitrap fusion and analysed by Scaffold DIA or Skyline.

Results: (Limit 900 characters)
4338 proteins were identified by using 5 different 10-plex TMT experiments (14 fractions of 2 hours) with a common reference pool. 1821 quantifiable proteins were common between the 5 experiments and 48 variant proteins were obtained. For the Label-Free experiment, individual samples were run for 5 hours identifying 3619 proteins, from which 2749 were quantifiable. 393 proteins were considered variant with MaxQuant and the results will be compared with PD 2.2 software. 55 of the selected proteins for the SRM experiment were quantifiable and 13 proteins were variants. DIA experiments (50 windows of 10 amu) using Scaffold DIA and Skyline will be compared with the 3 other Methods: 11 proteins were commonly statistically variants between the 4 different techniques at specific time points and 8 of them behave exactly the same along the time course for the quantitative techniques tested.

Conclusions (Limit of 400 characters)
For this project, TMT gave the highest number of protein identifications, Label-Free analysis gave the highest number of quantifiable proteins and DIA gave the best results for the reproducibility. Label free analysis using precursor intensity was the most valuable method despite the longest time of analysis. We found that the use of Scaffold DIA is promising for the analysis of DIA data.

Novel Aspect: (Limit of 150 characters)
Different quantitative proteomics methods and the use of a new released software for DIA allows better understanding and confidence in the mechanism of skin reconstruction.
Cystic Fibrosis (CF) is a genetic disorder, caused by mutations in the CFTR gene [1]. Deletion of phenylalanine 508 (F508del) is the most frequent mutation. Dr. Pedemonte’s group has selected molecules with a role in F508del-CFTR processing and degradation. After their silencing, an increase of CFTR at the cell membrane was measured [2], [3]. Using SWATH workflow [4], it is possible to analyze changes at proteome level. The first step is the creation of an ion library.

Methods:

We characterized the proteome of CFBE41o-, the in-vitro model for cystic fibrosis pharmacological research [5]. CFBE41o- cells were cultured and transfected as already described [2]. From cell lysates, we performed both an in-gel digestion and an in-solution digestion. The in-solution digestion was performed on both Proteominer [6] wash and eluted fractions and the resulting peptides were then fractionated offline (8 fractions) with a high pH/low pH strategy [7].

Results:

Raw data were analyzed with ProteinPilot software using the Paragon algorithm [8]. Spectra were searched against the Homo sapiens reference proteome, reporting 71599 proteins. Search was performed against both target and decoy databases. Carbamidomethylation of cysteine (CAM) was set as fixed modification and only four variable modifications were allowed, maximum one per peptide (methionine oxidation, asparagine and glutamine deamidation and conversion of glutamine and glutamate to pyroglutamatic acid). This search, done using the FDR calculation protocol described by Tang in 2008 [9], returned 5935 proteins and 72031 distinct peptides at a FDR of 1%.

To ensure high quality to our library, we imported in the library builder only MRM data of proteins with a maximum global FDR of 1%. Moreover, we removed the peptides shared by more than one protein. After these filtering steps, we produced an ion library with the spectral information on 5627 proteins.

Conclusions:

Thanks to this ion library, we were able to quantify more than 2000 proteins in a total lysate from a single 2 hours LC-MS/MS run. The ion library we generated will enable us to detect changes at protein level in response to the silencing of molecules known to be involved in CFTR processing.
Novel Aspect:

We built the first SWATH ion library dedicated to the most used cell model for CF research. Unlike the pan-human ion library [10], our library contains CFTR and some of CFTR interactors.

References

Introduction:
Quantitative (phospho)proteomics is often used to elucidate cellular responses on media supplements [1]. Especially for non-model species, certain aspects besides LC-MS set up, like sample preparation, databases and evaluation workflow strongly impact the outcome of signaling studies. Adapted workflows then lead to impacted phosphorylation sites and signaling pathways. Here we outline critical steps based on our experience with chinese hamster ovary (CHO) cell analysis.

Methods:
CHO cells were cultivated in chemically defined media. For SILAC experiments, media are supplemented with either lLys/lArg or hLys/hArg. After protein digestion, phosphopeptides were enriched with different strategies. Measurements were performed by nLC-ESI Orbitrap MS and data was evaluated utilizing two different pipelines based on MaxQuant/Perseus and Proteome Discoverer (PD). The obtained SILAC data was also quantified by LFQ for cell line comparison.

Results:
Sample preparation workflow
An optimized lysis buffer solubilizes membrane proteins and allows a direct digest. The resulting new 6-step workflow decreases the variation of identified phosphorylation sites between techn. replicates.

Database searches
Combination of two databases (well annotated mouse; incomplete C.griseus for CHO) leads to the highest identification rate compared to individual DB. However, the mix of identifiers for both species complicates software evaluation. Merging of data for e.g. pathway mapping hence is to great extend manually. An intermediate step was necessary for mapping different protein identifiers to homologous ones.

Quantification methods
The SILAC-based data analysis yields in substantially more identification of modified peptides then LFQ-based approaches, where only 75 % were identified. However, LFQ offersto describe cell-specific ground phosphorylation status and protein expression comparisons. As the current version of PD supports LFQ, we summarize pros and cons in comparison to MaxQuant.

Conclusions
Several aspects besides the LC-MS set up showed to have strong impact on signaling analysis of non-model organisms such as CHO. We highlight the importance and limitations of sample preparation steps, referred protein databases and quantification methods and propose applicable solutions. With this improvements, based on complementary LFQ + SILAC phosphoproteomic data, a comprehensive view on CHO cell signaling in dependence on media supplements was possible.

 Novel Aspect:
Optimized procedures allowed us to reach a high coverage of less-abundant phosphoproteins in CHO cells, which results in a full view on signaling events in this non-model organism.

References
Introduction:

Distilled spirits production using S. cerevisiae requires understanding the mechanisms of yeast cell resistance to alcohols. Mutations in genes of ubiquitin-proteasome proteolytic system, such as RPN4, may result in appearance of the strains exhibiting hyperresistance [1]. Indeed, RPN4 controls expression of proteolytic genes involved in metabolic pathways [2]. In this work we elucidate the RPN4-dependent mechanisms of yeast cell resistance to alcohol.

Methods:

Three S. cerevisiae strains: wild-type (WT) BY4742, RPN4-Δ (with RPN4 deletion), and YPL mutant with decreased proteasome activity, were grown up to log phase in stressed (7% ethanol) and normal conditions. Samples were subjected to LC-MS/MS analysis followed by protein identification, quantification (X!Tandem/MPscore), statistical evaluation of changes in protein expression, and pathways analysis (STRING).

Results:

PCA results have shown the protein grouping according to strains, biological and technical replicates. No technical bias was found for analyzed data. Next, protein levels were compared in pairs: no stress vs. ethanol stressed for each strain (WT, YPL, RPN4-Δ). Proteins that passed the corrected (Benjamini–Hochberg FDR) t-test's p-value of 0.05 were defined as differentially expressed. We found the following pathways affected significantly by the ethanol effect on the cells: protein folding and unfolding, rRNA modification, as well as carbohydrate and nucleotide metabolism. Thus, the ethanol elicits complex cellular response that involves known players like chaperones and metabolic enzymes, as well as the new ones like enzymes of nucleotide metabolism and rRNA modification. Some of these genes are RPN4-dependent. Detailed analysis of differentially expressed proteins allowed identifying the candidates that determine the phenotype of the cells' alcohol resistance.

Conclusions:

Comparative proteome analysis of three different strains of S. cerevisiae revealed differences in yeast response to ethanol at proteome level and suggested gene candidates that are potentially responsible for developing alcohol resistance in the cells. These genes can be targets for further studies using methods of molecular genetics.

Novel Aspect:

The importance of RPN4-regulated non-proteasomal genes as novel factors involved in cellular response to ethanol was revealed for the first time using LC-MS/MS.

The work was supported by the Russian Foundation for Basic Research (grant #18-04-00692) and Russian Science Foundation (project #14-14-00971).

References

Introduction

Regulation of gene expression is controlled by mRNA translation and thus defines the functional proteome. Experimental methodologies are needed that can rapidly capture and reveal the actual nascent proteomes. In addition, such methods must be able to measure changes in nascent protein abundances quantitatively in response to pharmacologic or environmental cues.

Methods

We have developed a protocol using an analog of puromycin, a tyrosine-tRNA mimic, called O-propargylpuromycin (OPP), to identify nascent proteomes rapidly. OPP is cell permeable and catalytically incorporated into nascent polypeptide chains as a specific covalent label during elongation resulting in premature termination. These polypeptides are biotinylated using click chemistry, then isolated and measured by tandem mass spectrometry[1].

Results

We have chosen the erythropoiesis paradigm known to be dependent upon an intricate balance involving maintenance of erythroid precursors and their rapid differentiation to accommodate hematopoietic stresses. In this study, we employed extensively self-renewing erythroblast (ESRE) cells to define the earliest proteomic program unique to the decision between expanding erythroid precursors and terminal maturation. Using OPP-ID, we discovered a network of genes involved in splicing, nucleic acid secondary structure unwinding, and cell cycle regulation which are rapidly repressed in the absence of dexamethasone. While we observed genes previously known to be associated with early hematopoietic precursor states (such as Ddx18, Stag2 and Pold1), we also identified multiple genes such as Prpf6, Sart1, Psen2, and Mycbp2 which have not been previously implicated in erythropoiesis.

Conclusions

This OPP-ID strategy provides rapid experimental view of changes in gene expression at the nascent protein level. This opens a new window for the determination of the actual proteomic changes occurring in response to a variety of cues that direct cellular fates such as proliferation or differentiation.

Novel aspect

This OPP-ID strategy has the potential to ask innumerable questions on the composition and rapid dynamics of proteomic networks at the organismal level.

Quantitative Label-Free Proteomics in a Novel Cellular Knock Out Model of Methylmalonic Acidemia

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Keywords: methylmalonicacidemia, quantitative proteomics, mass spectrometry, bioinformatics, CRISPR-CAS9

Introduction:
Methylmalonic Acidemias (MMAs) are severe autosomal recessive inborn errors of metabolism caused by deficiency of the mitochondrial methylmalonyl-CoA mutase (MUT) enzyme, which converts methylmalonyl-CoA into succinyl-CoA, a Krebs cycle intermediate. As consequence, methylmalonic acid accumulates in body fluids as biomarker of the disease. Long-term complications can include cognitive deficit, chronic kidney disease, liver and kidney failure.

Methods:
A stable MUT-knock out (KO) cell model was generated by using CRISPR-CAS9 genome editing technology on the embryonic kidney (HEK) 293 cell line. Methylmalonic acid was measured in KO cells by liquid chromatography-tandem mass spectrometry (LC-MS/MS) in the multiple reaction monitoring (MRM) mode. The proteomes of 4 biological MUT-KO and 4 wild type (WT) cell samples were compared using a quantitative label-free approach by LC-MS/MS, according to MaxQuant software.

Results:
The goodness of this new cellular model, in which MUT protein was completely knocked out, was tested measuring the levels of methylmalonic acid inside the cells. By contrast, it has not been detected in WT cells, as expected. Bioinformatics analysis was carried out downstream the proteomic experiment in order to select the significant differentially expressed proteins between the two conditions, among those identified in the 8 samples. The analysis showed a number of 178 differentially expressed proteins, which passed all the statistic tests (results from Perseus platform). Applying a more stringent parameter (the identification of each protein with at least 5 peptides in at least 3 of 4 samples per condition), the number of significant proteins was further reduced to 69, of which 39 down-regulated and 30 up-regulated in the MUT-KO condition.

Conclusions:
The selected proteins are globally involved in energy metabolism and oxidation-reduction processes and most of them have mitochondrial localization, supporting the idea of mitochondrial impairment in MMAs. The rescue of MUT protein will be performed in KO cells in order to specify if the deregulated proteins are strictly linked to MUT absence and highlight unknown molecular mechanisms underlying the cellular damage and tissue alterations in MMAs.

Novel Aspect:
This is the first cellular model of methylmalonicacidemia generated by using CRISPR-CAS9 genome editing technology.
Introduction:
Polio (poliomyelitis) once a widespread, occasionally paralytic disease is almost eradicated. Key to this success has been broad vaccination programs with oral poliovirus vaccine (OPV) but the endgame requires a switch to inactivated vaccines (IPV). IPV potency is assayed in vivo (immunogenicity) or in vitro (antibody based) by relative comparison to international references; improved IPV testing methods are desired.

Methods:
Reference Antigens (RA), Inactivated Polio Vaccine (IPV), monovalent bulks, and combination vaccines (DTaP-IPV) were disulfide cleaved and capped then digested with trypsin. The peptides were separated on a C18 column connected to Orbitrap Fusion MS collecting MS/MS in data dependent mode with HCD fragmentation. The data was analyzed with Proteome Discoverer 2.1 by searching a custom database and quantification of proteins based on top 3 precursor intensity.

Results:
We compared numerous polio standards and vaccines using proteomics and label free quantitation. The database search required generation of a custom database consisting of individual polio capsid proteins and proteins derived from the other components of the combination vaccine. A recent RA with established DU/ml values was used to prepare a calibration curve and the equation relating precursor intensities to DU was determined. The calculated DUs in the samples were then compared to the results obtained by the manufacturers by antibody based Methods. The DU values obtained by this method are higher than labelled amounts for the combination vaccines, IPV, and reference antigens while the bulks were lower than the labelled content. An independent preparation of the calibration curve RA has calculated DU values close to expected. Identification and quantification of polio, diphtheria, pertussis, tetanus proteins in combination vaccines is possible.

Conclusions:
The label free quantitation of Reference Antigens using optimized MS methods and bioinformatics software provides results that are closely aligned with those from antibody based Methods. The advantage of the proteomics approach is that unexpected or adventitious proteins in the sample are also identified and quantified without prior knowledge of their presence. The disadvantage of the proteomics approach is the lack of proof regarding the protein folding.

Novel Aspect:
A rapid proteomics and bioinformatics approach has been developed for polio vaccine antigen quantitation.
References
1118 - TMT-BASED QUANTITATIVE APPROACH DISCLOSES MONACOLIN K-INDUCED PROTEOME ALTERATIONS IN TRIPLE NEGATIVE BREAST CANCER CELLS.

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Keywords: Quantitative Mass Spectrometry, TMT-labeling, Natural compounds, Nutraceuticals, Triple Negative Breast cancer (TNBC).

Introduction: MS-based Proteomics on Natural Compounds
MS-based proteomics represents a clever tool to disclose unclear bioactivities of small molecules such as natural compounds in nutraceuticals [1]. The latter are rising as significant building block in prevention or/and treatment of numerous diseases [2]. Monacolin K (MNK), abundant in red yeast rice extract, is used to lower hematic concentration of cholesterol. Recent scientific evidences link statins to antiproliferative and apoptotic effects in a wide panel of cancers [3].

Methods: TMT-based quantitative proteomics
A kinetic proliferation assay was performed using IncuCyte ZOOM® system to find out the IC50 of MNK to set up proteomics experiments.
Proteomes obtained upon different treatments of MDA-MB231 cells with MNK were digested and pooled together, after former TMT 10-plex™ labeling of the related peptide mixtures [4][5]. The obtained multiplexed pool was prepared for the UPLC-MS/MS analysis of proteomes and phosphoproteomes. Bioinformatic analysis were carried out by MaxQuant and Perseus [6][7].

Results: Monacolin K affects proteome and phosphoproteome of MDA-MB231
MDA-MB231 cells were treated with different concentrations of MNK and growth kinetic curves unraveled an IC50= 4.02±1.23μM.
This time-course experiment was the groundwork to drug treatments for the proteomics approach: MDA-MB231 cells were treated with MNK at 5 μM or 0.5 μM and harvested after 30’, 6h or 24h. Control experiments without MNK were harvested in parallel. Then, cells were lysate and proteomes were digested. The obtained tryptic mixtures were chemically labeled with amino-reactive tandem mass isobaric tags (TMT 10-plex™). Labeled peptides were pooled together and the multiplexed sample was simplified upon HpH-fractionation. An aliquot of each fraction was used to obtain the phosphoproteome upon Fe3+-NTA affinity chromatography. NanoUPLC-MS2 experiments were carried out both for proteomes and phosphoproteomes. Raw MS data were analyzed with MaxQuant software to identify and quantify proteins. Statistical analysis of MQ results was performed by Perseus. When MDA-MB231 are treated with MNK 5μM, some alterations in their proteome and phosphoproteome have been found.

Conclusions: Monacolin K regulates proliferative pathways in TNBC cells
MNK has an antiproliferative effect on MDA-MB231, as shown by growth kinetic curves. MS-based proteomics unrevealed the potentiality of this bioactive metabolite, abundant in red yeast rice, in modulating expression and phosphorylation of proteins involved in cellular pathways responsible for cellular proliferation. These findings configure Monacolin K as an interesting metabolite in adjuvant therapies for the treatments of triple negative breast cancer.

Novel Aspect:
Exploiting TMT-10plexTM, a quantitative MS-based proteomics approach was used to investigate antiproliferative effect of the natural compound Monacolin K on MDA-MD231 cells.

References
372 - HIGH TNALING PATHWAY MONITORING BASED ON FAST LC-PRM ANALYSES

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Title: High throughput signaling pathway monitoring based on fast LC-PRM analyses

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Keywords
Parallel reaction monitoring; signaling pathway; high throughput

Introduction
Targeted analyses based on HRAM parallel reaction monitoring (PRM) measurements have opened new opportunities in quantitative proteomics. Here PRM was combined with fast capillary-flow LC separation to accelerate the execution of analyses. The setups developed turned out to be ideally suited to high throughput monitoring of signaling pathways in the context of clinical research applications, typically encompassing thousands of samples.

Methods
The analyses were performed on a Thermo Scientific™ Q Exactive™ HF mass spectrometer operated with several PRM-based acquisition schemes (using instrument programming interface in some cases). Chromatographic separation were carried out using a Thermo Scientific™ UltiMate™ 3000 RSLC system equipped for capillary flow and a Evosep-One system (Evosep). Various gradient lengths and MS parameter settings were employed to analyze samples of various complexities.

Results
Fast LC methods have been designed with the objective to increase the analytical throughput, therefore requiring both higher flow rate (typically 2-3 µL/min) and gradient length reduction, while maintaining acceptable duty cycle. Different experiments have been developed for conventional PRM, dynamic retention time-PRM (dRT-PRM), and internal standard triggered-PRM (IS-PRM) analyses of ten to a few hundred endogenous peptides. Another IS-PRM method variant, relying on the concomitant measurement of each pair of internal standard and endogenous peptides in a single scan, was explored to further shorten analysis time. The final methods differentiated themselves by their unique combination of acquisition scheme, experiment scale, gradient length, and MS parameter settings. They were applied to the analyses of samples of high complexity, e.g., digests of cell lines, optionally mixed with
Yeast digest, and samples of moderate complexity obtained through multiplex immunoprecipitation targeting proteins of AKT/mTOR pathway.

Conclusion

A detailed comparative evaluation of the quantification performance (e.g., quantification precision, accuracy, and sensitivity) obtained for various experimental configuration was carried out. This in-depth investigation enabled the establishment of a reference baseline demonstrating sensitive quantification of a few dozens of peptides in 100 samples within one day under high acquisition efficiency (typically through dRT-PRM and especially IS-PRM mode).

Novel aspect

Sensitive PRM-based signaling pathway monitoring with the unique combination of analytical throughput and experiment scale required for clinical research applications.
367 - QUANTITATION OF AMYLASE/TRYPsin-INHIBITORS IN VARIOUS WHEAT SPECIES
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Keywords: α-amylase/trypsin-inhibitors, non-celiac gluten sensitivity, ancient and modern wheat species, stable isotope dilution assay, intensity based absolute quantitation

Introduction:
The “ancient” wheat species einkorn, emmer and spelt are claimed to have sensory and health benefits compared to “modern” bread and durum wheats [1]. To date, it is not known if ancient wheat species contain lower contents of α-amylase/trypsin-inhibitors (ATI), which have been associated with intestinal inflammation and symptoms typical of non-celiac gluten sensitivity (NCGS) [2]. Therefore, the aim of this study was to develop a method to quantitate ATI in wheat.

Methods:
ATI were extracted from eight wholegrain flours of the five wheat species with salt solution. Proteins were reduced, alkylated and digested with trypsin. Peptides were analyzed by LC-MS/MS and five ATI-types were quantitated by stable isotope dilution assays (SIDA). For the validation of trueness, the results of SIDA were compared with an independent LC-MS/MS system and data evaluation by intensity based absolute quantitation algorithm (iBAQ).

Results:
Precision (< 8%), recovery (80–120%) and limits of detection (< 5 mg/kg) showed the good performance of the method. The accuracy of SIDA was confirmed by iBAQ, which yielded comparable results. In contrast to the other wheat species, only three of eight einkorn cultivars contained ATI in very low concentrations (0.2 ± 0.1 mg/g). On average, all spelt (4.7 ± 0.5 mg/g) and emmer cultivars (5.0 ± 0.5 mg/g) had higher ATI contents than bread wheat (3.7 ± 0.3 mg/g) and durum wheat (4.2 ± 0.9 mg/g). The ATI content was not correlated with the total crude protein content (r = 0.01). Finally, differences in the distribution of ATI-types were found. The percentages of 0.19 and 0.28 ATI to CM-type ATI was balanced (52%/48%) in bread wheat and spelt, whereas CM-types were predominant (25%/75%) in durum wheat and emmer.

Conclusions:
We developed a novel sensitive LC-MS/MS method based on SIDA for the quantitation of ATI in ancient and modern wheat species. Applying the new method, the assumption that ancient wheats contain less ATI than modern ones was disproven for spelt and emmer. The results indicated that different wheat species can be distinguished by the distribution of ATI. Comparison with iBAQ showed that one internal standard was sufficient for SIDA.

Novel Aspect:
The ancient wheat species spelt and emmer contain more ATI than modern ones. Therefore it is not confirmed that ancient wheat species might be more suitable for NCGS patients.

References
Introduction:
Diabetes, a disease altering the normal insulin production, affects 422 million of people globally and research into its mechanisms and treatment is a growing research area. Current quantifications for insulin and its analogues, such as ELISA, have flaws in terms of performance and throughput. Liquid chromatography–mass spectrometry (LC-MS/MS) approaches have recently been developed as alternative strategies for analysis of biomolecules, including insulin.

Methods: Insulin immunocapture followed by LC-MS/MS
Sample preparation includes capturing of insulin (aspart and human) using immobilized (on magnetic beads) biotinylated antibody from insulin spiked (rat or human) plasma. Insulin is then eluted and subjected to the Glu-C digestion prior LC-MS/MS analysis using Waters UPLC Peptide CSH C18 combined with Sciex Qtrap 6500+.

Results: Method validation for quantification of insulin aspart and human insulin
All investigated calibration curves were characterized by a high correlation coefficient (R2 ≥ 0.99). For Insulin aspart a method was developed from 1.75 to 700 ng/mL. Good selectivity of the method was confirmed with only 2.6% relative analyte response in the blank sample. Quality control samples of 1.75, 5.25, 36.8 and 525 ng/mL were analyzed (in fivefold for each level), with high accuracy (97%, 97% 101% and 95% respectively), demonstrating good precision of the method (CV = 4%, 6%, 11% and 22% respectively).

The assay for human insulin quantification is under development and currently viable for quantification of human insulin in BSA/PBS for 0.35 ng/mL to 15 ng/mL. For this assay we have the ultimate goal to go lower to 70 pg/mL in human plasma, which is necessary for insulin quantification assays according to the clinical guidelines. By improving the selectivity of method by antibody and internal standard selection, and by LC-MS parameter optimization, this goal is considered to be feasible, on which the method is currently under development.

Conclusions
With significant reduction of matrix interference by immunoaffinity sample preparation and with sensitivity of Sciex Qtrap 6500+, a low level of quantification of 1.75 ng/mL and 0.35 ng/mL for insulin aspart and human insulin, respectively, is achieved. To set the LLOQ to 0.07 ng/mL the blank response has to be reduced. To achieve that, additional tests are on the way: to use another more selective antibody and to investigate other selective MS transitions.

Novel Aspect: Outlook of the method
This research investigates the possibilities to overcome the inability of current insulin quantification of human and animal origin and insulin drug analogues by developing a LC-MS assay instead. At Charles River we go towards the implementation of LC-MS protein quantitation to set a sensitive, selective and reproducible method for the quantification of insulin according to the clinical guidelines that is soon to become a fast, reliable and routine analysis.
References

World health organization, www.who.int
Introduction:
Many signaling pathways are activated when proenzymes are proteolytically cleaved releasing the active enzyme (e.g. auto-activation of Factor XII by contact with negatively charged surfaces, starting pathways of coagulation and inflammation). In this work, we use migration profiles [1] for differential analysis of Factor XII contact activation by dextran sulfate (DXS) and poly-phosphate (polyP) and downstream effects.

Methods:
Human Plasma was activated using DXS or polyP. An immunoprecipitation with anti-FXII antibody was performed on the activated plasma samples and a control sample. Eluates were loaded onto a 10% SDS-page. Each of the obtained lanes was cut into 19 bands for in gel tryptic digestion and LC-MS/MS analysis. Migration profiles were obtained by plotting the relative intensity of the identified peptides against the corresponding empirical molecular weight.

Results:
The obtained migration profiles show a distinguished pattern for the FXII peptides in plasma activated with PolyP or DXS and in the control. When plasma is activated by DXS, it shows a clear separation of the heavy chain from the light chain, this separation is not observed in the case plasma was activated by polyP and in the control. In both cases of activated plasma, the downstream proteolytic processes are activated. Compared to the control, the activation of plasma kallikrein (PK) and release of bradykinin from high molecular weight kininogen (HK) is clearly demonstrated. These results corroborate previous findings by Engels et al. that suggest activation of FXII by polyP to be due to conformational changes, and not by cleavage at the activation site (R353-V354) [2].

Conclusions:
Migration profiles of all identified proteins from differentially activated plasma allowed a comprehensive study of the activated proteolytic pathways. It’s shown that the use of different activators has an impact on the mechanism of FXII activation. These findings might provide an explanation for FXII activation by polyP resulting in coagulation and activation of the kallikrein-kinin system, whereas by DXS only activates the kallikrein-kinin system [3].

Novel Aspect:
The method presented is a useful tool to understand the differential activation of FXII and can be applied on a wide array of fields where proteolysis is an issue.

References:
Engels R.; Journal of Thrombosis and Haemostasis, 12, 1513-1522 (2014)
Weidmann, H.; BBA - Molecular Cell Research, 1864, 2118-2127 (2017);
Keywords: myelin, FASP, automation, quantification, neuropathies

Introduction:
Myelin, a plasma membrane specialization of glial cells, facilitates rapid signaling in the nervous system. A key tool to approach failing glia-axonal support in neurological disorders is the proteome analysis of myelin, but membrane protein content and dynamic range pose technical challenges (reviewed in [1,2]). We show advances in label-free quantification of myelin proteins and applications to a mouse model for a heritable peripheral neuropathy.

Methods:
Myelin was solubilized with ASB-14 and subjected to FASP-based digestion automated on a liquid handling workstation (Tecan Freedom EVO 150). Data were acquired on a QTOF LC-MS-system (Waters Synapt G2-S) using three different data-independent acquisition (DIA) strategies: MSe, without ion mobility separation (IMS); UDMSe, with IMS; DRE-UDMSe, with IMS and dynamic range enhancement. Label-free protein quantification was performed with the IsoQuant software.

Results:
As a proof-of-principle for robustness and accuracy of quantification, we first compared CNS myelin from wildtype mice (CnpWT/WT) with myelin from mutant mice heterozygous for the CNP gene (CnpWT/null). In three independent experiments, each comprising 24 MS runs (three biological replicates per condition, duplicate digestion and injection), we determined the ratio of the abundance of CNP CnpWT/WT:CnpWT/null as 1.98:1 (CV < 10%), exactly matching the ratio of 2:1 as expected from CNP gene heterozygosity. In UDMSe runs, however, levels of high-abundant myelin proteins including CNP were misquantified due to the compression of the dynamic range caused by IMS of peptides. This issue was addressed by DRE-UDMSe, a novel data acquisition method in which a deflection lens is used to cycle between full and reduced ion transmission during one scan. All three data acquisition methods were applied to PNS myelin from mice deficient for periaxin (Prxnul/null), a model for a demyelinating neuropathy of the Charcot-Marie-Tooth type (CMT-4F).

Conclusions:
While MSe and UDMSe are well suited for accurate quantification of highly abundant proteins and deep proteome coverage, respectively, DRE-UDMSe provides a compromise between dynamic range and identification rate needed for routine quantification of myelin proteins. Analysis of Prxnul/null mice systematically revealed altered abundance of potentially disease-relevant PNS myelin proteins and thus allows insights into myelin-related pathology in this model.

Novel Aspect:
development and automation of a customized FASP procedure; improved label-free quantification of myelin proteomes; routine proteomics phenotyping of mutant mice

References:
Simplifying the Use of Ion Libraries During Data Processing of Data Independent Acquisition Proteomics Data

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Keywords: DIA, SWATH, proteomics, libraries, cloud computing

Introduction

As the use of data independent acquisition (DIA) grows in proteomics research, the need for improved data processing workflows increases. The most common data processing workflow is to use spectral ion libraries to drive targeted extraction of peptide / fragment areas from the data, using the m/z and retention time information contained in the library. Increasing the size and quality of the ion library has been shown to increase the proteins reliably quantified from a dataset. Retention time (RT) correlation between ion library and the dataset is another key factor that determines quality of data extraction. Simplifying how ion libraries are built and used during data extraction was explored in this work.

Methods

Two algorithms were explored to simplify the data extraction workflow for SWATH® Acquisition data within the OneOmics™ Project cloud processing pipeline, the automatic merging of related ion libraries followed by auto RT calibration. During library merging, ProteinPilot™ Software group files are combined by selecting the largest file as seed library, then peptides from smaller libraries are merged in using a non-linear calibration strategy. New peptides are added to existing proteins and new proteins are added if not present in seed library. During SWATH processing, endogenous peptides are automatically selected across time bins and the best peptide are chosen based on precursor intensity and ID confidence. Best scoring peak groups are used for RT calibration.

Results

Previous data has shown that increasing the size of the ion libraries used provides improved protein quantitation. Moving from ion libraries generated from 1D data dependent acquisitions to libraries generated from 2D fractionation experiments to pan proteome libraries provides a >100% gain in peptides quantified (~60% gain in proteins) to >300% peptide quantified over 1D libraries (>100% gain in protein). Therefore the ability to easily combine and align ion libraries for greater depth of sample interrogation would provide a significant workflow benefit. To evaluate the quality of the retention time alignment during SWATH processing using the new AutoRT calibration function, it was compared to using the typical RT calibration process using a set of spiked standards peptides. In 33/48 tests, AutoRT approach found similar or more proteins quantified than the standard approach and similar linear fit equations, indicating the new, easier to implement process was as good or better than the currently used standard peptide workflow.

The quality of library merging on SWATH results was explored with a series of small datasets using non-linear retention time alignment. By merging libraries from replicates of a 1D dataset, a 20% gain in peptides was observed. The gain in peptides identified and quantified by SWATH is primarily attributed to an increase in peptide coverage rather than improved ion selection.
Full pipeline was then used on some biological datasets (PBMC and Mouse cell lines) to measure performance, where a series of libraries were available to merge. Results will be discussed.

Conclusions
Non-linear retention time alignment was shown to be effective for merging of libraries with identical or differing gradient lengths resulting in better results.

Novel Aspect:
Simplifying DIA data processing in the cloud by pipelining ease of use algorithms
Introduction:
Alzheimer’s Disease (AD) is a neurodegenerative disorder characterized by memory loss and cognitive decline. (1) The current AD treatment methods are aim to prevent cognitive loss. There is no treatment to slow down progression of the disease or stop it. (2) In this study, the effects of new natural compounds on cognitive impairment will be examined and localization of the compounds will be demonstrated by mass-spectrometry based approach.

Methods:
The natural compounds were administered at 2 different doses to 12 months old transgenic mice (5XFAD) for 7 days i.p. (n = 8). Non-transgenic littermates (LM) were used as the control group. Morris Water Maze test was performed to measure cognitive impairment. The MALDI-IMS technique was applied to demonstrate whether the natural compounds pass the blood brain barrier and determine the localization of the compound in the brain regions.

Results and Conclusion:
Cognitive changes related to learning and memory were measured by Morris Water Maze test after injection of low and high doses of 6 different compounds. When compared to control groups, it was observed that 2 of the 6 compounds positively affected both the learning process and memory. It was demonstrated that the compounds were localized in certain brain regions after they have passed the blood brain barrier by utilizing MALDI-IMS.

Novel Aspect:
This is the first study to show the regional localization of the novel native compounds administered on the 5XFAD mouse model.

Reference:
SIMULTANEOUS DUAL PROTEOME ANALYSIS TOWARDS UNDERSTANDING NEUTROPHIL INTERACTION WITH THE HUMAN-PATHOGENIC FUNGUS ASPERGILLUS FUMIGATUS

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Keywords: Aspergillus fumigatus, neutrophil extracellular traps, dualproteome, phosphoproteome, host-pathogeninteraction

Introduction:
Invasive aspergillosis caused by the fungus Aspergillus fumigatus is a life-threatening infection in immunocompromised hosts. Neutrophils are indispensable in eliminating the fungal pathogen by various strategies, i.a. phagocytosis and neutrophil extracellular traps (NETs) [1, 2]. To discover how fungal hyphae activate pathogen-specific responses of neutrophils and to explore fungal counter-defense mechanisms we conducted a quantitative dual proteome study.

Methods:
Neutrophils forming NETs against A. fumigatus were compared with chemically induced NETs. Hyphae trapped by NETs were compared against those controls grown under equal conditions devoid of neutrophils. LC-MS/MS-based bottom-up proteomics was used to quantify proteins based on the MS2 reporter ion intensities of multiplexed unique peptides labeled with tandem mass tags (TMT). Protein-protein interactions (PPIs) were studied using a network modeling approach.

Results:
We were able to quantify 454 differentially abundant proteins of 843 proteins in total on the fungal side and 335 differentially abundant proteins of 1906 proteins in total on the host side. Neutrophils induce fungal proteins involved in the cell wall stress response, toxic secondary metabolite production, and the unfolded protein response. A. fumigatus modulates the trace metal ion utilization. Neutrophils and NETs also strongly repress the fungal protein biosynthetic machinery. Analysis of the neutrophil response upon fungal confrontation revealed an altered stoichiometry of alternative and core histones and a modulation of the nuclear envelope. Neutrophils also induce antimicrobial proteins and a repression of proteins involved in calcium-dependent membrane scaffolding. Furthermore, the neutrophil-fungal interaction caused an impairment of the energy metabolism on both sides. PPI network modeling revealed the induction of an autophagy pathway in A. fumigatus and a regulatory role of CDK1 during NETosis.

Conclusions:
We investigated the responses of human neutrophils to A. fumigatus hyphae using a simultaneous dual proteome analysis. The challenging approach provided us with an ample dataset of differentially abundant proteins on both sides. We generated a model of interaction that allowed us to reveal protein interaction partners and regulatory circuits governing the counter response of A. fumigatus and the mechanism of hyphal induced NETosis.

Novel Aspect:
Our study revealed the fungistatic effects of NETs and the host-pathogen interplay on the molecular level.
References:
Introduction:
Ischemic stroke is classified into large artery atherosclerosis (LAA), cardioembolism and small vessel occlusion. In this study, LAA that is the most common cause of stroke is analyzed compared to healthy subjects. Patients with LAA stroke is approximately 85%. To increase efficacy of diagnosis and recovery, our aim is to discover biomarker of LAA using individual serum of patients.

Methods:
We conducted proteomics analysis of serum protein in patients with LAA stroke. 52 patients and 43 healthy subjects are used to identify global proteins and to compare level of serum proteins. Individual samples was analyzed using Sciex 5600 TripleTOF. Identification and quantification of proteins were conducted by information dependent acquisition (IDA) mode and SWATH-MS acquisition.

Results:
As a result, differentially expressed proteins by more than 2 fold were 149. Differentially expressed proteins by more than 2-fold was related with GO processes such as regulated exocytosis, exocytosis, secretion by cell, immune response and regulation of response to stress. And, alternative complement pathway were top scored. Among differentially expressed proteins that is related with alternative complement pathway vitronectin, complement factor B, complement component C7, and complement component C5 had the most statistically significance. Vitronectin, complement factor B and complement component C7 were upregulated and complement component C5 was downregulated in patients with LAA stroke.

Conclusions:
A proteomics approach discovered associations of LAA stroke with several immune responses compared to healthy subjects. This study highlights four alternative complement pathway-related proteins including vitronectin, complement factor B, complement component C7, and complement component C5. These four proteins contribute to immune responds and B cell and T cell activation and can be potential biomarker to understand LAA stroke.

Novel Aspect:
The proteomics analysis of serum of patients with LAA stroke showed alternative 4 immune-related proteins can help to distinguish subtype of stroke from healthy subjects.

References:
831 - DEVELOPMENT OF A SIMPLE AND EFFICIENT QUANTIFICATION METHOD OF HUMAN FOLLICULAR FLUID (HFF) PROTEOME

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Keywords: human follicular fluid, proteomics, SWATH-MS, MED-FASP, Total Protein Approach (TPA)

Introduction:
Human follicular fluid (hFF) is a potential source of biomarkers of the oocyte quality, which could be assayed in a non-invasive manner prior to oocyte fertilization. In our previous study [1], we investigated the proteome and peptidome of hFF by SWATH-MS methodology [2]. Here, we combine and compare that methodology with MED-FASP procedure and quantitative MS measurements by TPA [3] to develop an efficient quantification method of hFF proteins.

Methods:
Sample of pooled hFFs from patients undergoing the IVF procedure was acquired from the INVICTA Fertility Clinic in Gdansk. Samples after prior ultrafiltration (>10 kDa fraction) or without preliminary treatment were digested by two distinct protocols: i) in-solution trypsin digestion [1]; ii) FASP or MED-FASP [3]. Afterwards, samples were analyzed by two distinct MS methods: i) SWATH-MS [2]; TPA [3].

Results:
We have developed a methodology for hFF proteome and peptidome identification and SWATH-MS quantification strategy. To increase the size of the spectral library used for quantification, we applied extensive multistep fractionation consisting of ultrafiltration, optional immunodepletion, and high pH RP-HPLC. We managed to identify 400 proteins with high confidence: 302 in High Molecular Weight Fraction (HMWF, >10 kDa), and 161 proteins in peptidomic Low Molecular Weight Fraction (LMWF, <10 kDa). In order to improve our method, we intend to conduct comparative experiments of two sample preparation procedures: previously used in-solution digestion and FASP or MED-FASP. Our preliminary results suggest that both protocols allow for high confidence discovery of slightly different sets of proteins. Moreover, ultrafiltration prior digestion improved the outcomes. We plan to carry out more experiments to further test both protocols along with two quantification methods: SWATH-MS and TPA.

Conclusions:
Our methodology enables a relatively simple quantitative measurement of a high number of proteins in one run. A time and cost efficient fractionation method, such as ultrafiltration significantly improves the qualitative and quantitative analyses of hFF, at the same time allowing for investigations of scarcely studied peptidomic fraction (LMWF). MED-FASP is a promising sample preparation strategy with a high potential of application in hFF proteomics.

Novel Aspect:
This is a first work employing MED-FASP and TPA strategy for hFF proteome identification and quantification, as well as their combination with SWATH-MS.

References:
Introduction:
Biological toxins are relevant in the security, health and food sectors. Development of new analytical methods for rapid and unambiguous identification of low toxin concentrations in various complex matrices is essential. We reported previously a method based on LC-ESI-MS/MS for abrin quantification in food and environmental matrices [1]. Here, immuno-MALDI-TOFMS was evaluated for rapid and chromatography-free quantification of abrin.

Methods:
Magnetic beads coated with abrin-specific antibodies were mixed with 500 µL of food samples spiked with the toxin. After harsh washing conditions with PBS-tween, analytes were eluted from beads by incubation with 0.1% TFA. Abrin was heated for 15 min at 95 °C in RapiGest SF 0.05% for denaturation and digested with trypsin in a bath-type sonicator. Peptides were co-crystallized with CHCA matrix on a disposable plate for MALDI-TOF analysis using a Bruker UltrafleXtreme.

Results:
Up to 29 peptides of abrin isoforms were detected by MALDI-TOF MS in a pure standard and the 4 most intense, different to the best ESI peptides, were selected. Isotope-labeled peptides were added for quantification. Immuno-capture conditions were improved for MALDI-TOF MS, including additional washes of antibody-coated beads. On-beads digestion resulted in high interfering signals from antibody peptides, and abrin was therefore eluted before digestion. Trypsin amount was reduced 10 times and digestion time increased to 1 hour in the bath-type sonicator. Using the new protocol, interfering peaks and ionization suppression in apple juice and milk samples were decreased significantly, resulting in spectra with predominant abrin peptide signals at 250 ng/ml. At this concentration, about twenty peptides of abrin isoforms were detected in the two food matrices. On-going parallel evaluation with LC-ESI-MS/MS showed sensitivity in the same range with limit of quantification in milk at 10 and 40 ng/ml for LC-ESI-MS/MS and MALDI-TOF, respectively.

Conclusions
Bottom-up proteomics and immuno-MALDI-TOF MS allowed quantification of abrin and its isoforms in complex matrices, without time consuming peptide chromatography or extraction resulting in simple and fast (< 5 min) MS analysis. The method was found specific, reliable, and sensitive in milk. Further optimizations are on-going to reach equal or better limit of quantification than LC-ESI-MS/MS and to reduce the total analysis time by automatization of the workflow.

Novel Aspect:
Evaluation of MALDI-TOF and LC-ESI-MS/MS for rapid and sensitive quantification of abrin in complex matrices.
References

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804 - A TARGET FISHING AND SHOTGUN PROTEOMICS APPROACHES TO STUDY THE EFFECT OF A NEW DRUG AGAINST M. TUBERCULOSIS

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Keywords: Quantitative proteomics, Target fishing approach, Click chemistry, Drug effect, M. tuberculosis.

Introduction:
Mycobacterium tuberculosis (MT) is the causative agent of tuberculosis. The development of more effective and shorter MT treatments requires the killing of both MT growing and non-growing bacilli. Today, mass spectrometry based proteomics can address challenges that cannot be approached by genomic analysis. The study of the effect of a drug and the molecular mechanisms contributing to disease pathogenesis can be performed using quantitative proteomics.

Methods:
A target fishing proteomic approach for the in vitro study of the effect of a new drug, TP053, on MT was performed. The target fishing experiment, which is based on click chemistry, was used to understand if an active metabolite was able to bind a possible target in MT. Moreover, in order to explore the possible pathways affected by the new drug, MT cell lines were treated with TP053 and a shotgun proteomic approach was used to identify modulated proteins.

Results:
The TP053 treatment negatively affected the redox reactions, intermediary metabolism and DNA. There is a down regulation of proteins linked to growth processes and generation of metabolites but also a down regulation of carbon and lipid metabolism and ATP generation proteins. All this data are in agreement with the inhibition of aerobic respiration which is a well known NO target.
There is also a modulation of enzymes involved in lipid metabolism and an over expression of many ribosomal proteins, most of them belonging to the 30s ribosome unit.
Using the target fishing approach we were able to identify a direct interaction of TP053 with 30S ribosomal protein S13.

Conclusions:
By a shotgun proteomic approach and a target fishing experiment, it has been demonstrated that the activated TP053 is responsible for a global perturbation in the mycobacterial cells through the release of nitric oxide, as well as by a direct interaction with 30S ribosomal protein S13.

Novel Aspect:
A target fishing experiment coupled to shotgun proteomic approach allowed to study the effect of a new drug and the interaction drug-proteins.
648 - A COST-EFFECTIVE APPROACH TO PRODUCE 15N-LABELLED AMINO ACIDS EMPLOYING CHLAMYDOMONAS REINHARDTII CC503

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Keywords: Chlamydomonas reinhardtii, 15N-labelled amino acids, GC–MS.

Introduction:
The synthesis of isotopically labelled compounds from enriched stable isotopes is an expensive and difficult task. Both chemical and biochemical methods have been proposed to produce labelled molecules, but few cost-effective methods have been described [1]. Previously reported biochemical methods to prepare labelled amino acids have only considered their use as hydrolyzed proteins to grow cell cultures or in proteomics [2].

Methods:
The aim of this study was to use the microalgae Chlamydomonas reinhardtii to produce, at laboratory scale, 15N-labelled amino acids with a high isotopic enrichment. To do that, a culture medium containing 15NH4Cl was used. The labelled proteins biosynthesized by the microorganism were extracted from the biomass. Then, the 15N-labelled amino acids were obtained after a protein hydrolysis with HCl and their isotopic enrichment was measured by GC–MS [3].

Results:
No differences in cell growth were observed when they were feeding with natural abundance N or 15N, demonstrating an absence of isotopic effects. On the other hand, the use of the strain CC503 cw92 mt+ and 15NH4Cl in the culture medium reduces the incorporation of natural abundance nitrogen, as this strain is not able to incorporate nitrates or other nitrogen containing compounds. Moreover, this wall deficient mutant facilitated the extraction of proteins, leading to higher extraction yields (32.5±4.0%) than those obtained for a wildtype strain 137c (12.7±3.8%). The average isotopic enrichment obtained for the 16 detected proteinogenic amino acids was 99.56±0.05%, demonstrating the lack of natural abundance nitrogen incorporation through the whole procedure. The amino acid content ranged from 18–90 μg/mL, depending on the occurrence of the amino acids present in the proteins. A derivatization procedure based on the use of MTBSTFA was optimized to convert the amino acids into volatile compounds.

Conclusions:
Sixteen 15N-labelled amino acids were obtained with an extremely high enrichment from the strain Chlamydomonas reinhardtii CC503. As the effective incorporation of 15N has been proved, other labelled biomolecules should be present in the cells, and this biomass could be employed to isolate 15N-labelled nucleic acids and other metabolites in future experiments. Additionally, multiple labelling could be performed if 13C were supplied to the culture media.

Novel Aspect:
An innovative, simple, and fast method to produce isotopically labelled amino acids, which could be used for future quantitative proteomics studies, is presented.

References
Keywords: Spirulina, phycobiliprotein, microbial fermentation, Lactobacillus plantarum, Orbitrap

Introduction:
Arthrospira is a cyanobacteria with a high nutritional and therapeutic potential. It is a source of essential and non-essential nutrients and bioactive compounds with anti-inflammatory and antioxidant activity[1]. Large proportion of Arthrospira dry weight comprises phycobiliproteins of high bioactive value[2, 3]. Microbial fermentation can contribute to products nutritional and bioactive efficiency due to new metabolite production and transformation[4].

Methods:
Arthrospira samples were fermented with L. plantarum culture, protein extraction of fermented and non-fermented samples followed. Yeast culture was treated with protein concentrates and later extracted. Protein content and pathway alterations were analyzed by Bradford assay and HF Orbitrap. Liquid chromatography tandem mass spectrometry was used to determine protein changes and Proteome Discoverer for protein identification against the UniProt database.

Results:
Exposure of yeast cells to fermented or non-fermented protein extracts affected yeast cell protein content. A higher protein concentration was determined in yeast cells treated with fermented and non-fermented Arthrospira extracts compared to control. Furthermore, label free high mass resolution proteomics enabled us to determine protein changes during microbial fermentation in Arthrospira water-phase protein extracts.

Conclusions
Arthrospira microbial fermentation using Lactobacillus plantarum culture offers great possibilities for protein efficiency and uptake improvement. During lactic acid fermentation proteins are modulated with enhanced digestibility and bioavailability and adding bioactive properties to the food component. This method enables us to develop a food product with an upgraded functional value.

Novel Aspect:
Further analysis of lactic acid fermentation of Arthrospira is needed to specify its effects on products nutritional and bioactive properties.

References
PROTEOMIC ANALYSIS OF UNCONVENTIONAL PROTEIN SECRETION

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Keywords: autophagy-related protein 7, unconventional protein secretion, epithelial ovarian cancer, secretome

Introduction:
The cancer secretome comprises numerous proteins that promote tumorigenic processes [1]. A substantial number of proteins lack signal peptides and are released via unconventional mechanisms[2]. Herein we analyzed the contribution of autophagy-related (ATG) based unconventional protein secretion to the microenvironment of epithelial ovarian cancer cells.

Methods:
Epithelial ovarian cancer cells (OV-90, ATCC) were transfected with siRNA targeting human ATG7 or control siRNA. A label free quantitative approach was applied to analyze secreted proteins of ATG7 deficient cells and ATG7 intact (control) cells. Conditioned media was collected over 24 hours. Samples were digested using FASP[3] and analyzed by nanoLC-MS/MS (Orbitrap MS). Raw data processing was performed with MaxQuant.

Results:
ATG7 siRNA transfection efficiently reduced ATG7 protein levels. The secretome was highly enriched with extracellular localized proteins. Approximately one-third of these proteins contained a signal peptide as predicted by SecretomeP. Multivariate analysis of the data further demonstrated changes of the secretory protein profile upon siRNA mediated ATG7 knockdown. A subset of proteins emerged that were not quantified in the conditioned media of ATG7 deficient cells, including proteins involved in cell-cell adhesion.

Conclusions:
Besides the well-known function of ATG7 in the elongation reaction and the formation of the autophagosome[4], the present proteomic approach shows the contribution of ATG7 protein to the extracellular release of a subset of proteins in epithelial ovarian cancer cells.

Novel Aspect:
Novel insights into the complexity of the epithelial ovarian cancer cell microenvironment.

References
Absolute quantification of proteins relies on externally quantified peptide standards which are spiked in the sample [1]. For the quantification of large numbers of proteins, the QConCat strategy [2] efficiently provides multiple internal standard peptides concatenated in one proteolytically cleavable artificial fusion protein. We applied this strategy for absolute quantification of the currently known lysosomal proteome using targeted mass spectrometry.

Methods:
12 QConCats were expressed in E. Coli using stable isotope labeled amino acids and purified via a His tag at their C-Terminus. Tryptic digestion was performed by in gel digestion, in solution digestion in the presence of 1 M GCl or RapiGest as well as by the FASP [3] and SP3 [4] strategies. The generated peptides were desalted and analyzed using LC-MSMS. Data analysis was performed by Mascot and scheduled MRM transitions were established using Skyline [5].

Results:
Based on in house and published large scale proteomics studies, we selected 422 unique tryptic peptides of lysosomal proteins acting as representatives. We were able to cover 143 lysosomal proteins representing 94% of the known lysosomal proteome [6]. The selected peptides were used to design 12 QConCats which were reverse translated and generated by gene synthesis. After expression in E.Coli and His tag purification of the QConCats, we optimized tryptic digestion. We tested in gel digestion, in solution digestion using RapiGest 1M GCl as well as the FASP and SP3 approaches. RapiGest gave us the lowest number of missed cleavage sites and was therefore used for sample processing. A mixture of all QConCat peptides was analyzed by LC-MSMS using an Orbitrap Velos and QTrap 5500/6500 mass spectrometers. For 417 of the 422 peptides we defined MRM transitions and are currently in the process of assigning specific retention times. In an initial study, we absolutely quantified 48 lysosomal proteins from mouse embryonic fibroblast using the first 4 QConCats.

Conclusion:
We successfully designed, expressed and purified 12 QConCats providing internal standard peptides for absolute quantification of the majority of currently known lysosomal proteins. We optimized purification and proteolytic digestion of the QConCats. Assay development for targeted quantification by MRM is almost finalized. Our assay will allow for the first time to perform absolute quantification of the known proteins of a whole organelle.

Novel Aspect:
The approach allows for the first time the absolute quantification for almost all proteins of an organelle, the lysosome.

References
233 - THE KEY TO BIG BIOBANKS: ANALYZING 24 PROTEOMES PER DAY BY MICRO-FLOW SWATH® ACQUISITION AND SPECTRONAUT PULSAR ANALYSIS

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Keywords: DIA, SWATH, high throughput proteomics, micro-flow, FFPE

Introduction:
Micro-flow chromatography provides a similar workflow sensitivity compared to nano-flow separations but greatly increased robustness enabling high throughput proteomics for 100’s of samples. In combination with data-independent acquisition methods (DIA) such as SWATH® acquisition, 1,000s of proteins can be quantified with high data completeness and high quantitative precision. Here, we show the potential of the workflow for 105 cancer samples.

Methods:
FFPE colon tissue samples (10 healthy, 95 cancer) were obtained from a public repository. Proteins were extracted as described previously [1]. Tryptic digests were spiked with iRT peptides (Biognosys) and separated on a NanoLC 425 system (SCIEX) by a 43min gradient at a flow rate of 5µl/min coupled to a TripleTOF® 6600 system (SCIEX) operated in SWATH mode. Run time per sample was 1h. Data were analyzed in Spectronaut Pulsar (Biognosys) with a project specific library.

Results:
The sample preparation resulted in average in >100µg peptides per 10µm slice, which is plenty for the micro-flow SWATH® acquisition workflow. The whole dataset (105 samples) was acquired in only 4.5 days. Data analysis in Spectronaut Pulsar took one additional day demonstrating the large potential of the workflow for even more samples. In total, about 4,500 protein groups were quantified at 1% peptide and protein FDR (3,600 in average per run) providing a sufficient depth for biological interpretation of the data. Data analysis revealed that a larger number of proteins were expressed in the cancer cohort. Unsupervised clustering of the data separated the healthy and the cancer cohort. Interestingly two samples of the healthy cohort clustered together with the cancer cohort. Within the cancer cohort, three major groups were identified, potentially different cancer subtypes. The data will be further biologically interpreted using ingenuity pathway analysis (IPA).

Conclusions:
This study demonstrates, how high throughput proteomics can be used to analyze large sample sets from tissue biobanks. Application of the micro-flow SWATH® acquisition workflow combined with Spectronaut Pulsar data analysis generates data from these large resources in a timely fashion and therefore pave the way to a better understanding of health and disease.

Novel Aspect:
Micro-flow/SWATH® acquisition and Spectronaut Pulsar analysis enables efficient and comprehensive analysis of large sample cohorts (>100 samples).

References:
Introduction:
Avian pathogenic E. coli (APEC) gives rise to colibacillosis which result in economic losses in the production system and reduced animal welfare. At the same time, an extensive use of antibiotics to combat infections promotes selection of resistant bacteria. We investigated the proteome of an E. coli ST117 strain during infection of the mucus layer of oviduct to suggest potential genes (essential and redundant) as targets in development of antibiotics or helper drugs.

Methods:
A combination of reducing agent and a number of enzymes was used to purified and enriched bacteria from mucus in quantities enough for LC-MS based quantitative proteome analysis. Peptides from infecting bacteria and the host (in the same sample) were labelled with TMT isobaric tags and mixed with peptides derived from proteins of ST117, grown in culture media (log and stationary phase), labelled with complementary TMT tags.

Results:
By combining reducing agent and selected enzymes we successfully purified and enriched bacteria from mucus of infected hens. We identified over 2000 bacterial proteins and were able to quantify approx. 1500 of them across all the samples. This relatively high number of positive hits was greatly facilitated by spiking TMT labelled peptides derived from proteins extracted from ST117 grown in culture media into these obtained from infection experiments. The abundant spiked in TMT labelled peptides served as factor to isolate E. coli derived peptides for fragmentation in the LC-MSMS analysis, as otherwise peptides from co-isolated proteins of the host highly suppressed the signal of peptides originating from isolated bacterial proteins.

Conclusions
The approach presented here is suitable for detection and quantification of relatively high number of proteins of bacteria isolated from infected oviduct of hens, where proteins of the latter highly dominate the sample. Apparently, this approach could be adopted to investigate pathogen/host interactions, or proteomes of mixed population of different organisms.

Novel Aspect:
Improved method for isolation of mucus infecting bacteria. Combination of TMT labelled peptide populations derived from pure cell culture and isolates from infected hosts.
Introduction:
Traceability of protein and peptide quantifications to the International System of units is still a challenge in analytical chemistry. Due to the lack of certified reference materials peptide purity assessment must be carried out through amino acid analysis. This procedure is based on the addition of isotopically labelled amino acids before the application of a time-consuming acid hydrolysis.

Methods:
The only commercially available peptide certified reference materials SRM 998 (Angiotensin I from NIST) and CRM 6901-a (C-peptide from NMIJ) were used for validation purposes. The hydrolysis time was optimized for both materials applying either classical thermal heating or focused microwaves during hydrolysis. Labelled amino acids were spiked to the samples prior hydrolysis with HCl 6N and the peptides were quantified by Isotope Dilution Mass Spectrometry.

Results:
The quantification of the peptides was performed by GC-MSMS employing 5 different isotopically labelled amino acids (Valine, Isoleucine, Leucine, Proline and Phenylalanine), which were previously characterized in terms of concentration by reverse isotope dilution using natural abundance certified analogues. The isotopic enrichment of the labelled amino acids was determined as reported elsewhere [1]. The hydrolysis time required to achieve a complete hydrolysis was optimized for SRM 998 and CRM 6901-a when applying thermal heating at 130°C or focused microwaves. Finally, the purity of four isotopically labelled proteotypic peptides of two protein glaucoma biomarkers was assessed applying the optimized conditions. A final validation of the methodology was carried out by quantifying both proteins in human serum samples by isotope dilution and LC-MSMS. An accurate assessment of the peptide purity can be demonstrated if no statistical differences are obtained in the protein concentrations obtained by each labelled peptide.

Conclusions:
The application of focused microwaves significantly reduces the time required to achieve a complete hydrolysis of peptide standards. Besides the hydrolysis of peptide certified reference materials, the determination of proteins in real samples by LC-MSMS through proteotypic peptides allow an additional validation of peptide purity assessment within the optimized hydrolysis method.

Novel Aspect:
Focused microwaves to decrease peptide hydrolysis time. Validation with reference materials and tryptic peptides applied for absolute quantification of proteins.

References
Introduction

Krabbe disease (KD) is an autosomal-recessive sphingolipidosis caused by a deficiency of galactosylceramide beta-galactosidase, and is characterized by an accumulation of psychosine in the nervous system. In this study, we applied a quantitative microproteomic workflow for the characterization of the central and peripheral nervous system of the Twitcher mouse (a mouse model for KD). The analyses focused on the corpus callosum, motor cortex and sciatic nerves.

Methods

The brains and sciatic nerves of 5 Twitcher and 5 littermate wild type mice were extracted and snap frozen. Cryosectioning and laser capture microdissection was then used to isolate small 1mm² regions of interest from each mouse brain. The workflow combined SP3 digestion, TMT labeling and high-pH fractionation followed by LC-MS/MS analysis using a nanoLC-Orbitrap Fusion system. Data analysis was performed using Proteome Discoverer 2.1, Perseus and WebGestalt.

Results

We performed three TMT-10plex experiments to separately compare the proteomes of the sciatic nerve, corpus callosum and motor cortex of 5 TWI and 5 WT mice. Overall, more than 3000 protein groups were quantified in each of the three datasets. Principal component analysis was performed on the protein expression levels and the TWI and WT mice fell into two distinct clusters, well separated by PC1 in all datasets. A two-sided t-test (FDR=0.01) was performed to compare the expression values of specific proteins. We found 75, 14 and 386 protein groups differently regulated between TWI and WT mice in the corpus callosum, motor cortex and sciatic nerves datasets, respectively. Gene ontology analysis revealed several pathways upregulated in the TWI mice related to inflammatory response (i.e. antigen processing and presentation, and complement pathway) and to phagocytosis and lysosomes.

Conclusions

The comparison between TWI and WT mice proteomes highlighted many proteins with distinct expression profiles. Most of the enriched biological processes in the TWI mice were related to immune response, accumulation of lysosomal proteins and reduced nervous system development. These initial results will be further investigated and validated in order to gain new insights into the molecular mechanisms of KD.

Novel Aspect

This work is the first reported mass spectrometry-based in-depth proteomic characterization of the Twitcher mouse proteome to study Krabbe disease.

References

Keywords:
Proteomics, FAIMS, PTM, TMT quantification

Introduction:
Proteomic analyses are often limited by the overwhelming proportion of confounding background ions that compromise the identification and quantification of low abundance peptides. Gas phase ion fractionation using high field asymmetric waveform ion mobility (FAIMS) can reduce sample complexity[1], and improve quantitative proteomic measurements[2]. Here we report a new FAIMS interface that further expand the sensitivity and depth of proteomic analyses.

Methods:
LC-MS/MS experiments were performed with and without FAIMS on an Orbitrap Fusion mass spectrometer. We used cylindrical FAIMS electrodes (1.5 mm gap) with both electrodes set to 100°C, pure nitrogen as carrier gas and a dispersion voltage (DV) of -5000V. LC-FAIMS-MS/MS experiments were conducted by stepping through 2-4 different CVs in a single run. Cell extracts were reduced, alkylated and digested with trypsin. TMT at 1:2 label excess was done in HEPES buffer, pH 8.3.

Results:
To evaluate the advantages of FAIMS, we first compared the number of identification obtained for triplicate injections of a 500 ng HEK293 digest analyzed by LC-MS/MS with and without FAIMS. These analyses revealed that FAIMS provided a 30% enhancement in identification with 39791 peptides (2876 protein groups) compared to 31397 peptides (2414 protein groups) without FAIMS. We next used isobaric peptide labeling with TMT to profile the dynamic changes in protein abundance for HEK293 cells exposed to heat shock. A different TMT label was used for each 1h interval for a treatment period up to 9h. Samples were analyzed using both synchronous precursor selection (SPS-MS3)[3] and LC-FAIMS-MS/MS. SPS-MS3 enabled the quantification of 12400 peptides corresponding to 1229 proteins, of which 375 proteins showed dynamic changes in abundance. FAIMS further enhanced the comprehensiveness of quantitative proteomics by more than 2-fold with 30848 peptides assigned to 2646 proteins, of which 902 proteins showed dynamic changes in abundance.

Conclusions
A new FAIMS interface was coupled to the latest generation of Orbitrap instruments and enabled unparalleled sensitivity in proteomics analyses where multiple CVs can be combined in a single LC-MS/MS run. The enhancement in ion transmission and duty cycle of the extended the depth of proteomic analyses by at least 30% and expand the comprehensiveness of multiplex quantitative proteomics using isobaric peptide labeling by at least 2-fold compared to SPS-MS3 analyses.

Novel Aspect:
Novel FAIMS interface combined with an Orbitrap tribrid MS improves the sensitivity of proteome analyses and the comprehensiveness of quantitative proteomic measurements.

References

Draft
Pfammatter S., Bonneil E., Thibault P., Journal of Proteome Research, 50(11), 1181-95 (2016)

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Introduction

Traditionally, metabolic pathways are quantitatively characterized by correlating gene expression profiles to protein abundances. However, owing to poor congruence between them, an alternative approach was needed to resolve this issue.

In *C. elegans*, metabolic transitions are crucial for development and survival. They are tightly regulated by tweaking dynamically the components of the pathways, but very less is known on how absolute protein amounts can contribute to this regulation.

Methods:

53 Metabolic enzymes from central carbon metabolism of *C. elegans* were quantified by MS Western method [1]. Unique quantitpic peptides were selected from sequences of metabolic enzymes and from reference protein BSA and expressed as a single chimera in ΔLysΔArg double auxotroph E.coli strain supplemented with isotopically labelled arginine and lysine. The isotopically labelled chimera, full length BSA & whole worm extracts were co-digested and analyzed by LC-MS. The abundances of tryptic peptides from the labelled chimeric protein and the unlabelled target protein were compared and absolute molar quantities of target proteins were calculated.

Results:

53 metabolic enzymes spanning glycolysis, gluconeogenesis, TCA and glyoxylate shunt were accurately quantified in fmole/worm using a single isotopically labeled 300 kDa chimera protein standard comprising, in total 200 quantitpic peptides. Molar concentrations of endogenous enzymes determined with CV <10% allowed to determine stoichiometric ratios between subunits of multiprotein complexes and between individual members of metabolic pathways. To the best of our knowledge, for the first time this work provided a quantitative overview of the molecular organization of 4 metabolic pathways at the protein level. Although the abundance of enzymes is independently regulated, their molar abundances were ranging from 0.03 fmole/worm to 4 fmole/worm and within each pathway, differed less than 4-fold. High accuracy of the protein quantification allowed to confidently determine concentration difference of 2-fold, typically disregarded by label free quantification. By comparing molar abundances of metabolic enzymes in several mutants, this work provided molecular insight into the metabolic regulation at different stages of *C. elegans* development, including the dormant dauer stage.

Conclusions:

Absolute quantities of 53 metabolic enzymes provided crucial evidences of tight regulation of metabolic pathways. The changes in the specific gluconeogenic enzymes primed towards the conclusion that dormant worm uses gluconeogenesis as their survival strategy by exploiting all possible metabolic bypasses [2].

Novel Aspect:
Comprehensive absolute quantitation of 53 metabolic enzymes spanning 4 major pathways in one experiment with a CV < 10 %
Detailed study of isoform specificity in pathway regulation.
Easy estimation of subunit stoichiometry of enzyme complexes.
Unique repository for system biologists for pathway studies.

References:

APPLICATION OF MANUAL CHROMATOGRAPHY DEVICE FOR FRACTIONATION OF COMPLEX PEPTIDE SAMPLES PRIOR NANOESI-MS/MS

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Keywords: microgradient device, peptide sample fractionation, basic reversed phase chromatography, nanoLC-MS/MS

Introduction:
Proteomic analyses using mass spectrometry often rely on suitable chromatography separation of complex peptide mixtures. In some cases, multidimensional chromatography application is even needed for a deeper insight of proteome. A combination of basic reversed phase chromatography fractionation with subsequent acidic reversed phase chromatography coupled to ESI-MS/MS analysis may be applied.

Methods:
Manual gradient chromatography device was used for fractionation of complex peptide mixture. Mobile phases were composed of acetonitrile in 20 mM ammonium formate at pH 10. Microcolumn (250 um i.d., 50 mm length) contained core-shell particles (Kinetex EVO C18, 2.6 µm) and sample was fractionated either into 17 or 33 fractions that were further dried and analyzed using nanoLC-MS/MS (Q-Exactive).

Results:
The nanoLC-MS/MS analysis of a complex peptide mixture without fractionation enabled identification of 1975 protein groups. The fractionation of this sample using manual chromatography device in basic reversed phase chromatography revealed identification of up to 6197 protein groups. Not only the number of identified proteins was increased, but also the number of identified peptide sequences was increased about 4 times.

Conclusions
The manual chromatography device fractionates low amounts of peptides (tens of micrograms) into several fractions that provide a much deeper inside into the analyzed proteome after the nanoLC-MS/MS analysis. The device is advantageous for its simplicity of application, speed of fractionation (about 45 min per samples) and its low cost.

Novel Aspect:
Manual chromatography device for peptide sample fractionation prior to nanoLC-MS/MS analysis.
868 - A CHEMICAL PROBE FOR UNDERSTANDING HOW PROTEOME FOLDEDNESS CHANGES UNDER PROTEOSTASIS STRESS

Gavin Reid (1) - Dezerae Cox (2) - Yuning Hong (3) - Danny Hatters (2)

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Keywords: proteostasis, protein folding, chemical proteomics

Introduction:
The components of the cellular quality control network responsible for maintaining proteostasis, some of which function to protect the folded state of proteins, are now well understood. However, it is still unknown which proteins in the proteome are most vulnerable to unfolding, misfolding, or aggregation, in response to diverse proteostasis stresses.

Methods:
Here, we demonstrate the application of a thiol-specific probe, tetraphenylethene maleimide (TPE-MI), coupled with stable isotope labelling and MS-based quantitative proteomic analysis, for measuring changes in the reactivity of free cysteine residues that are normally buried in the core of globular proteins but become exposed upon unfolding due to proteostasis stress1.

Results: (Limit 900 characters without spaces)
We have demonstrated the potential of this method to (i) probe the kinetics of single-protein folding/unfolding for endogenous proteins on a proteome-wide scale, and (ii) monitor the specific changes in cellular proteome foldedness that occur in response to a variety of stressors (e.g., ER stress (tunicamycin), Heat Shock, HSP-70 inhibition, proteasome inhibition, or overexpression of a non-native misfolded protein, barnase2). Notably, we show that different stress conditions result in little overall similarity in changes in foldedness, and share only a small subset of common vulnerable core machinery.

Conclusions
TPE-MI is demonstrated as a promising tool to probe proteostasis at both the proteome wide and individual protein scales. This knowledge will contribute to our understanding of disorders characterised by proteostasis imbalance, and will assist in targeting those proteins most prone to misfolding under stress conditions.

Novel Aspect:
This approach enables analysis of folding/unfolding kinetics for endogenous proteins on a proteome-wide scale, without expression of specific protein reporters.

References
Introduction
Label free quantification (LFQ) and isobaric labelling quantification (ILQ) are the two most popular quantification workflows in discovery proteomics. Here, we wanted to objectively compare the two workflows given a fixed amount of LC-MS time. For ILQ, we chose TMT 10-plex, for LFQ we chose single shot data-independent acquisition (DIA). We joined forces with a laboratory at FLI where ILQ is well established to compare the two workflows.

Methods
We generated a gold standard set of ten samples composed of a complex mouse cerebellum background spiked with the UPS2 protein standard (Sigma) in five different concentrations. LC-MS data was acquired on an Orbitrap Fusion Lumos (Thermo) in both laboratories and with both workflows. For TMT an MS3 method was used, for DIA a method as described in (Bruderer et al. 2017) was chosen. Data were analyzed with Proteome Discoverer (Thermo) and Spectronaut (Biognosys).

Results
In the four experiments (two workflows in two laboratories) we identified 48,500 (FLI TMT), 47,700 (BGS TMT), 51,800 (FLI DIA) and 53,500 (BGS DIA) peptides. On the protein level this corresponded to 6,500 (FLI TMT), 6,200 (BGS TMT), 5,700 (FLI DIA) and 5,900 (BGS DIA) identifications. 16 (FLI TMT), 15 (BGS TMT), 14 (FLI DIA) and 17 (BGS DIA) UPS2 spike in proteins were detected. Generally, protein identifications were higher with TMT and protein sequence coverage higher with DIA. The quantitative performance in detection of differential abundance was very similar with a slight edge towards LFQ-DIA at both sites (FLI and BGS) and for two criteria used (partial area under the curve or true positives in the top 200 candidates).

We also performed an ILQ-TMT experiment with very deep pre-fractionation (24 fractions). This illustrated that ILQ-TMT is particularly suited for very deep proteome coverage. In this experiment we were able to quantify 8,800 protein groups, 160,000 precursors and 26 of the UPS2 proteins.

Conclusions
The quantitative performance of both methods was very similar in both laboratories. It turned out that for the analysis of the DIA data it was beneficial to use both quantitative information levels available in DIA, at MS1 and MS2 level. We see the strength in LFQ-DIA particularly in a single shot workflow for dozens to thousands of samples and for ILQ-TMT particularly for very deep coverage of a few samples.

Novel Aspect
Comparison of isobaric labelling workflow with single shot label free DIA quantification on a gold standard sample set
Introduction:
Mass spectrometry-based proteomics has become a powerful technology for the identification and quantification of proteins. We recently introduced the Parallel Accumulation Serial Fragmentation (PASEF) method on TIMS QTOF instrument, delivering high robustness, speed and sensitivity, for shotgun proteomics. Here we show reproducible and accurate quantification using the PASEF acquisition method and the optimized PEAKS and MaxQuant software packages.

Methods:
The peptide mixtures (< 200 ng) were separated with a nanoElute (Bruker Daltonics) nano-flow LC on-line coupled to a high-resolution TIMS QTOF (Bruker Daltonics). Chromatographic separation was carried out at a flow rate of 400 nI/min using a 30-90 min linear gradient. LC-MS/MS data were acquired using the PASEF method. Data analysis was performed using PEAKS studio (Bioinformatics Solutions Inc.) and MaxQuant (Jürgen Cox, Max Planck Institute of Biochemistry).

Results:
200 ng of a complex peptide mixture derived from a mammalian cell line in the single-run format were analyzed using a 90 min gradient and optimized PASEF parameters. More than 5300 proteins families were identified in each run with a good reproducibility when comparing the protein identities between replicates. Comparing label free intensities between technical replicates shows excellent reproducibility with a R2 = 0.98 using either a 90 min or with a 30 min gradient. To evaluate the reproducibility of PASEF, we determined the coefficient of variation of the label free intensities. Also here good reproducibility could be shown. For the evaluation of the accuracy of quantification over a wide concentration range we performed a spike-in experiment. We spiked E.coli digest in different ratios (1:2, 1:5 and 1:10) into HeLa digest and injected 100 ng sample material and measured each concentration in triplicate analyses. With all different concentrations, expected ratios could be determined.

Conclusions

tims-PASEF allows to perform reproducible, accurate and high sensitive label-free quantitation from shotgun proteomics samples.

Novel Aspect:
Label free quantification using PASEF.
Introduction:
Ratio compression reduces dynamic range in multiplexed quantitative proteomics studies, particularly for high-resolution MS2 Methods: SPS-MS3 has been highly successful for improving quantitative accuracy by reducing interference-based compression. Building on this, we tested whether the use of high-Frequency Asymmetric Ion Mobility Spectrometry (FAIMS) could further reduce interference in multiplexed quantitative analyses on an Orbitrap Fusion Lumos.

Methods:
Three complex samples were used to test the utility of FAIMS to reduce interference: (1) Triple-knockout yeast (TKO, 100% yeast), (2) canonical interference standard (CIS, 46% yeast), and (3) a novel small change interference model (SCI, 10% yeast). Known peptide ratios across channels ranged from 1.5-fold to infinite. Peptides were analyzed on an Orbitrap Fusion Lumos (Thermo Scientific) for analysis with or without a FAIMS source (three CVs tested: -40, -60, -80V).

Results:
Thermorecently developed a FAIMS source with improved measurement sensitivity/speed and FAIMS was previously shown to reduce interfering precursors by ion-mobility-based separation[1]. Here, we tested if Thermo’s new FAIMS source could reduce co-eluting interfering ions to improve quantitative accuracy for multiplexed samples. We compared HRMS2 with and without FAIMS using a CIS (labeled yeast dilutions with/without mouse background), and strikingly observed a 55% reduction in quantitative interference. We identified 2288 total yeast peptides with FAIMS-HRMS2 compared to 2423 total yeast peptides for HRMS2 without FAIMS (94%) indicating similar PSM sensitivity with or without FAIMS.

For the TKO sample [2], a 9-plex with triplicates of three KO strains (Δmet6, Δura2, Δpkf2), FAIMS improved the average interference free index by 22% (reduced avg. interference by 62%) compared to the same method without FAIMS. Additionally, we observed a 2.2-fold increase in total peptide identifications for FAIMS-HRMS2 compared to FAIMS-SPS-MS3.

Conclusions
We observed that FAIMS-SPS-MS3 (with the SCI model) had the least quantitative interference (most accurate ratios), followed by non-FAIMS-SPS-MS3 and closely thereafter by FAIMS-SPS-MS3. Non-FAIMS-SPS-MS3 had the highest levels of interference and ratio compression. Total peptide identifications for FAIMS-SPS-MS3 were 54% higher than for SPS-MS3. Our findings suggest that Thermo’s new FAIMS source would be a valuable tool for any multiplexed quantitative workflow.

Novel Aspect:
FAIMS (HRMS2 or SPS-MS3) consistently improved quantitative accuracy without sacrificing peptide identifications for multiplexed samples compared to standard Methods:
References

For information please contact: scientific@imsc2018.it
Title: A targeted multiple reaction monitoring method for quantifying Nav1.6 protein in HEK293 cells and mouse hippocampus

Authors: Tung M (1,2), Kwan R(1), Xi C(1), Li J(1), Dang C, (1,3) and Sojo, L E (1,2)

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Keywords: Nav1.6, targeted proteomics, MRM, Human Embryonic Kidney (HEK) 293, Scn8a hippocampus

Introduction:
Nav1.6 expression and activity in hippocampus are shown to mediate seizure resistance (1). HEK293 cells expressing human sodium channel Nav1.6 are used to screen compounds targeting Nav1.6 for the discovery of drug candidates to treat epilepsy. Expression of Nav1.6 proteins is traditionally analyzed by Western blot analysis. Here we show that targeted multiple reaction monitoring mass spectroscopy can provide protein quantitation using stable isotope dilution of unique Nav1.6 peptides.

Methods:
Membrane proteins were extracted from Nav1.6 expressing HEK293 cells, and from Scn8aN1768D/+ and WT mouse hippocampus (2) using Mem-PER Plus kit (Thermo 89842). Detergents were removed using spin columns (Pierce 87777). Protein (80 µg) from Nav1.6 HEK293 cells and 240 µg of Scn8a and WT hippocampal protein were subjected to in-solution tryptic digestion (Thermo 89895). Samples were dried and reconstituted in 50 µL 1:1 ACN:water + 0.1% TFA for UHPLC-MS/MS analysis.

Results:
A total of 39 tryptic peptides, based on an in-silico digestion of a complete human Nav1.6 protein were targeted for MRM analysis of Nav1.6 expressed in a stably transfected HEK293 cells as well as in mouse hippocampus. Out of the 39 peptides, 11 were detected with at least three specific fragment ions. The remaining peptides were present, but not all fragments were detected. From these 11 peptides, seven were unique to Nav1.6. The other four peptides shared homology with other sodium channels. Peptide DSLFIPIR (480.781 m/z, 598-605) was selected for Nav1.6 quantification due to its intense and robust signal. Light and heavy (internal standard) versions of DSLFIPIR were synthesized and used to quantify Nav1.6 using an isotopic dilution method. Nav1.6 HEK293 cells showed Nav1.6 expression of 3.3 fmol/µg of total membrane protein. Scn8aN1768D/+ and WT mouse hippocampus showed 0.41 and 0.39 fmol/µg of total membrane protein, respectively.

Conclusions:
A simple and fast turnaround proteomic work-up was tested for detecting and quantifying Nav1.6 expression in a stably transfected Nav1.6 HEK293 cell line as well as in Scn8a mouse hippocampus. The success of these Nav1.6 proteomic studies enables the use of proteomics to screen and select optimal cell lines for epilepsy drug development. Changes in Nav1.6 expression in mouse hippocampus could also be monitored using proteomics. The same approach can be applied to other ion channels of interest.

Novel Aspect:
This is the first time that quantification of Nav1.6 protein expression in HEK293 cell lines and mouse hippocampus is reported.

References:
1. Christopher D Makinson et al., Role of the hippocampus in Nav1.6 (Scn8a) mediated seizure Resistance, Neurobiol. Dis. 2014, 68, 16–25.
Introduction:
Several approaches are available for performing MS-based quantitative proteomics. These include label-free and label-based methods [1]. However, which quantification strategy is best suited for identification of biologically relevant changes at the protein level is still not clear [2]. In this study, the analytical performance of three different strategies for relative protein quantification using shotgun proteomics have been compared: LFQ, DML and TMT.

Methods:
Two protein mixtures were analyzed, both containing the same amount of human proteins, but different protein amounts from E. Coli (1.5-fold difference) and soybean (3-fold). After protein denaturation and tryptic digestion (5 technical replicates), peptides were divided in three aliquots: one was used for label-free analysis; the second was labeled by TMT, while the third was derivatized by dimethyl labeling. All samples were analyzed by LC-MS/MS on a Q-Exactive.

Results:
Data were processed for statistical analysis (MaxQuant/Perseus) aiming to find out the best conditions for acceptable diagnostic accuracy. For 5 replicated samples per protein mix, a total of 2495 (LFQ), 1385 (DML), and 1803 (TMT) proteins with minimum 2 peptides (FDR 0.01) were quantified. Parameters such as specificity, precision and sensitivity were evaluated for each of the three quantification strategies. Differentially expressed proteins between protein mix A and B for LFQ and TMT datasets were identified by using a corrected Student t-test (permutation-based FDR < 0.05, S0=0.2). Instead, DML data were processed using the following criteria: one sample t-test, S0=0.2, p-value <0.05. The diagnostic accuracy has been evaluated for all the three datasets: LFQ data returned 95% specificity, 71% precision, and 87% sensitivity; samples labeled with TMT obtained 100% specificity, 96% precision and 51% sensitivity; DML labeling instead gave back the following values: 96% specificity, 64% precision, 79% sensitivity.

Conclusions
Since LFQ and DML provided comparable diagnostic accuracy, in our analytical pipeline LFQ was preferred because it provided deeper proteome coverage and faster sample preparation. TMT provided excellent precision (low number of false positives), though sensitivity was poor, especially at low fold changes (1.5-fold).

Novel Aspect:
Comparison of the diagnostic accuracy of the three main categories in quantitative proteomics: label-free, isobaric tagging and label-based with full MS quantification.

References
1371 - PROTEOMIC AND METABOLOMIC ALTERATIONS IN THE RAT BRAIN AFTER ISCHEMIC STROKE

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Keywords: ischemic stroke, proteomics, metabolomics

Introduction
Stroke is one of the major causes of death worldwide, and in non-lethal cases often leads to permanent symptoms[1]. Understanding the biological phenomena occurring in the brain after stroke on a molecular level is crucial for the development of new treatments to alleviate these symptoms. The aim of our study was to characterize the effects of stroke on the proteome and metabolome of rat brain at a timepoint in which the brain’s response to stroke has been active for days.

Methods
Rats were subjected to 60 min cerebral ischemia or a sham operation as a control. The peri-infarct areas were dissected 4 days later, and the samples were homogenized and divided into two aliquots. Sample preparation was done separately for proteomics and metabolomics, and the untargeted analyses were performed with Thermo EASY-nLC 1000/Q Exactive MS and DionexUltiMate 3000 UHPLC/Orbitrap Fusion MS, respectively. Data was analyzed with MaxQuant, MZMine2, and R.

Results
Hierarchical clustering and PCA of the data indicated major differences between the sample groups, and in the case of PCA, higher between-sample variability in the stroke group than in the control group in both proteomics and metabolomics. Across the samples, in total 2,403 proteins were quantified, of which 277 were upregulated and 167 downregulated in the stroke samples relative to the control samples (q < 0.01). In line with the literature, inflammatory response-related proteins were upregulated in the brain after stroke[2]. Further processes identified by the bioinformatic analysis include e.g. increased translation and decreased energy metabolism in the stroke samples. In metabolomics, 470 features were significantly different (q < 0.01, fold change > 2) between the sample groups. Based on MS/MS identifications, the abundance of e.g., sugars and sugar phosphates, unsaturated fatty acids and a wide variety of other lipid species increases in the brain after stroke, whereas the abundance of nucleosides (adenosine, guanosine) decreases.

Conclusions
Ischemia causes extensive damage to the brain, and with the consequent reperfusion, induces various biological processes. Our work identified major alterations in the brain after stroke on both proteomic and metabolomic level, including proteins and metabolites related to inflammation, protein synthesis, and energy metabolism.

Novel Aspect
This study integrates proteomics and metabolomics data from the peri-infarct brain area of rats subjected to cerebral ischemia and sampled several days post-stroke.

References
Introduction:
Accurate quantification of peptides and proteins rely on the availability of certified reference materials. Here we investigated the use of ultraviolet (UV) and mass spectrometric (MS) detection for the simultaneous determination of the quantity and identity of peptidic impurities present in peptide standard solutions. The data combined with amino acid analysis can provide accurate determination of the SI-traceable concentration of peptide calibrants.

Methods:
Peptide solutions were SI-traceably quantified by amino acid analysis using exact matching isotope dilution mass spectrometry (EM-IDMS). The relative amount and the identity of the impurities was determined by UV-MS. Relative response factors (RRF) were verified by varying the injection volumes of the stock solution. The amino acid results were corrected for peptidic impurities. The results were confirmed by quantitative NMR (qNMR).

Results:
The SI-traceable concentration of the peptide solutions can be determined by peptidic impurity corrected amino acid analysis (PICCA). The peptide and its impurities must absorb UV light and the impurities must be baseline-separated. The peptidic impurities were sequenced by MS/MS. The main impurities are deletion products and related sequences with post translational modifications on unstable amino acid residues. The determined purity of the synthetic peptides used in this study is indirectly proportional with the length of the peptide sequence. The validation of the method for the determination of the purity of potential peptide calibrators will take place by applying it to the determination of the purity of oxytocin as part of an international comparison study. Quantitative NMR results complement the findings of the UV-MS results.

Conclusions:
The relative (detector) response ratios for the impurities vs the main compound can be determined without the use of solid impurity standards, and the determination of the purity of a peptide solution by the evaluation of the UV chromatogram is possible. The agreement between the purity results determined by UV and MS using the orbitrap mass analyzer suggests linear behavior for the orbitrap.

Novel Aspect:
Determination of purity of synthetic peptides is achieved with the use of universal (UV) and mass spectrometric detection and quantitative NMR.
Introduction:
The multi-drug resistant bacteria A. baumannii belong to the group of human opportunistic pathogens responsible for most common life threatening nosocomial infections. The resistance of A. baumannii to the last-resort antibiotic colistin is increasing and, recently, a high rate of colistin dependence has been demonstrated [1, 2]. However, the mechanisms of antibiotic resistance and dependence are not yet fully understood.

Methods:
Colistin-susceptible clinical isolate of A. baumannii and its subpopulation that exhibited a colistin-dependent growth pattern were studied. The whole-genome sequencing (WGS) and the shotgun proteome analysis by high-resolution mass spectrometry with following bioinformatics analyses were performed to characterize the molecular mechanisms of antibiotic dependence in A. baumannii.

Results:
We utilized a proteogenomic approach to explore the molecular mechanisms of colistin dependence. The bioinformatics analysis of the obtained WGS data identified multiple antimicrobial resistance genes which were identical in colistin-susceptible and colistin-dependent subpopulations; however colistin-resistant genes were not detected by ResFinder server. WGS data were used to construct a custom strain-specific six-reading frame database, which was used for the proteomic analysis. We found that 183 proteins were expressed differently in the two phenotypes and 119 of them were downregulated in the colistin-dependent subpopulation. The significant part of these proteins are metabolic enzymes, chaperones, outer membrane proteins, efflux pumps and porins. Notably, in addition to novel proteins, several of differentially expressed proteins are known to be involved in colistin-resistance mechanisms.

Conclusions:
Our study shows that the significant part of proteome is modified in colistin-dependent subpopulation of A. baumannii. This proteogenomic study also indicates that the colistin-dependence mechanisms are closely related to colistin-resistance. Our research provides a new analytical platform and a foundation for further research of multiresistant A. baumannii.

Novel Aspect:
This is the first study to use proteogenomics approach for identification of the molecular mechanisms behind antibiotic dependence in A. baumannii.

References:
Title: TMT Quantification Using Spectral Libraries in SpectroMine

Authors: Lynn Verbeke, Tejas Gandhi, Oliver Bernhardt, Lukas Reiter

Introduction:
SpectroMine can search both data-dependent (DDA) and data-independent acquisition (DIA) data using in silico generated search spaces (from FASTA files) or spectral libraries. The advantage of using libraries is the greatly reduced search space, leading to a speed-up and improved sensitivity/reproducibility [1]. Here we set out to see if a library-based targeted approach can lead to improvements, both in identification and quantification, in a TMT-experiment.

Methods:
We used a published data set of 7 TMT10-plex experiments, all from different mouse tissues. Every experiment consists of 12 fractions of 5 controls and 5 nicotine-treated samples [2]. We combined all 7 experiments to generate a library in SpectroMine with 1% false discovery rate at PSM, peptide, protein group level. SpectroMine also identifies the TMT reporter ions, in MS2 or MS3 scans. These are used to calculate differential abundances between conditions.

Results:
The generated library has a size of 673'204 fragments, 112'587 precursors, 10'120 protein groups (11 hours for processing 84 runs). We used this library to search the brain experiment. In the case of library searching, SpectroMine uses normalized retention time (iRT) [3] available from the libraries to further improve the search: only peptides within an expected retention time window will be matched to the scans. This resulted in 48'126 precursors and 7'627 protein groups (20 min). With a Q-value of maximum 0.05 and a minimal absolute log ratio, we found 18 candidates. When searching this data with the mouse FASTA file, we find 39'098 precursors, 6'617 protein groups (2 hours). Applying the same criteria, leads to only 16 candidates. Comparing the quantities of the candidates we obtained with those of the paper, resulted in correlations of 0.98 for both the FASTA as well as for the library approach.

Conclusions
When using a library instead of a FASTA file to identify TMT-labeled peptides, we managed to get an increase of 23% at precursor level and of 15% at protein group level. On top of that, quantification can still be applied by extracting the reporter ions from the MS3 scans. Very similar quantification results are obtained, showing that the library-searching approach of DDA-data can be combined with TMT-quantification.

Novel Aspect:
Library searching of TMT-labeled data, while making use of normalized retention time (iRT) to increase identifications while maintaining quantitative accuracy.

References
Roland Bruderer, Oliver M. Bernhardt, Tejas Gandhi, Lukas Reiter, Proteomics, 16, 2246 (2016).

For information please contact: scientific@imsc2018.it
Keywords: inflammation markers, feces, selected reaction monitoring, targeted proteomics

Introduction:
Inflammatory bowel disease (IBD) and food allergies represent a wide group of intestinal disorders that may lead to chronic inflammation of the gastrointestinal tract (e.g., Crohn’s disease, celiac disease). Food allergies are abnormal reactions to an allergen presented in food, however, the cause of IBD remains unclear [1,2]. Quantification of specific inflammation markers in feces allows to characterize an inflammation and estimate its progress in the digestive tract.

Methods:
Proteins were extracted from both lyophilized and fresh fecal samples for comparison, using 1x phosphate saline buffer and centrifugation. The supernatant was subjected to bottom-up proteomics workflow. We have tested isolation of neutrophils and eosinophils from feces and characterized their CD markers. Proteotypic tryptic peptides were selected, inspected for isoforms and SNPs and quantified using UHPLC-SRM analysis.

Results:
We have developed and validated a multiplex protein assay based on ultra-high performance liquid chromatography (UHPLC) and selected reaction monitoring (SRM) mass spectrometry for the absolute quantification of six inflammatory proteins (i.e., ECP, EDN, CAL S100A8, CAL S100A9, F-MPO and A1AT) in feces. Inflammation proteins were quantified in both lyophilized and fresh fecal samples. Higher concentrations of inflammation proteins were quantified in lyophilized samples, most likely due to the contribution from the lysis of neutrophils and eosinophils, which contain these inflammation proteins and occur in feces. Lyophilization allowed us to normalize the concentration of each protein to dry weight of lyophilized feces and to achieve a lower limit of quantification. Certain inflammation proteins were cross-quantified using ELISA and results of both methods were correlated.

Conclusions:
We have developed a UHPLC-SRM method for quantitative analysis of selected inflammation proteins in feces to identify an inflammatory status and potentially a specific type of inflammation. We have cross-validate quantitative results of newly developed UHPLC-SRM assay to the results obtained using ELISA as a reference method.

Novel Aspect:
A novel multiplex method applicable to study development of gastrointestinal immune system, development of IBD disease and food allergies.

References:
Keywords: TMT, proteomic standard, QC, method development

Introduction:
Quantitative proteomics strategies using Tandem Mass Tags (TMT) enable sample multiplexing and precise measurement of protein abundance. However, co-isolated ion interference can suppress accurate ratio quantification and thereby mask true differences in protein abundance across biological systems [1]. To be able to detect and diagnose co-isolation interference, we developed the Thermo Scientific™ TMT11plex™ labeled peptide reference sample.

Methods:
Lysates from four strains of Saccharomyces cerevisiae were prepared and labeled with TMT11plex reagents and mixed in equimolar ratios. Peptides from each knockout strain were labeled in triplicate, while the parental line was labeled in duplicate. The samples were then analyzed on a range of Thermo Scientific™ Orbitrap™ instruments using LC-HCD MS2 or MS3 fragmentations. Data analysis was performed with Thermo Scientific™ Proteome Discoverer™ 2.2 software.

Results:
To develop a widely applicable TMT11plex standard, we modified the selection of yeast strains from Paulo et al. 2016 [1] and included the parental strain BY4742 as reference channels. We show the yeast triple knockout (TKO) standard has less than 20% variability with lot to lot reproducibility. We then used the TMT11plex standard to establish a standardized workflow for liquid chromatography, MS acquisition on Orbitrap mass spectrometers, and data analysis software. Synchronous precursor selection (SPS) based methods provided the best accuracy and precision as compared to MS2 Methods: Depending on precursor isolation purity and notches (fragments) selected for MS3, ratios for knock out proteins can be still distorted. We then implemented an additional filter into the Proteome Discoverer software quantitation node to exclude precursors if more than the user defined number of isolated fragments were not from the identified peptide. Implementation of a new filter significantly improved quantitation accuracy and precision for all proteins.

Conclusions:
We demonstrate that the TMT11plex standard can be used an LC-MS system suitability standard to measure and optimize protein/peptides identification, acquisition, and data analysis methods to limit co-isolation interference, while also diagnosing MS instrument status by monitoring mass accuracy, ion injection time, and reporter ion signal to noise. The TMT11plex TKO standard provides users a tool to measure and assess different mass spectrometry approaches.

Novel Aspect: We present a widely-applicable, high quality TMT11plex yeast peptide standard for LC-MS quality control and application development.

References:
447 - SOLVING THE YELLOW MYSTERY OF PAPAVER NUDICAULE WITH AN INTEGRATED -OMICS APPROACH

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Keywords: biosynthesis, integrated -omics approach, nudicaulins, Papaver nudicaule

Introduction:
Indole alkaloids are a widespread group of secondary metabolites with huge structure variety. Nudicaulins, yellow flower pigments from Papaver nudicaule (Island poppy), are unique representatives of this compound class, combining structural elements from indole and flavonoids.[1] Previous studies identified indole and pelargonidin glucosides as final precursors.[2] The aim of our work is to understand the biosynthesis of the nudicaulins.

Methods:
Our approach on the biosynthesis of nudicaulins is based on four pillars: NMR studies on their chemistry, UPLC-HRMS and LC-UV/Vis based metabolomics, transcriptomics, and DIGE-based quantitative proteomics.

Results:
The development of the P. nudicaule flowers was divided in five stages. Theses stages were monitored by transcriptomics, proteomics, and metabolomics approaches. Thereby, the most important genes and their products involved in the phenylpropanoid/ polyketide as well as indole biosynthetic pathway were identified. Relevant metabolites were semi-quantified over the flower development.

Additional experiments showed that the final nudicaulin formation from indole and pelargonidin glucosides is also possible in vitro under acidic pH conditions. This raises the question about the exact mechanism of this reaction in vivo, which is a work in progress.

Conclusions:
Based on transcriptomic, proteomic, metabolomic, and NMR studies we propose a biosynthetic pathway of nudicaulins in yellow petals of P. nudicaule. This opens new insights into this unique class of indole alkaloids.

Novel Aspect:
With the help of a multi method approach, our investigation provides access to the unique biosynthetic pathway of nudicaulins, the yellow pigments of P. nudicaule petals.

References:
Universal S-Trap sample processing: standardized reproducible sample workup from <ug to >mg scales for all sample types

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Keywords: sample preparation, digestion, sample cleanup, bottom-up proteomics, FFPE

Introduction:
Sample preparation has long hampered bottom-up proteomics. Proteins’ vastly different solubilities and myriad extraction protocols yield very different data depending on what proteins are [not] extracted. S-Trap sample processing[1] solves this problem by integrating: 1) complete 5% SDS protein solubilization; 2) total protein denaturation (pH < 1 and > 70% organic); 3) simultaneous sample concentration/cleanup; and 4) rapid reactor-type proteolytic digestion.

Methods:
S-Traps were obtained from Protifi (www.protifi.com, Huntington NY). S-Trap micros were used from sub-ug to 100 ug max, S-Trap minis from 100 – 300 ug, S-Trap midis > 300ug, and 96 well plates for high throughput applications. Briefly, all samples were extracted with 5% SDS, fully denatured, captured on the S-Trap where they were cleaned of all contaminants including Laemmli sample buffer, PEG, detergents, urea, reduction and alkylation reagents, etc. and digested.

Results:
S-Trap sample processing allowed unbiased protein analysis including poorly soluble proteins (membrane proteins, chromatin and muscle, etc.) and was successfully applied without alteration to all samples including tissues, FFPE blocks, cell lines, biological fluids, etc. Using only standard lab equipment, S-Traps enabled reproducible sample processing with < 10% CVs from protein to peptides in around 1 – 2 hrs from sub-microgram to multi-milligram scales.

Conclusions
S-Trap processing is highly reproducible (CVs typically < 10%) and independent of the properties of the proteins under study. Without change, its harmonized protocol is applicable to all kinds of samples from liquid biopsies (serum and dried blood spots [DBS]) to FFPE blocks to tissues or cell lines. S-Trap processing increases protein recovery, affording analysis of very small sample quantities, and can enable simultaneous proteomics and metabolomics.

Novel Aspect:
Use one protocol for all samples from <ug to >mg scales greatly improves reproducibility and ease of research. Throughput is greatly improved by 1 hr digests. The ability to remove contaminants is especially helpful to core labs.

References

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A STREAMLINED WORKFLOW FOR HIGH-THROUGHPUT, PRECISE, AND COMPREHENSIVE LARGE-SCALE QUANTITATIVE PROTEOMICS ANALYSIS

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Keywords: label-free quantitation, data-independent acquisition (DIA), high-throughput, reproducibility, robustness

Introduction:
A reproducible and robust global proteome profiling workflow is required to confidently determine putative protein marker panels. In this work, we illuminate the impact of key components of the DIA workflow, including chromatography, MS resolution & accuracy, spectral library quality, as well as the statistical analysis to demonstrate the label-free quantitation advantages of the HRMS1 – DIA workflow on a complex multi-proteome sample set.

Methods:
Experiments were performed on a Thermo Scientific™ Q Exactive™ HF mass spectrometer using a method consisting of full scan MS with interspersed DIA acquired for qualitative analysis. Chromatographic separations were performed using a Thermo Scientific™ UltiMate™ 3000 RSLC system, equipped for capillary-flow using a 60-minute gradient. The samples consisted of three separate proteomes (HeLa, yeast, and E.coli), which are mixed in various proportions.

Results:
The quality and breadth of the spectral library has a critical impact on the detection of peptides and proteins from DIA data sets. Therefore an exhaustive spectral library is created using multiple off-line high pH reverse phase fractions of each proteome. Quantification by DIA is based on the precursor level in the high resolution full scan. Scan cycles consisting of multiple full-MS1 scans with interspersed DIA scans are employed to improve the precision of MS1 based quantification. For robust and reproducible chromatographic separation, a 60-minute capillary LC-DIAMS method is developed without compromising sensitivity along with high-throughput. The median CV of all the experiments are < 10%. A roll-up statistics strategy is applied to calculate peptide p-Value, improving quantitation precision to 95% by removing the interferences from the real quantitative signals. As a result, the ratios of the mixed three proteomes accurately reflect the expected values.

Conclusions
An HRMS1–DIA workflow solution has been developed by streamlining each key component to achieve ease-of-operation and reproducible label-free quantitative performance in a high-throughput manner. The initial data demonstrate >7000 proteins were reliably quantified. For a higher proteome coverage, a 120-minute DIAMS method based on the nano-LC separation is developed, where ~20% more protein groups (> 8000) were quantified.

Novel Aspect:
With the novel and complete HRMS1-DIA workflow ca. 10,000 protein groups are identified with 1% FDR and ca. 8000 protein groups are quantified with 95% quantitation precision.

References

For information please contact: scientific@imsc2018.it
Introduction:
A high-throughput and streamlined analytical workflow using high resolution MS1-based quantitative data-independent acquisition (HRMS1–DIA) is standardized with well-defined experimental steps, and systematically applied to a set of samples. The study was benchmarked across multiple Cancer Moonshot sites worldwide utilizing same instrument platform, procedures, and software, and demonstrated to be stable in a 24/7 operation mode for 7 consecutive days.

Methods:
Experiments were performed on a capillary chromatographic separation with 1h gradient online coupled toQ Exactive HF mass spectrometer. HRMS1-DIA method contains multiple MS1 scans with interspersed DIA MS2 scans. The samples consisted of three separate proteomes mixed in various proportions. HeLa digest served as a quality control (QC) sample. Spectral libraries were created by performing LC-MS/MS analysis of multiple off-line high pH reverse phase fractions.

Results:
>250 QC files and >360 mixed proteome sample files are acquired by the participated laboratories in a 24/7 operation mode for 7 consecutive days.
With the 1 hour capillary LC-HRMS1 DIA workflow, > 5,000 protein groups from > 40,000 peptides of the HEla QC sample are consistently identified with 1% FDR across all sites and all 7 days. The peptide intensity correlation of all the 250 QC files is > 0.85, demonstrating ultrahigh inter-laboratory and inter-day reproducibility.
> 7,000 protein groups from > 50,000 peptides of the mixed proteome sample are reliably quantified across all 11 international labs and 7 days. > 80% proteins are overlapped identified and quantified across the laboratories. Additional to the 1% FDR, a rollup strategy is developed and applied to remove the non-reliable peptide quantitation ratios, to successfully enhance the quantitation precision to > 90%. As a result, the label-free
quantitation ratios of the mixed three proteomes accurately reflect the expected values at each laboratory across all 7 days.

Conclusions
To elevate discovery proteomics to translational research in the pipeline of precision medicine, large-cohort studies are essential in discovery and verification of protein biomarkers. Yet, reproducible proteome profiling and quantitation in different laboratories remains a challenge. This multi-site study across the globe demonstrates the high-throughput, reproducibility, and scalability of the standardized HRMS1-DIA workflow for large-cohort studies.

Novel Aspect:
Multi-site reproducibility, robustness, and method transfer evaluation for HRAM MS-based global proteome profiling using a standardized DIA analytical workflow
A.03 LIFE SCIENCES - POST TRANSLATIONAL MODIFICATIONS

747 - IMPROVEMENT OF PROTEOMIC ANALYSIS TARGETING MULTIPLE POST-TRANSLATIONAL MODIFICATIONS USING IMMUNOPRECIPITATION WITH PAN-PTM ANTIBODIES.

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Keywords: post-translation modification, systems oncology, immunoprecipitation, PTM crosstalk

Introduction:
It is widely known that post-translational modification (PTM) is important for regulation of protein activity and several cellular functions. Therefore, abnormality in those modification is closely related to oncogenesis. Thus, understanding of global PTM status significantly contribute to cancer biology and drug discovery against cancer. So far, several PTM proteomics were conducted by using immunoprecipitation with pan-PTM antibodies.

Methods:
One of examples is phosphotyrosine proteomics. When performing large-scale phosphoproteomics, phosphorylated tyrosine sites account for less than 1%. To increase identification of phosphotyrosine sites, we optimized enrichment step of phosphotyrosine peptides with immunoprecipitation, and achieved highly sensitive detection of phosphotyrosine peptides (more than 1,000 sites) from samples without pervanadate treatment [1].

Results:
In this study, we examined optimization for enrichment step of peptides containing other PTM sites. Newly developed protocol is commonly applicable for enrichment of several types of PTMs. We successfully measured several PTM status from single sample.

Conclusions
We concluded that optimization for enrichment of each type of PTM peptides is crucial for comprehensive proteomics of multiple PTM.

Novel Aspect:
This technique would lead to understand complexed crosstalk between multiple PTM signalings and find novel therapeutic targets.

References
1. Abe Y et al., Journal of Proteome Research,16(2), 1077-1086 (2017).
INTRODUCTION:

Bacillus cereus is a food-borne pathogen responsible of toxico-infections in humans. During the course of infection the survival and growth of the bacteria depend on its capacity to maintain an intracellular redox state in response to environmental changes. Its virulence depends on the production of virulence factors secreted in the extracellular milieu. In order to determine the key factors that contribute to redox homeostasis and virulence, we have performed high-throughput shotgun proteomics.

METHODS & RESULTS:

In the studies cellular proteome and exoproteome time courses were monitored by nanoLC-MS/MS. When required comparisons were performed between wild-type and the mutant of interest cells. The roles of the redox balance sensor Rex [2] and the organic hydroperoxide stress resistance system OhrRA [1] in the machinery that maintains intracellular redox state was deciphered by next-generation proteomics. Their role in toxinogenesis was highlighted through a detailed analysis of exoproteome [1, 2]. Exoproteome analysis also highlighted that toxins and other extracellular virulence factors contained oxidized methionine residues [3]. We showed that oxidation of Met residues in extracellular proteins is regulated by methionine sulfoxide reductase AB (MsrAB), a key factor of the antioxidant system, suggesting that secretion of virulence factors could contribute to maintain intracellular redox homeostasis [4]. Furthermore, interactions of bacteria genomic information and their recently acquired plasmids were reported once again through the proteomics viewpoint [4]. We also contributed to identify by proteogenomics a new key virulence factor that we named EntD which was revealed to be pivotal for pathogenesis [5], meanwhile it was missed by automatic annotator software.

CONCLUSIONS:

Bacillus cereus is well able to survive to various environment conditions. The bacteria exploits its metabolic flexibility to grow and produce extracellular toxins. We have shown that Met residues in exoproteins and their recycling by MsrAB serve as an antioxidant strategy. This project illustrates the potential of next-generation proteomics in microbiology.

REFERENCES

1086 - THE ROLE OF THIOLS IN REDOX BIOLOGY: HPLC-MS CHARACTERIZATION OF CYSTEINE DERIVATIVES GENERATED BY INTERACTION WITH COLD PLASMA JETS, POWERFUL SOURCES OF NITROGEN AND OXYGEN SPECIES.

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Keywords: thiols, redox biology, cold physical plasmas

Introduction
The proteome is a sensor and mirror of the redox state in mammalian cells [1]. Cysteine is the amino acid most sensitive to oxidation since its thiol group forms different biologically relevant chemotypes [2]. In this study, possible derivatives of cysteine formed by impact with cold physical plasma as means to produce oxygen and nitrogen centered reactive species (RONS) [3] were surveyed by high resolution MS/MS in order to predict the potential impact of plasma in vivo.

Methods
Different concentrations of cysteine standard solutions were treated by various cold plasma jets with respective parameter variation. The unknown compounds were then separated using an HILIC column in an HPLC system, and identified by using a high resolution q-TOF mass spectrometer. A relative quantification was made by relating the intensities in each spectra to the one of the L-cysteine-13C3, 15N at known concentration.

Results
A large number of oxidation products from cysteine were identified and the potential pathway of formation relative to RONS generation by the plasma was described. Modifications were mostly oxygen driven and located on the thiol moiety, with the cysteine sulfonic acid as major, but not ultimate, compound. By distinct modulation of the plasma parameters, e.g. nitrogen shielding of the effluent or addition of water to the feedgas, the formation of specific cysteine bioactive derivatives such as nitrosocysteine, cysteine-S-sulfonate, or cystine di- and tetra-sulfoxides were increased. By varying the plasma parameters, cysteine’s biochemical impact could be modulated ultimately reflecting back on the biological impact of cold plasma treatment in redox related conditions, e.g. wound healing [3].

Conclusions
Cold plasmas are valid tools to produce RONS and simulate molecular changes occurring in oxidative environments. It was observed that cysteine product profile reflects treatment conditions, allowing cysteine variants with potential relevance in redox signaling to be characterized. These observations imply a profound impact on protein thiol groups when treated by oxidizing conditions like plasma in vivo, with consequences on cellular redox signaling pathways.

Novel Aspects
Rare but potentially biologically relevant cysteine derivatives were observed after physical plasma treatment, allowing a forecast of thiol oxidation products in vitro.

References
A rapid, multiplexed kinase activity assay using iTRAQ labeling and MALDI-TOF/TOF MS

Keywords: multiplexed, kinase assay, MALDI-TOF/TOF, insulin stimulation, inhibitor

Introduction:
Substrates from chemically synthesized peptides have been used for in vitro phosphorylation using purified kinase or cell lysates. Because of its high specificity and high throughput, MALDI-TOF-MS has been a powerful platform for the detection of phosphorylated peptide substrate from enzymatic reactions. However, for screen assay, a direct readout and comparison among different experimental conditions without using internal standard would be preferred.

Methods: The peptide substrates labeled with 8-plex iTRAQ reagents were separately added in 8 different experimental conditions. For kinas activity comparison among 8 samples, the lysates of each experimental conditions containing individual iTRAQ-labeled peptide substrates were pooled for protein removal using SPE, followed by enrichment of phosphorylated peptide substrates using TiO2 plate and MALDI-TOF analysis.

Results: We found that abundant proteins in cell lysate can severely affect the purification efficiency of phosphorylated peptides on TiO2 plate. Therefore, SPE approach was first optimized to remove abundant proteins without significant influence of the recovery of phosphorylated peptides from the TiO2 plate. To enable our platform applicable in insulin stimulation and cancer drug inhibition, a list of the peptide substrates was designed. The protein amount and incubation time for kinase assay of insulin stimulation and drug inhibition were optimized. By labeling peptide substrates with 8-plex iTRAQ reagents, protein kinase activities in 8 samples can be directly compared by the mass tags on their fragmented ion spectra. The kinase activity results from our multiplexed-kinase activity MALDI-TOF assay are consistent with the results from western blots analysis.

Conclusions:
We have successfully developed a rapid and quantitative measurement of multikinase activity based on MALDI-TOF. In this assay we combined 8-plex iTRAQ labeled peptide substrates for quantification with a phosphorylated peptide substrate purification plate to rapidly determine multikinases activities in cell lysates. The effect of insulin stimulation and an inhibitory drug on the cellular protein kinase activity were successfully evaluated.

Novel Aspect:
This study is the first report to use iTRAQ-labeling strategy combined with TiO2 plate and MALDI-TOF/TOF for sensitive and high throughput multikinase activity analysis.
Keywords: Peptidomics, Database Search, Proteomics

Introduction:
In peptidomics and proteomic study, a large portion of the MS/MS spectra acquired by the data dependent acquisition (DDA) approach cannot confidently match to a specific peptide sequence. To improve the peptide and protein identification performance, a new database search approach was proposed. Performance and application of this approach were studied and discussed.

Methods:
The processed DDA analysis spectra were searched against a protein database using semi-tryptic peptide specific (sTPS) and non-enzyme specific (NES) setting in the identification tryptic peptides and endogenous peptide, respectively. The 1st to the 10th peptide matching scores for each of the MS/MS spectrum were used to develop new peptide matching score.

Results:
In this study, we showed that the score distributions of 1st and 2nd ranked hits were similar if the 1st ranked hits matched to random sequences but distributions were differentiated if the 1st ranked hits matched to forward sequences. Because the differences of the 1st and 2nd ranked score for random matched hits are smaller than the true positive hits, the use of delta score (DS) can make the true positive hits to be more discriminated from the random hits. We further developed a new scoring method named confidence scoring (CS), which considered the 1-10th matching scores of each spectrum. For the identification of tryptic peptide using NES search, the peptide identification sensitivity of CS was 12% and 2% higher than the Mascot score and DS, respectively. In the sTPS search, the number of the identified peptides of CS and DS was 12% and 25% higher than the Mascot score, respectively.

Conclusions
A new approach for performing the tryptic and endogenous peptide identification was developed. This new approach not only can improve the identification performance of endogenous peptides but also on tryptic peptides. In the analysis Tomato peptidomics, we demonstrated that the majority of the endogenous plant peptides are derived from the C-terminal of precursor proteins.

Novel Aspect:
This method does not require complex computational steps and can significantly improve the sensitivity for detecting tryptic and endogenous peptide.
Introduction:
Mass spectrometry is suitable to investigate protein post-translational modifications (PTMs). Any modifications on any proteins are detectable as mass shifts. Combinations of the PTMs are often called as ‘codes’ and they are thought to have different biological meanings [1]. Histones are representative proteins that have the code. Since the codes can be formed between PTMs on distant amino acids, typical LC-MS/MS analysis using trypsin often loses their combinatorial information.

Methods:
Histone H3 was purified from cells by histone extraction and HPLC fractionation. Histone H3 tail 1-49 amino acid long peptides are digested with AspN. LC-MS/MS with electron transfer dissociation (ETD) was performed using the Orbitrap Fusion with high-resolution MS/MS and Ion mobility separation-MS/MS analysis was performed using the timsTOF Pro. Data was analyzed with MASCOT and a developing software in house.

Results:
Histone H3 tails have multiple positional isomers of PTMs that is difficult to separate by liquid chromatography. Consequently, the obtained MS/MS spectra had the mixed ones of the positional isomers of modifications. A in-house software have been developing by using de Bruijn Graph to resolve the mixed spectra.
We are examining the effects of ion mobility separation [2]. We have obtained several species that have different drift time from one precursor, which might be positional isomers.

Conclusions:
Advanced data analysis and ion mobility separation might improve combinatorial PTM identification.

Novel Aspect:
We are developing novel software to improve PTM identification of histone middle-down analysis.

References
PROTEOME-WIDE EFFECTS OF SINGLET OXYGEN PRODUCED BY NEXT GENERATION IRIDIUM ANTI-CANCER METALLODRUGS

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Keywords: anticancer, photodynamic therapy, proteomics, Fourier Transform Ion Cyclotron Resonance (FT-ICR) MS, post-translational modification

Introduction:
New metal-based anticancer drugs have been the focus of next-generation chemotherapy. Novel Ir(III) based drugs have been designed to minimise side effects on healthy cells, while increasing selectivity and efficacy towards cancer cells by inducing reactive oxygen species (ROS) when triggered with blue visible light. The catalytic production of 1O2 with Ir-complexes, and effects on target species within cells were investigated to uncover their mechanism of action on a proteome-wide scale. [1]

Methods:
FT-ICR MS analysis was conducted using a 12T Bruker SolariX (Bruker Daltonik GmbH, Bremen, Germany) FT-ICR mass spectrometer. nLC-MS/MS experiments was conducted via coupling an EASY nano-LC II system (Proxeon, Hemel Hempstead, UK) to the SolariX FT-ICR MS, separation was achieved using a water/acetonitrile gradient and a C18 reverse phase column. All data was analysed with Data Analysis v4.2 (Bruker Daltonik GmbH, Bremen, Germany) and the Mascot database search engine.

Results:
Activation of the Ir metallodrugs caused production of reactive singlet oxygen species and extensive oxidation of nearby biomolecules. A whole range of oxidation products, up to 6 per peptide, were observed. The high resolution and mass accuracy and extensive MS/MS capabilities of the FT-ICR MS allowed unambiguous identification of the oxidation sites and the modifications induced. 1O2 was found to cause many methionine oxidation events (to sulfoxide and sulfone), but also oxidation of tryptophan residues, producing diagnostic kynurenine and 3-hydroxy kynurenine moieties. Histidine residues were oxidised to 2-oxo-histidine, which is commonly observed in 1O2 oxidation events. Bottom-up nLC-MS/MS experiments of drug-treated cancer cells revealed two key targets; aldose reductase (AR) and heat shock protein 70 (Hsp70). Quantitative nLC-MS/MS showed the increase in oxidised products in the drug-treated samples, calculated as 3.0-fold up-regulation with AR and 5.8-fold up-regulation with Hsp70, together with 9 proteins up-regulated along the glycolysis pathway.

Conclusions:
New Ir-based anticancer drug was designed to be highly and selectively cytotoxic to cancer cells when activated with visible blue light. Generation of 1O2 was confirmed by observing diagnostic oxidised species in peptides. Damage to specific cellular proteins, AR and Hsp70 was discovered in cells, induced by photodynamic therapy. 9 proteins along the glycolysis pathway were found up-regulated, which happened in the mitochondria of cells, disrupting the energy source of cancer cells.

Novel Aspect:
Top-down and bottom up FT-ICR MS/MS approaches shed light on new anticancer, catalytic photoactivated Iridium complex targets and proteome effects

References:

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Quantitative phosphoproteomics identifies alterations of Doublecortin-like Kinase 1 (DCLK1) in neural progenitor cell differentiation

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Keywords: Quantitative phosphoproteomics, Neurosphere differentiation, LC-MS/MS, DCLK1, BioID

Introduction
Oligodendrocytes are the myelinating cells of the central nervous system and originate from neural stem cells through differentiation mechanism which is only incompletely understood.[1] It has been shown that kinases play important roles in this differentiation process.[2-3] Therefore, in this study, we performed a large-scale phosphoproteomics experiment to investigate phosphoproteome alterations during the differentiation process.

Methods
Samples were harvested at 3 time points of differentiation, labeled using 3-plex dimethyl, and separated into 12 fractions using SCX chromatography. Phosphopeptides were enriched and analyzed by LC-MS/MS. For follow-up experiments, proximity biotinylation by DCLK1-BioID fusion proteins was performed: 8 BioID constructs were generated resembling distinct splice variants of DCLK1. Samples were biotin- and phosphopeptide-enriched and analyzed by LC-MS/MS.

Results
Neural progenitor cells were differentiated over 2 weeks to oligodendrocyte precursor cells. Samples were harvested at three time points: 0, 7, and 14 days of differentiation. Harvested samples were lysed, digested, dimethyl labeled and fractionated using LC-SCX fractionation. Phosphopeptides were enriched using TiO2 and analyzed by LC-MS/MS. 6963 unique phosphopeptides were quantified covering 9053 phosphorylation sites. In total, 1118 phosphosites were up- or downregulated at least 2-fold. Among the most highly upregulated candidates, several phosphorylated peptides of DoublecortinLike Kinase 1 (DCLK1) were found. Phosphomimetic studies showed that DCLK1 phosphorylation alters its stability. We therefore employed BioID[4]-DCLK1 fusion proteins to investigate DCLK1 substrates and interaction partners. In these experiments the phospho-enriched fractions revealed 2574 phosphosites representing 2086 phosphopeptides from 1229 phosphoproteins while the flow through yielded 33623 peptides from 4616 proteins.

Conclusion
9 residues of DCLK1 were found to be differentially phosphorylated during the differentiation process. Site directed mutagenesis revealed that phosphorylation in the SP-rich domain of DCLK1 altered its stability. Applying a combination of BioID-DCLK1 fusion proteins and phosphopeptide enrichment, Neurofilament medium polypeptide (NEFM), Filamin-A (FLNA), and Fatty acid synthase (FASN), mTOR, and CAMKK1 were found as possible DCLK1 interaction partners.

Novel Aspect
First phosphoproteomic analysis of neural progenitor cell differentiation identifying differential phosphorylation of DCLK1 and follow up by BioID fusion proteins.

References:
Guardiola-Diaz HM., Ishii A., Bansal R., Glia., vol. 60, 476-486
82 - COMPREHENSIVE NISTmAb REFERENCE SPECTRAL LIBRARIES FOR RELIABLE AND ROBUST IDENTIFICATION OF POST-TRANSLATIONAL MODIFICATIONS IN HUMAN AND RECOMBINANT IGG ANTIBODIES

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Abstract for THE 22nd INTERNATIONAL MASS SPECTROMETRY CONFERENCE (IMSC) 2018

Comprehensive NISTmAb reference spectral libraries for reliable and robust identification of post-translational modifications in human and recombinant IgG antibodies

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Key Words:
Monoclonal antibodies, post-translational modifications, spectral library, 1D/2D LC-MS, glycation

Introduction:
We report an alternative peptide and PTM identification method based on matching experimentally derived spectra in a comprehensive library of the NISTmAb IgG1κ. We show that this method is advantageous over the database tools for reliably determining low levels of biologically modified species in antibody drugs. The simultaneous detection of these modifications, challenging for conventional methods, can be achieved without using special settings or procedures.

Methods:
500μg of NISTmAb was denatured using various chemicals and solvents in 50μL of 100mmol/L Tris HCl buffer at room or 85 °C. Parameters for 13 protocols were used during denaturing, reduction, and alkylation of the NISTmAb. Each sample was digested with trypsin for 0.25, 2, and 18h. 1D/2D LC-MS was used for various digestion conditions. Initial identifications were made using MS-GF+ search engine against a fasta-file containing the sequence.

Results:
We created a high-resolution mass spectral library of all identified peptides produced in the tryptic digests of a humanized IgG1κ mAb reference material (NISTmAb). The reference library contains over 12,600 high-quality tandem spectra of more than 3,300 peptide ions identified and validated by accurate mass, differential elution pattern, and expected peptide classes in peptide map experiments. These include a variety of biologically modified peptide spectra and method-induced artifacts obtained from 1D/2D LC-MS/MS analyses. The 12 varieties of spectra included in the library are derived from peptides of unmodified major classes, N-linked glycosylated, oxidized, deamidated, modified N-/C terminal, glycated, metal-ion adducted, overalkylated, and other types of modifications. A complete glycation profile was obtained for the NISTmAb with spectra for 58% and 100% of all possible glycation sites in the heavy and light chains, respectively. The site-specific quantification of methionine oxidation in the protein is described.
Conclusions:
The library of annotated spectra of all NISTmAb peptide ions acquired in this work, along with the library search software, is freely available. The general utility of this library is demonstrated by the analysis of Humira, where 60% sequence coverage for constant regions of this commercial mAb. The NISTmAb library platform may be used as a tool for facile identification of the primary sequence and PTMs for human and recombinant IgG antibodies.

Novel Aspect:
Facile, reliable, and simultaneous identification of low levels of biological modifications in mAbs by advanced NISTmAb reference material spectral library.
207 - SEAGAL: THE METHOD TO FISH FOR UNCOMMON PHOSPHORYLATIONS

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Keywords: phosphorylation, enrichment, gallium, immunopurification

Introduction:
Traditional methods for phosphopeptide purification are reported to introduce strong bias and hence detected phosphorylations only partially reflect the true phosphoproteome distribution of the cell [1]. Importantly, part of phosphoproteome, especially, N-linked phosphorylations (i.e. histidine, arginine, lysine) are generally out of the scope of conventional enrichment methods, despite high interest in those type of phosphorylation.

Methods:
The mixture of phosphopeptides was modified by the recently developed by dimetallic gallium complex [2] in 20 mMtriethylammonium bicarbonate (pH 8). Modified peptides were enriched by immunopurification using an immobilized antibody against the introduced metal complex and analyzed by LC-MS/MS. Raw data analysis was performed in Thermo Proteome Discoverer 2.2, including home-built data deconvolution and pre-processing step and conventional database search.

Results:
The developed dimetallic complex is highly specific for the phosphate moiety, irrespective of its nature, thus, the same method allows simultaneous enrichment of peptides having O-linked (Ser, Thr, Tyr) and N-linked (His, Lys, Arg) phosphorylation. The modification and enrichment are performed at close to neutral pH (optimal range 5 – 8), thus, avoiding destabilization of N-linked phosphorylation and providing less bias of the phosphoproteome. Modified peptide and all fragments containing the modification display distinct isotopic pattern preventing efficient data analysis by conventional proteomics tools. To solve this issue, data pre-processing software was developed as a processing node for Thermo Proteome Discoverer. The source code and complete description of algorithms are made available for the community. Alternatively, the introduced modification could be removed by pyrophosphate in a simple one-step process compatible with conventional LC-MS-based proteomics.

Conclusions:
The proposed phosphopeptide enrichment approach is based on specific phosphate-capturing dimetallic complex and immunopurification of complexed phosphopeptides. The enrichment can be performed at neutral pH, preserving labile modifications. The present proof-of-concept study clearly indicates the potential of the method, however, further optimization is necessary to apply the method in biologically-relevant cases.

Novel Aspect:
The presented method allows for obtaining comprehensive information on O- and N-linked phosphoproteome simultaneously at neutral pH.

References:
Pan, C., Gnad, F., Olsen, J. V., Mann, M. Proteomics, 8, 4534-4546 (2008)
ANALYSIS OF PROTEIN-PHENOLIC COMPOUND MODIFICATIONS USING ELECTROCHEMISTRY COUPLED TO MASS SPECTROMETRY

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Keywords: Electrochemical oxidation, phenolic compounds, adduct formation, EC-MS, phenol-protein interaction

Introduction:
Posttranslational modifications of proteins are associated with influencing the essential structural and regulatory properties of each protein. For example, one of these post-translational modifications is the covalent addition of other food compounds to diverse side-groups of (food) proteins or peptides [1]. Influences on the biological properties such as the enzyme activity [2], the nutritional protein quality [3], and changes in the allergenicity of the proteins/protein derivatives, seem to be more or less obvious.

Methods:
Electrochemical oxidation of phenolic acids (e.g., chlorogenic acid) was performed using a preparative electrochemical thin-layer cell consisting of a boron-doped diamond working electrode. A solution of the phenolic acid was injected into the electrochemical cell and a constant potential was applied [4,5]. Detection of oxidation products was performed using an ESI-MS ion trap mass analyzer in negative ion mode. Subsequently, a second flow system was used to combine both analyte solutions, phenolic acid and mixture of tryptic peptides of a dietary protein e.g., alpha-lactalbumin, to generate and detect potential adducts.

Results:
After optimizing the reaction parameters for the four phenolic compounds selected, various oxidation products were detected and identified by means of ESI-MS. By connecting a flow system with a tryptic peptide solution of digested dietary proteins, oxidation products of the phenolic substances, and further reaction products of phenolic oxidation products and tryptic peptides were identified. It has been shown that an electrochemically induced oxidation of the model phenols is possible as well as a reaction of the oxidation products with selected, previously tryptically digested dietary proteins. A preparation of the reaction products in higher concentrations could be generated by preparative LC. Structural elucidation of the resulting products by means of NMR are carried out.

Conclusions:
Electrochemistry coupled with mass spectrometry was successfully used to investigate the redox potential of dietary phenolic acids such as chlorogenic acid. It was possible to create optimized conditions for the oxidation of chlorogenic acid to generate a wide variety of oxidation products. Electrochemically oxidation of chlorogenic acid, caffeic acid, ferulic acid, and sinapinic acid generated a variety of reactive intermediates. Feasibility of this technique for the research topic was shown.

Novel Aspect:
The young field of electrochemical coupled mass spectrometry (EC-MS) is a suitable system for generating and identifying potential metabolites. Target compounds can be identified directly by mass spectrometry. After separation of generated metabolites, structural elucidation can occur in following steps.

References:
# A NEW PHOSPHOPROTEOME ENRICHMENT STRATEGY IN SMALL-SCALE USING PHOSPHO-SPECIFIC ANTIBODIES WITH ONLINE MHER-NANOLC-ESI-MS/MS

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Keywords: Phosphoprotein enrichment, phospho-specific antibodies, MHER, nLC-ESI-FT orbitrap-MS/MS

**Introduction:**

Phosphoprotein is involved in cellular processes such as proliferation, cell signaling, and apoptosis.[1-3] Mass spectrometry is commonly used to identify both phosphorylated protein(s) and its site(s). However, phosphoproteins in MS analysis have several limitations due to low abundant and low ionization efficiency caused by negative charge of phosphates in a positive mode.[4-5] Herein, we introduce a MW sorting-based new strategy for phosphoproteomics.

**Methods:**

The tryptic peptides from MCF7 were mixed with phospho-antibodies (pAbs, above 150 kDa), and followed by which the mixtures introduced into the MHER (50-100 kDa in MW-cutoff) with continuous flow rate of 5 uL/min. During breakthrough run, ordinary peptides having no affinity with pAb are first washed out through membrane wall of HF. The remaining peptides bound to pAb can be simultaneously exited via online tryptic digestion in MHER for next nLC-ESI-MS/MS run.

**Results:**

For MHER-based phosphopeptide enrichment, we optimized the experimental conditions according to the kind of pAbs, reaction time between peptides and pAbs, reaction temperature, reaction ratio, and the length of MHER. From our experimental results, the enrichment yield of phosphopeptieds was dramatically enhanced when used the mixture of phospho-serine, -threonine and -tyrosine antibodies at the ratio of 1:1 (proteins/pAbs, w/w) reacted for 1 hours at 4 °C. Finally, we found total 11282 phosphorylation site from 5673 phosphopeptides of MCF7 cells utilizing online MHER-2D-nLC-ESI-MS/MS and confirmed that the pAbs-based enrichment coupled with online MHER can be the best way with the small amount of proteins (~10 ug) for phosphoproteomics than conventional methods [e.g., immobilized metal affinity chromatography (IMAC), titanium dioxide (TiO2), and filter aided sample preparation (FASP)] with a large amount (above 500 ug).

**Conclusions**

We found that the developed pAbs-based online MHER is highly selective, precise, and suitable for phosphopeptide enrichment with the small amount of proteins, compared to that of conventional methods (IMAC, TiO2, and FASP). Additionally, our phosphopeptide enrichment strategy with online MHER is a promising tool for phosphoproteomics, and for the development of therapeutic agents.

**Novel Aspect:**

The developed a MHER-based phosphoproteome enrichment platform is one of advanced strategy for understanding the biological functions of protein PTMs study.

**References**

Keywords: Histone modifications, epigenome, ETD, UVPD

Introduction:
Mass spectrometry is a powerful tool for protein post-translational modifications (PTMs) analysis. A state-of-the-art mass spectrometer distinguishes very small mass differences. Histone has many PTM sites and their theoretical PTM combinations can be above trillion. Semi-bottom-up and middle-down proteomics are used for PTMs identification or decision of their combinations. Here, we used middle-down proteomics and compared various fragmentation methods.

Methods:
Purified histone or histone H3 HPLC fraction were digested with AspN to obtain 49 or 23 aa length of H3 or H4 tails. They were fragmented with CID, ETD, or UVPD method and were analyzed by Mascot database search for PTM identification. The identification number and the identified kinds of PTMs were compared. The search was performed after deconvolution into the masses of singly charged ions.

Results:
Although all fragmentation methods enabled PTM identifications, ETD showed relatively accurate identification in the middle-down proteomics. However, it was difficult to differentiate citrullination from z, z+1, z+2 ions generated by hydrogen rearrangement in ETD. In addition, the molecules of molecular weights above 5kDa with multiple charged ions showed deformed isotope pattern of distributions, which make it difficult to determine monoisotopic ions. We have developed an algorithm to determine monoisotopic ions by matching to theoretical isotope distributions and compared with the Advanced Precursor Detection (APD) in the Orbitrap Fusion Lumos. Moreover, CID and UVPD were compared and the CID was suitable for shorter peptide analysis. Longer peptide analysis was better in UVPD and the UVPD generated relatively frequent b, y series ions.

Conclusions:
Appropriate combination of ETD, CID, and UVPD and selection of the optimized fragmentation method according to molecular size and charge might improve the accuracy of histone PTMs analysis. Further, the PTM site locations are examined.

Novel Aspect:
We have compared the fragmentation methods to reveal complicated histone PTMs.

References
Greer et al. Proteome Res. 17, 1138–1145 (2018)
Introduction:
Post-translational modifications (PTMs) on monoclonal antibodies (mAbs) are potential critical quality attributes that define therapeutic efficacy and safety [1]. Mass spectrometry based peptide mapping (PM) is the gold standard methodology for the in-depth study of PTMs on mAbs. Artificial PTMs, however, may be induced during sample preparation for PM, e.g. asparagine deamidation [2]. The current work describes the effect of digestion pH and incubation time on Trastuzumab PTMs. Trastuzumab, marketed as Herceptin, is a humanized IgG1 mAb used in the treatment of HER2 positive breast cancer.

Methods:
The regular trypsin digestion protocol for peptide mapping involved the use of RapiGest, dithiothreitol (DTT), and iodoacetamide (IAM) in Tris-HCl buffer at pH 7,5. The low pH protocol, based on a commercial kit from Promega, made use of guanidine-HCl, Tris(2-carboxyethyl)phosphine (TCEP), IAM, trypsin, and low pH resistant rLys-C. Peptide mapping was performed on an Agilent Technologies 1290 Infinity UHPLC and Agilent Technologies 6540 Accurate-Mass Quadrupole Time-of-Flight LC/MS. Peptides were separated on a Waters Acquity UPLC CSH 130 C18 (2,1 x 150 mm, 1,7 µm) analytical column operated at 60°C at a flow rate of 0,4 mL/min using 0,1% formic acid in water or acetonitrile.

Results:
Post-translational modifications of Trastuzumab peptides produced under regular alkaline, and low pH digestion conditions were identified and compared. Aspartate isomerization, methionine oxidation, N-glycosylation, N-terminal cyclization, and asparagine deamidation were PTMs of primary interest. Digests with incubation periods of 2, 4, 6, and 16 hours were obtained and analyzed by LC-MS. Results show the progression of artificial modifications, especially asparagine deamidation, using regular trypsic digestion conditions with longer incubation periods up to 16 hours at 37°C. On the contrary, the low pH digestion protocol showed less identified artificial modifications while providing longer peptides and higher sequence coverage. Moreover, modifications not associated with sample preparation were identified, such as oxidation, deamidation, glycosylation, and cyclization of certain peptides.

Conclusions:
The accurate determination of PTMs is important to ensure the quality of therapeutic mAbs. Artificial PTMs introduced by sample preparation using alkaline trypsinic digestion can be reduced with shorter incubation times while providing sufficient peptide profile and protein sequence coverage. Acidic digestion effectively prevents modifications caused by sample preparation.

Novel Aspect:
Post-translational modifications of Trastuzumab under different digestion conditions were identified and compared. Shorter incubation times for regular trypsin digestion or the use of low pH digestion conditions is advised.

References

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IDENTIFICATION AND CHARACTERIZATION OF O-XYLOSYLATION AND O-GLYCOSYLATION OF RECOMBINANT PROTEIN THERAPEUTICS BY MASS SPECTROMETRY

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Keywords: antibody fusion proteins, post-translational modification

Introduction:
To connect the different functionalities of antibody fusion proteins in a single molecule G4S linker are often used. O-XYlosylation is a recently described post-translational modification (PTM) of a heterogeneous glycan structure on the serine of the G4S linker [1, 2]. Content, structure vary between different therapeutic proteins and expression hosts. O-Glycosylation is a known PTM with well described glycan structures [3, 4] and located on serine or threonine.

Methods:
Intact measurement: Protein samples were N-deglycosylated and desalted by size exclusion chromatography. ESI mass spectra were recorded on a Q-TOF instrument (maXis, Bruker) equipped with a nano ESI source (TriVersa NanoMate, Advion).
Peptide Mapping: Protein samples were denatured and reduced, carboxymethylated and digested by trypsin. LC/ESI-MS/MS was done on an Orbitrap Fusion system (Thermo). Peptides were separated using RP HPLC (Waters NanoAcquity).

Results:
Here we describe mass spectrometry based methods to detect, differentiate and locate O-Xylosylation and O-Glycosylation in recombinant protein samples. We will show that intact measurement of deglycosylated antibody derivates using ESI-TOF-MS is an easy and reliable method to quickly get an initial impression of present protein modifications which exist with significant amount. A major disadvantage of this method is that detected O-Xylosylation signals are often overlaid by other modification or adduct signals or detected peaks are not fully resolved. Measurements after deglycosylation and reduction can provide remedy regarding peak resolution issues. But, as we will show, LC/MS-MS peptide mapping using an Orbitrap Fusion is the method of choice to get a more detailed view on the modification, to locate the particular modification site in protein sequence and to make an exact estimation of the O-Xylosylation and O-Glycosylation content.

Conclusions:
Detection and quantification of O-Xylosylation and O-Glycosylation in antibody fusion proteins using the combination of intact measurement and peptide mapping.

Novel Aspect:
Detection of O-Xylosylation and O-Glycosylation of recombinant protein therapeutics.

References
958 - SPECIFIC MASS TAG OF METHYLATED AMINO ACID VALIDATES THE SUMOYLATION SITES BY HCD ON ORBITRAP MASS SPECTROMETER

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Keywords: SUMOylation, methylated, HCD, Orbitrap

Introduction:
The identification of SUMOylation sites by mass spectrometry is not easy because of the large SUMO chain remnant attached to peptides after tryptic digestion. Therefore, the widely adopted method is still the site-directed mutagenesis. Here, we will demonstrate the utility of specific mass tag from the methylated terminal to facilitate and validate the identification of SUMOylation sites by HCD on Orbitrap mass spectrometer without mutagenesis.

Methods:
The digested peptides are reductively methylated using formaldehyde-d2 and subsequently subjected to LC/MS/MS analysis with LTQ-Orbitrap Elite. The digested peptides were analyzed in data-dependent mode by LC/MS/MS. The data-dependent acquisition method consisted of ten sets of HCD/CID scans. Here, the HCD spectrum is used for monitoring specific a1 ions, and the CID spectrum is used for peptide sequencing.

Results:
The signal enhancement for a1 ions of dimethyl labeling peptides in the CID of Q-TOF instrument had observed and applied for identifying the N-terminal amino acid.1-3 In this study, we identified the spectra of SUMOylated peptides by monitoring two a1 ions of HCD spectrum, one is from the SUMO chain remnant and the other is from the SUMOylated peptide. The optimal collision energy of HCD for monitoring two a1 ions simultaneously was 40%. For peptides sequencing, the identification of the MS/MS spectra generated by the SUMOylated peptide using the Mascot database search is still difficult. To address this problem, the candidate spectra selected by specific mass tag were submitted to do the sequence match by the software “ChopNSpice”.4 The results showed the specific mass tag of methylated amino acid could dramatically narrow down the candidates of possible spectra, thus the number of identified spectra were more than the one without spectra selection before ChopNSpice search.

Conclusions:
We use a di-SUMO-2 protein as a model to demonstrate the method and successfully applied to identify the SUMOylated sites on the p53 protein. The results show the methylated mass tag (two specific a1 ions) is suitable for screening SUMOylated spectra in complex protein mixture. This method is more sensitive than direct database search, and it also greatly reduced the false identification.

Novel Aspect:
The method could dramatically narrow down the candidates of possible spectra and is more sensitive than direct database search for SUMOylation sites identification.

References:
Introduction:
Post translational modifications (PTMs) are important indicators of change in cells. Two of the top four most abundant PTMs are deamidation and phosphorylation and the location of the phosphorylation site is an important factor in understanding the effect of this modification on the activity of proteins.

Results:
Traditional LC-MS methods can sometimes struggle with the identification of positional isomers of phosphorylated peptides as they are identical in mass and may have not separate by traditional reverse phase LC. Phosphorylated peptides are often polar, and elute early in reverse phase chromatography (especially when more than one phosphorylation site is present on the peptide) which can make them difficult to detect by trap-elute LC-MS. LC-MS methods also struggle to identify and quantify Aspartate and iso-Aspartate isomers (associated with deamidation) as they have the same mass and similar fragmentation patterns which often result in false positive identifications.

Conclusions:
As CE separates analytes based on their charge and size (a different mechanism to LC) it can often overcome some of the separation challenges seen by LC. In this poster we will show how CESI-MS has been used to tackle these challenges and how it compares to LC-MS and find out how it has been applied to real biological samples in proteomics research.

Novel Aspect:
Separation of isobaric and/or co-eluting post-translational modifications by CE-MS
Introduction:
Transforming growth factor β-induced protein (TGFBIp) is an extracellular matrix (ECM) protein composed of an NH2-terminal cysteine-rich domain (CRD) and four fasciclin-1 (FAS1-1 to FAS1-4) domains. Previous studies have shown that TGFBIp is cross-linked to type VI and type XII collagen through a reducible bond [1,2]. TGFBIp contains 11 cysteine residues and is thus able to form five disulfide bonds, leaving a single cysteine residue available for the cross-link.

Methods:
Recombinant human TGFBIp was digested with Glu-C and trypsin to get cleavages between all cysteine residues except Cys84 and Cys85. The generated peptides were separated by strong cation exchange (SCX) followed by reversed-phase high-performance liquid chromatography (RP-HPLC) and analyzed by mass spectrometry (MS) and NH2-terminal sequencing. For verification, the disulfide bond pattern of TGFBIp from human cornea was also analyzed by MS.

Results:
The NH2-terminal CRD contains six cysteine residues, and one of these (Cys65) was identified as the candidate for the reducible cross-link between TGFBIp and the ECM molecules type VI and type XII collagen. This residue was not engaged in any disulfide bond, but was modified by a cysteinylation. The cysteinylation can be exchanged with a cysteine residue of another protein forming an intermolecular cross-link. In addition, the CRD contained two intradomain disulfide bonds (Cys49-Cys85 and Cys84-Cys97) and one interdomain disulfide bond to the FAS1-2 domain (Cys74-Cys339). The cysteine residues in the FAS1-3 domain (Cys473 and Cys478) were shown to form an intradomain disulfide bond. Finally, an interdomain disulfide bond between the FAS1-1 and FAS1-2 domains (Cys214-Cys317) was identified. The disulfide bond pattern was verified in human corneal tissue and Cys65 was also found in a cysteinylated form.

Conclusions
The results contradict the predicted assignment of the CRD as an emilin domain [3] and we have renamed the domain to the cysteine-rich domain of periostin and TGFBIp (CROPT). The interaction to the ECM is through Cys65 of the CROPT domain. The interdomain disulfide bonds indicate that the NH2-terminus of TGFBIp adopts a compact globular fold. This together with the disulfide bond pattern has recently been validated by a crystal structure of TGFBIp [4].

Novel Aspect:
We identified Cys65 of the renamed NH2-terminal CROPT domain to be the residue forming a reducible bond to other ECM molecules such as type VI and type XII collagen.

References
Garcia-Castellanos R., Nielsen N. S., Runager K., Thøgersen I. B., Lukassen M. V., Poulsen E. T., Goulas T., Enghild J. J.,
Introduction:
Diatoms are unicellular algae that use highly specialized proteins to produce nano silicified cell walls. These proteins, termed silaffins, share no homology across diatom species. Silaffins are heavily modified [1] and their lysine residues bear polyamine chains [2]. However, their structure and physiological role remain elusive.

Methods:
Total biosilica extracts from Thalassiosira pseudonana, T. oceanica and Cyclotella cryptica species were isolated as described [3], subjected to acidic hydrolysis and derivatized with AccQ•Ultra Reagent (Waters). Lysine derivatives were quantified by LC-MS and confirmed by MS/MS on Q-Exactive HF mass spectrometer (Thermo Scientific). Modifications were mapped to protein sequences by GeLC-MS/MS [4] on OrbitrapVelos mass spectrometer (Thermo Scientific).

Results:
We developed a method to identify and quantify polyamine-modified lysines and map them back to silaffin sequences. In total, 17 novel modifications were discovered, including acid-resistant phosphoester-containing polyamines. We demonstrated that the pattern of polyamine modifications reflects the phylogenetic proximity of the diatom species. Modified lysine residues were identified by polyamine-specific fragments in MS/MS spectra followed by iterative searches and deconvolution of raw MS/MS spectra. We localized 130 polyamine-modified sites in 26 proteins from three diatom species and revealed 3 consensus motives common to all three diatoms.

Conclusions:
We report a novel approach to identify, quantify and localize 130 polyamine modifications in three diatoms and demonstrated that they occurred at conserved consensus motifs, despite full sequences of the modified proteins were not conserved.

Novel Aspect:
Identification of consensus motifs of polyamine modifications

References:
805 - CHARACTERIZATION OF NIST MONOCLONAL ANTIBODY ON INTACT, SUBUNIT, AND PEPTIDE LEVEL WITH MONITORING OF CQA’S ON THE 6600

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Keywords: Mass Spectrometry, Biologics, Antibodies, MAM, Biopharmaceuticals

Introduction:
Monoclonal antibodies (mAbs) can be incredibly complicated with hundreds of residues, varying degrees of glycosylation and PTMs which may be critical to quality. MS can uncover a wealth of information of these mAbs with a synergistic workflow used to maximise data quality and fidelity. Here BioPharmaView™ and the 6600 characterize the NIST mAb on an intact, subunit, and peptide level. Additionally the MAM workflow in BioPharmaView™ has been used to control CQAs.

Methods:
All measurements were carried out in replicates on a 6600 Q-ToF equipped with IonDrive™ source and the high flow ExionLC™ system. For all analyses solvent A consisted water containing 0.1 % formic acid and Solvent B consisted acetonitrile containing 0.1 % formic acid. Intact and subunit analysis were performed with a C4 column with a flow rate of 200 ul/min at temperatures of 80°C and 60°C respectively. Peptide mapping was performed with a C18 column at 250 ul/min at 50°C.

Results:
As complexity of biopharmaceuticals increases the need for accurate reliable data acquisition becomes ever more important. The NIST mAb has five major intact glycoforms G0F/G0F, G0F/G1F, G1F/G1F, G1F/G2F, and G2F/G2F representing one glycan on each heavy chain Fc region. The ratios of these various glycoforms are highly regulated and have been measured accurately after reconstruction with BioPharmaView™. Due to the number of overlapping features at high charges states features can be difficult to discern at the intact level, these features can become clearer at the subunit level. The glycoforms on the subunit level are clearer to distinguish due to the reduced complexity of a single glycan per fragment. The subunit fragments were matched with the BioPharmaView™ software providing the same glycan ratios as on the intact level.

The NIST mAb was digested and analysed with the 6600 with a SWATH analysis. Data was analysed with BioPharmaView™ software with 100 % of the protein sequence observed at the MS level and validated via MS/MS fragmentation.

Conclusions
As biologics become increasingly heterogeneous the need for complete characterization across all points in upstream and downstream production increases. Sciex provides a complete workflow solution for LC-MS analysis of complex protein products with Multiple Attribute Monitoring of Critical Quality Attributes.

Novel Aspect:
Complete characterization of NIST antibody and glycoforms with MAM analysis in BiopharmaView™ software.

References

For information please contact: scientific@imsc2018.it
Keywords: Phosphoproteomics, signaling, kinase, DIA, Ti-IMAC

Introduction: Protein activity is mainly modulated by dynamic reversible post-translational modifications (PTMs) such as site-specific phosphorylation, which regulates essentially all cellular processes. Despite continuous improvements, global analysis of protein phosphorylation is still challenging due to its sub-stoichiometric nature and low abundance.

Methods: We present a high-throughput IMAC based enrichment method to routinely quantify ~7000 phosphopeptides in 15 min of LC-MS/MS time on a Q Exactive HF-X MS [1]. But we have now reached the sequencing speed limit for data-dependent analysis (DDA) for phosphoproteomics. To overcome this, we have used data-independent analysis (DIA) for phosphoproteomics based on project-specific spectral libraries.

Results: To directly compare DDA and DIA in a biological setting, we performed a global kinase-substrate analysis, where we treated EGF stimulated RPE1 cells with three different MEK inhibitors. Phosphopeptides were enriched from only 200ug of peptides per sample using Ti-IMAC in 96-well format and analyzed with both DDA and DIA using label-free quantification. For the DIA analysis we generated a project specific spectral library containing ~55,000 unique phosphopeptides. From the DDA analysis we quantified ~7000 phosphopeptides per sample, whereas from the DIA analysis, we were able to quantify ~20,000 phosphopeptides per sample with few missing values. From the DIA runs we were also able to reproducibly identify ~400 phosphotyrosine-containing peptides in each sample, which is comparable to antibody-specific phosphotyrosine enrichments. Reassuringly, we found the same biological kinase signature using both DDA and DIA with strong overrepresentation of proline-directed ERK substrate motif sites among the inhibited phosphorylation sites.

Conclusions:DIA holds great promise for rapid phosphoproteomics profiling with deep coverage and accurate label-free quantification with few missing values.

Novel Aspect: Rapid DIA-based quantitative phosphoproteomics of hundreds of cell states

References

For information please contact: scientific@imsc2018.it
Introduction:
Development of therapeutic mAbs includes real time and accelerated stability studies to address critical Quality Attributes. Typically, these include e.g. protein structure, formation of particles as well chemical modifications of amino acid residues. This case study presents results from LC-MS analyses of samples from a stability study of a humanised IgG, which showed a storage temperature/ duration dependent increase of unknown variants within HIC analyses.

Methods:
Samples stored at -70 °C/ +5 °C/ +25 °C for 12 months and +30 °C for 6 months were analysed by LC-MS ESI Q-TOF using a Waters UPLC-Class coupled to a Waters Synapt G2-S. Performed analyses covered top down and multiple middle down analyses of IgG and its subunits using reducing conditions and/or IdeStreatment. Further analyses included bottom up analyses like tryptic and chymotryptic peptide mapping and determination of free thiol levels and disulfide mapping.

Results:
LC-MS top down analyses resulted in detection of slightly increased protein fragment levels for samples stored at +25 °C and +30 °C, as well as detection of an additional hydrophilic light chain species without mass shift in middle down analyses. Bottom up analyses of these samples (+25 °C/ +30 °C) by peptide mapping resulted in slight increase of methionine oxidation, asparagine deamidation and aspartate isomerisation of DG motifs. Free thiol levels as well as scrambled disulfides in disulfide mapping showed slight increases at +25 °C and +30 °C. In-depth data evaluation of peptide mapping data indicated high levels of three additional retention time-shifted peptide species. These peaks were further investigated by LC-MS including electron transfer dissociation resulting in the identification of two discretenon-DG aspartate isomerisation sites in the variable domain of the light chain and one isomerisation site in the hinge region of the heavy chain.

Conclusions:
LC-MSETD analyses of samples stored at +25 °C/ +30 °C detected elevated isomerisation levels of three non-DG motif aspartates. Two of them are located in the CDR1 and CDR2, therefore impact on the target binding was suggested. Analyses like DLS, fluorescence spectroscopy, RALS, ITF, IR and UV absorbance detected no major differences. Thus only in-depth LC-MS analyses revealed the root cause of additional HIC peak. Identified isomerisation sites were assessed as cQAs.

Novel Aspect:
Unexpected high isomerisation levels for three Aspartates during the accelerated stress study have been identified.
1375 - HIGH RESOLUTION MASS SPECTROMETRY CHARACTERIZATION OF THE OXIDATION PATTERN OF METHIONINE AND CYSTEINE RESIDUES IN HUMAN MITOCHONDRIA VOLTAGE-DEPENDENT ANION SELECTIVE CHANNEL ISOFORMS

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Keywords:
Voltage dependent anion selective channel isoforms; Cysteine over-oxidation; Mitochondrial intermembrane space; High Resolution mass spectrometry

Introduction:
Voltage-dependent anion selective channels are a quantitatively relevant component of the mitochondrial proteome. They are located in the outer membrane, which separates the cytosol from the inside of the organelle [1] and are responsible for the exchange of ions and small molecules in and out of mitochondria. Since the mitochondrial intermembrane space has an oxidative potential, we have investigated the oxidation state of cysteine and methionine residues of hVDACs.

Methods:
Intact mitochondria, extracted from HAP1 cell culture, were reduced with DTT, alkylated by addition of iodoacetamide and then lysed. After centrifugation, the supernatant containing the mitochondrial proteins was loaded onto a hydroxyapatite column. The hVDACs enriched eluate obtained was desalted, in-solution digested using porcine trypsin or chymotrypsin, and the resulting peptide mixtures analyzed by UHPLC/High Resolution nanoESI-MS/MS.

Results:
Our results demonstrate that the all the three mitochondrial hVDACs, in physiological state, contain all the methionines partially oxidized to methionine sulfoxide. Furthermore, in the isoform 1 Cys127 is detected exclusively in the oxidized form of sulfonic acid, while the Cys232 is observed totally carboxyamidomethylated. In the hVDAC2, the cysteine residues 8, 13, 47, 133, 210, and 227 are only carboxyamidomethilated, Cys138 was identified trioxidized, and the cysteines 76 and 103 are partially in the form of sulfonic acid. Finally, also for the isoform 3 the results confirmed the presence of some cysteines (2, 8, and 229) reduced and the partial oxidation of Cys65 and Cys65. The peculiar behavior of Met and Cys residues of hVDACs may be related with the accessibility of the protein to a strongly oxidizing environment and may be connected with the regulation of the activity of this trans-membrane pore proteins.

Conclusions:
In this work, adopting the same procedure developed for the structural investigations of rVDACs [2,3], a detailed profile of the oxidation state of methionine and cysteine residues have been obtained in all the three human isoforms. Our results demonstrate that the mitochondrial hVDACs, in physiological state, contains methionines oxidized to methionine sulfoxide and cysteines in the form of sulfonic acid.

Novel Aspect:
The structural features elucidated by the present work may be helpful for a better understanding of the functional role of these proteins in the cell.

References
Introduction:
Analysis of protein phosphorylation by mass spectrometry serve as a powerful tool for biologists and clinical researchers. However, the abundance differences as well as the variety of phosphorylation sites are pushing the need for instruments with higher sensitivity and selectivity. Here we evaluate the performance of a dual trapped ion mobility separation (TIMS) - QTOF that provides an additional dimension of separation and higher peak capacity.

Methods:
Phosphopeptides were enriched from 50-200 µg of proteolytic digests, separated using 15 to 90 min nano-HPLC gradients (nanoElute, Bruker Daltonics) and analyzed on a timsTOF Pro instrument using the Parallel Accumulation Serial Fragmentation (PASEF) method (Bruker Daltonics). Post-processing analyses were performed in PEAKS (Bioinformatics Solutions Inc.) and MaxQuant (Jürgen Cox, Max Planck Institute of Biochemistry).

Results:
Applying the PASEF method on the timsTOF Pro instrument enables on average the selection of 12 precursors within 100 ms. In addition to the high speed, sensitivity in the timsTOF Pro is achieved by a close to 100 % duty cycle of the dual TIMS cell design, cleanup of MS/MS spectra by ion mobility and time and space focusing in the TIMS analyzer. Using the combination of speed and sensitivity in PASEF method we identified more than 66,000 peptide spectrum matches (PSMs) resulting in around 25,000 unique peptides and 16,000 unique phosphopeptides within a 90 min gradient run. In addition to m/z separation, phosphopeptides are separated by space to charge, which increases the peak capacity with the most pronounced effect on very short gradients. Together, the high speed of PASEF phosphopeptide separation in the m/z to Q/z space, combined lead to 7,000 and more than 10,000 unique phosphopeptides identified at 15min and 30 min gradient times respectively, at a speed of up to 500 unique phosphopeptides/min. Some of those separated phosphopeptides were isobaric.

Conclusions
TIMS-PASEF allows to increase phosphopeptide analysis depth and selectivity.

Novel Aspect:
High speed and sensitivity provided by PASEF for fast and comprehensive phosphoproteome analysis.
THE CITRULLINE EFFECT IN THE DISSOCIATION OF DEIMINATED PEPTIDES

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Keywords: citrulline, citrullination, citrulline effect, tandem mass spectrometry, peptide sequencing

Introduction:
Protein deimination is a post-translational modification of proteins converting certain arginines to citrullines. Overcitrullination could be associated with severe pathological conditions. Mass spectrometric analysis of the modified proteins is hindered by several problems. Based on our tandem mass spectrometric experiments, we report here a new and pronounced amino acid effect.

Methods:
Model peptides containing either one or two citrulline residues as well as their native, arginine-containing variants were synthesized. Their solutions were injected directly to the electrospray source of a high accuracy and resolution quadrupole-time of flight instrument and were analysed by tandem mass spectrometry using low-energy collision induced dissociation.

Results:
A preferential cleavage site was observed at the amide bond of citrulline and any other following alpha amino acid (Cit-Aaa) yielding intensive complementary b and/or y type ions. If citrulline was located at the C-termini of the peptides, an enhanced cleavage of Aaa-Cit amide bond was perceived instead. These phenomena were described by us for the first time [1] and was coined citrulline effect, analogously to ornithine effect reported previously [2]. Loss of isocyanic acid from the citrulline side chains observed earlier [3] was also significant and effective for confirming the site of modification.

Conclusions:
Fragmentation behaviour of citrulline containing peptides is similar to ones not containing them, however, significant intensity differences can be observed due to the newly identified citrulline effect. The loss of isocyanic acid is also a pronounced fragmentation pathway. Our results are in concordance with the mobile proton hypothesis.

Novel Aspect:
The citrulline effect facilitates the MS/MS confirmation of citrulline residues in a peptide sequence, besides the neutral loss of isocyanic acid.

Acknowledgement:
Gitta Schlosser acknowledges the support of the MTA Premium Post-Doctorate Research Program of the Hungarian Academy of Sciences (HAS, MTA).

Introduction:
Age-related diseases are associated with alterations in energy metabolism and with chronic inflammation. Research indicates that changes in energy metabolism are closely linked to gene expression via changes in histone acetylation profiles, possibly leading to a pro-inflammatory phenotype [1]. To investigate the histone acetylation dynamics by mass spectrometry, an efficient chemical acetylation reaction of unmodified histone lysine residues is necessary.

Methods:
Histones derived from RAW264.7 and 16HBE cells were chemically acetylated using stable isotope labeled acetic anhydride in combination with different buffer systems; ammonium bicarbonate (ABC), triethylammonium bicarbonate (TEAB) or sodium borate (SB). After in-solution tryptic digestion, peptides were analyzed by liquid chromatography tandem-mass spectrometry (LC-MS/MS). Chemical acetylation efficiency was assessed by using Xcalibur software.

Results:
LC-MS/MS analysis resulted in identification of histone proteins (H1, H2A, H2B, H3 and H4) with a high sequence coverage, between 86% and 98%. The average acetylation efficiency of 14 selected lysine residues of the core histone proteins H2A, H3 and H4 was significantly increased by using isotopically-labeled acetic anhydride in combination with either the TEAB and SB buffer (on average 99.6%) compared to the ABC buffer (94%). For some acetylation sites (for example H3K14, H3K56 and H4K20), no considerable differences in the acetylation efficiency were observed between the three different buffer systems. For other acetylation sites (for example H3K18, H3K79 and H4K20), the acetylation efficiency achieved by the TEAB and SB buffer was significantly increased compared to the ABC buffer. Complete acetylation of the histone 4 4-17 peptide, which contains four lysine residues, was significantly increased as well by using the TEAB and SB buffer (95%) compared to the ABC buffer (84%).

Conclusions
Chemical acetylation of unmodified histone lysine residues by using isotopically-labeled acetic anhydride in combination with TEAB or SB provided, in contrast to ABC, a sufficient acetylation efficiency (>95%). This acetylation method can be used to study the histone acetylation turnover in age related diseases. Furthermore, the method can be used to study the effects of histone deacetylase (HDAC) inhibitors on the acetylation status of specific lysine residues.

Novel Aspect:
Acetic anhydride in combination with SB results in an almost complete chemical acetylation of histone lysine residues. SB outcompetes the more commonly used ABC buffer.

References
Free radical oxidation in the human body causes the accumulation of oxidized forms of proteins, which have an impaired function and can also be used as the markers of many pathological conditions, induced diseases such as cancer, nephropathy, and cardiovascular diseases. High resolution mass spectrometry approach is promising for characterization of oxidative protein modifications by reactive oxygen species (ROS) [1].

Methods
Protein isolation, analyses of enzyme activity and the polymerization of fibrinogen were accomplished as described in [2,3,4]. After the oxidation of samples by ozone or HOCl, the proteins were hydrolyzed, using a suitable technique for each protein. HPLC-MS/MS experiments were performed on an Agilent 1100 nanoLC (Agilent Technologies, USA) coupled to 7T LTQ-FT Ultra (Thermo, Germany). PTM searching was carried out by PEAKS Studio software (v. 8.5, BSI, Canada).

Results
The site-specific oxidative modifications in the proteins (fibrinogen, plasminogen, fibrin-stabilizing factor) under induced oxidation in model systems with various oxidants were analyzed by HPLC-MS/MS method. Oxidative modifications have been found in some functionally significant amino acid residues, as well as in sites whose function remains unclear and should be further investigated. The variation of the coverage in native and oxidized samples is compared. These results obtained were also used to analyze the effect of oxidation on the structure of the protein molecules [5]. Interestingly, along with the sulfur-containing residues of Met and Cys, aromatic residues of Tyr and Trp, polar residues of Glu, Lys, and Asp and non-polar Pro residues, are also targets for the oxidants. The information obtained by biochemical methods in complex with HPLC-MS/MS data, make it possible to suggest a mechanism for the functional property loss of proteins underoxidation.

Conclusions
The data obtained make it possible to evaluate the availability of amino acid residues of proteins for oxidants under conformational changes in enzyme molecules, to reveal oxidation sites and the degree of oxidation of amino acid residues by using different concentrations of oxidants. Moreover it is one may suggest the mechanism for the functional property loss of proteins underoxidation [6].

Novel Aspect
High resolution mass spectrometry based approach was developed for the characterization of plasma proteins oxidative modifications by ROS.

Keywords: Proteins, ROS, PTM, fibrinogen, fibrin stabilizing factor, plasminogen, HPLC-MS/MS

References
Keywords: Post-translational modification (PTM), Cold atmospheric plasma (CAP), Mass spectrometry, Peptides

Introduction:
Oxidative PTM's of proteins and peptides play a role in many signaling pathways with potentially drastic changes in structure and activity of this molecule by distinct chemical groups [1]. Creating a library of such PTM's facilitates global mapping of PTM's in mammalian tissue and ultimately fosters understanding of complex redox controlled processes, e.g. wound healing. Here, cold atmospheric plasma (CAP) was used as means to simulate oxidative conditions on various peptides.

Methods:
Different CAP sources were used for a determined modification of the model peptides Bradykinin, Angiotensin, and the artificial peptide LYTFAHD. The resulting products were analyzed by LC-HRMS/MS-measurements (Q Exactive™) by direct infusion ESI or by LC-ESI coupling using a PepMap column. A workflow for the identification of the modified peptides with various MS software solutions was developed by using different algorithm for an optimal result validation.

Results:
Using manual annotation, a time dependent increase in number and quantity of PTM’s was observed for the plasma treatment. Single and double oxidation and hydroxylation were identified in high intensities. For Bradykinin, the observed adducts were compared with literature [2] showing that only up to five oxygen adducts were found after kINPen treatment instead of 10 as described for the COST-jet. In contrast, the synthetic peptide carried up to 10 oxidation adducts after 5 minutes of plasma treatment. The two amino acids phenylalanine and tyrosine were oxidized particularly efficient. Investigation of additional modification patterns are currently under way. After testing different evaluation softwares for a manual identification of the PTM’s, the next step will be the optimization of a semi-automatic workflow for PTM annotation and quantification by using isotopic labeled peptides.

Conclusions:
Cold physical plasma can act as a rich source for oxidative PTM’s of a peptide, with numerous modifications detected by LC-ESI-MS in the model used. Plasma sources differ in modification efficacy, e.g. the He-COST-Jet is more effective than Ar-kINPen, due to the presence of atomic oxygen. Using cold plasma, an oxidative and nitrosative focused PTM-library for peptides and amino acids is under construction and should ultimately forecast PTMs in complex samples.

Novel Aspect:
Usage of cold physical plasma as a tool to create an oxidation focused PTM library using relevant model peptides with the ultimate goal of predicting PTMs in complex samplers.

1234 - UNDERSTANDING EPIGENETIC CONTROL OF HISTONE MODIFICATIONS DURING DROUGHT ACCLIMATION IN SORGHUM USING TOP-DOWN MASS SPECTROMETRY

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Keywords: top-down mass spectrometry, plant, epigenetics, post-translational modifications, molecular biology

Introduction:
Sorghum is a cereal crop noted for its ability to survive water-limiting conditions. We are studying epigenetic control of drought responses in sorghum to understand how to equip similar crops to better survive climate change, and also potentially design ways to increase yield under low-water conditions. This study focuses on understanding changes in sorghum histone modifications through developmental stages and in response to drought stress in two genotypes.

Methods:
Sorghum leaf tissue was collected in the field at Kearney Agricultural Research & Extension Center (Parlier, CA, USA) and frozen in liquid nitrogen on-site. Nuclei were extracted from ground leaf powder and lysed. Histones were purified from the lysate with weak cation exchange chromatography and directly analyzed with reversed phase liquid-chromatography (Waters NanoAcquity) mass spectrometry (Thermo LUMOS Orbitrap). Data were processed with TopPIC.[1]

Results:
Sorghum plants from pre-flowering (Pref) and post-flowering (PostF) drought-tolerant genotypes, RTx430, and BTx642, respectively,[2] were replicated in 3 plots. All plots were watered prior to planting. The control and PostF plots were watered from the 3rd week. The Pref plots experienced 8 weeks of drought from planting to flowering. The PostF plots experienced drought after flowering in week 9 through harvest. We analyzed leaf histones from the 3rd, 9th and 10th week. LCMS experiments identified >300 histone proteoforms out of ~30 histone proteins (unique FASTA entry) per sample, including two distinct subtypes of H2A around 16 and 14kDa, respectively. Most notably, comparative analysis revealed significant differences in the relative abundances of truncated H4 proteoforms, which showed highly elevated levels only at week 9 for leaves from control and Pref plots from BTx642, and week 10 for Pref in RTx430. The distinct responses imply that H4 clipping is related to post-flowering development and epigenetic control of drought resistance.

Conclusions
Earlier studies have suggested regulatory functions of H3/H4 clipping on nucleosome conformation and dynamics.[3] However, it is challenging to characterize histone clipping and other combinatorial modifications using standard bottom-up proteomics methods.[4–6]. Herein, we demonstrate the potential of top-down MS in profiling combinatorial histone modifications to improve our understanding of these elusive epigenetic control mechanisms.

Novel Aspect: (Limit of 150 characters without spaces)
First comprehensive top-down study of histones extracted from sorghum plants grown in the field reveals significant changes of modifications related to drought resistance.

References
Keywords: G-quadruplex, conformational polymorphism, ion mobility mass spectrometry, Collision Cross section, 1HNMR

Introduction:
G-quadruplexes are guanine-rich sequences mostly found in promoters and telomeres associated with key cellular processes [1-2]. They are polymorphic in terms of number of tetrads, strands orientation, cation involved and loop arrangement [3]. Here we are using Ion mobility mass spectrometry (IM-MS) to characterize conformations of diverse sequence topology in native MS buffer [4]. This is further supplemented by 1HNMR, CD and UV melting.

Methods:
All IM-MS experiments were performed on Agilent 6560 IMS-Q-TOF in negative ion mode with Helium in the drift tube [5]. All circular dichroism (CD) and UV melting experiments were carried out on Jasco J-815 and SAFAS UV mc² spectrophotometer at 25°C. All 1HNMR experiments were performed on Bruker Avance 700 MHz with TXI probe. The theoretical CCS values for each charge states were calculated from Gaussian optimized conformations by modified EHSS method by Siu et al. [6-7].

Results:
CD in native MS buffer (100mM TMAA+1mMKCl) shows preservation of the high-[KCl] G-quadruplex conformation. This is further supported by the 1HNMR spectra of the imino proton (10-14 ppm) regions, which agree well with the literature. ESI-MS shows a major population of 2-K+ complex for all 3-quartet conformations, and also a 1-K+ complex (2-quartet) for a few human telomere sequences. The collision cross-section (CCS) distributions are broad for all hybrid and anti-parallel conformations, and narrower for parallel conformations. For a given sequence length and charge state, the loop configuration, quartet polarity, bulges and flanking bases give rise to significant differences in CCS. The starting PDB structures give a higher theoretical CCS values compared to experimental ones. The structure neutralized to match the experimental charge states were optimized in the gas phase at the semi-empirical PM7 level and closely match the experimental CCS values. EHSSrot (Siu parameters) or the TM are used to calculate the theoretical CCS values.

Conclusions:
Most examined G-quadruplexes fold with the same topology in native MS (1 mM KCl) and traditional high KCl content buffers. IM coupled with ESI-MS is able to distinguish different shapes formed in solution. With these well-characterized G-quadruplexes, we validate the gas-phase structure optimization with PM7 to generate 3D models from which to calculate the CCS values.

Novel Aspect:
G-quadruplexes fully characterized by CD, UV and NMR in MS-compatible solutions were used to validate the interpretation of IM-MS data in terms of structure.

References
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Keywords: DNA dissociation, LILBID-MS, soft ionization, native MS, non-covalent interaction

Introduction:

LILBID-MS is a soft ionization method for mass spectrometry in which non-covalent complexes are transferred into the gas phase via microdroplets of a native-like aqueous buffer, which are irradiated by a mid-IR laser pulse. This leads to an explosive expansion of the sample droplets and release of ions into vacuum. As a result of the desorption process, the ions receive an energy input from the laser pulse, which can be controlled via laser energy transfer parameters.

Methods:

This technique is able to detect intact ssDNA and dsDNA. Here we use DNA strands, which consist of 15-20 bp. Under soft laser conditions, we observe nearly no dissociation of the duplex DNA, while harsh conditions lead to the complete dissociation of the dsDNA. We can create melting curves of dsDNA with LILBID by use of a heatable droplet generator. Comparison of LILBID melting curves with UV/Vis shows how LILBID can monitor the solution behavior of DNA.

Results:

The tolerance of LILBID-MS towards different buffers and high salt concentrations enables the use of the temperature-independent MgHPO4 buffer. These buffer conditions ensure the stability of double helices without compromising the spectral resolution. By increasing the temperature of a sample we can achieve different dissociation states of dsDNA. To calculate the duplex ratio, the total peak areas of duplex signals were divided by the sum of all peaks. Plotting the calculated duplex ratio against the temperature of the solution allows the determination of an apparent melting temperature (Tm) of dsDNA because the shape of curves recorded by LILBID-MS mimics the sigmoidal fit of melting curves recorded by UV/Vis spectroscopy. The detected Tm reflects the length of the dsDNAs. Comparison of the apparent LILBID Tm with the measured UV/Vis Tm allows assessing the amount of dissociation caused by energy transfer during the desorption process.

Conclusions:

Double-stranded DNAs as non-covalent complexes are suitable for investigating dissociation caused by energy transfer to the molecules during LILBID desorption. Apparent melting curves from LILBID-MS correlate with melting curves recorded by UV/Vis spectroscopy, indicating that LILBID-MS can give reliable information on binding affinities of bimolecular compounds.

Novel Aspect:

The method of creating melting curves of dsDNA via LILBID-MS allows the assessment of the amount of transferred energy during the desorption process.

References

Introduction
The discovery of dynamic post-transcriptional modifications (PTMs) of ribonucleic acid (RNA) including N6-methyladenosines affecstranscriptome, emerging field of ‘epitranscriptomics.’ Although its analysis requires a general sequencing method of modified nucleosides on RNA, conventional methods fail to provide such information. We present here an MS3-based method to sequence positional isomers of methyl nucleosides in RNAs.

Methods
Base-methyl adenosines (mAs), cytidines (mCs), guanosines (mGs), and uridines (mUs) were analyzed by pseudo MS3 [1] with a Q-Exactive; a base anion generated by in-source collision-induced dissociation (sCID) of each nucleoside was fragmented by MS/MS to obtain its characteristic pattern. An RNase T1 digest of tRNA-Phe was sequenced by nano-flow liquid chromatography-MS/MS in data-dependent [2] and pseudo MS3 [1] modes. The data were processed by Ariadne [3].

Results
To sequence monomethyl nucleoside isomers in RNA, we extended the pseudo MS3 method [1]. We inspected sCID spectra of RNase-digested oligonucleotides containing mono-methyl nucleoside in detail and confirmed an ion at m/z 225.02 exclusively from 2’O-methylated oligonucleotides and another ion at m/z 148.07 specific for the methyladenine (mAd) containing oligonucleotides. To further distinguish those mAde isomers, we examined their fragmentations using commercially available mAs; we found each sCID generated mAd anion exhibited a characteristic product-ion pattern for each mA isomer. Likewise, we defined the MS3-derived signature patterns for mCs, mGs, and mUs. We then validated the method by determining the chemical structure of yeast tRNA-Phe, which is mono-methylated at 8 positions out of 76 nucleotides. The MS3-based analysis of its RNase T1 digest (500 fmol) unambiguously confirmed the site and positional isomerism of all monomethyl nucleosides in the tRNA. The method will be applied to sequence methyl nucleosides on any RNAs.

Conclusions
We have established signature fragmentation patterns for mAs, mCs, mGs, and mUs and, using them in the pseudo MS3 method, sequenced monomethyl nucleosides on RNA. In addition, the method should extend to the other nucleosides, e.g., dimethyl ones. Because the method is applicable to a sub-picomole quantity of RNA sample, it will serve as a useful tool for analyzing the structure/function of cellular RNAs and toward the comprehensive understanding of ephitranscriptome.

Novel Aspect
A pseudo MS3 analysis allows discrimination of a monomethyl nucleoside from its positional isomers with a sub-picomole quantity of RNA.

References

For information please contact: scientific@imsc2018.it
Introduction:
TEAD transcription factors mediate gene expression regulation through interactions with their DNA response M-CAT motif. They are active during growth and development and activate gene expression of proteins responsible for cell proliferation, differentiation or apoptosis. To understand the principles of interaction between TEAD1 and M-CAT motifs originating from regulatory regions of human genes, several structural mass spectrometry techniques were used.

Methods:
TEAD1 DNA binding domain (TEAD1-DBD) was expressed in E.coli. Fluorescence anisotropy was used to determine KD for TEAD1-DBD/M-CAT complexes. H/D exchange was carried out for DNA-free TEAD1-DBD or in complex with each M-CAT motif. DSA in non/13C-labelled forms was used for quantitative cross-linking. LCMS analysis was performed on 1ST solariX. Structural models were built using MODELLER and in vivo quantification of M-CAT occupancy was determined byChIP-Q-PCR.

Results:
According to KD values, tested M-CAT motifs can be divided into two groups one with ten times higher affinity to TEAD1-DBD than the other. H/D exchange results revealed differences in deuterium uptake in helix H3, part of helix H2 and in the loop connecting them, identifying this as DNA-binding region. In correlation with dissociation constants, protection from deuteration in higher-affinity complexes was more intense than in low-affinity complexes. Quantitative chemical cross-linking resulted in 14 distance restraints. Three of them were not affected by presence of DNA, two were favored in presence of M-CAT and eight constrains were discriminated by DNA binding. Cross-link formation ability of lysines K57 and K88 significantly decreased in complexed state. Again, the effect of high-affinity duplexes was more significant than the effect of low-affinity ligands. H/D exchange and cross-linking results allowed creation of TEAD-DBD/M-CAT structural models. The chromatin immunoprecipitation confirmed such observation in living cells.

Conclusions
Experimental data uncovers higher affinity of TEAD1 to 5'-3' than 3'-5' oriented M-CAT motifs and enables molecular docking of TEAD1/M-CAT complex. Based on structural models TEAD1 recognizes its response motif by the shape of DNA major groove and the complex is stabilized by specific aminoacid-nucleotide interactions. TEAD1 binds to inverted M-CATs 180° degrees rotated, less and weaker bonds are formed. Such observation correlates with the in vivo situation.

Novel Aspect:
Structural mass spectrometry sheds a light on the tumorigenesis orchestrated by TEAD1 transcriptional factor.

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THE SIMULTANEOUS DETERMINATION OF INTRACELLULAR NUCLEOSIDE TRIPHOSPHATES AND CYCLIC DINUCLEOTIDES WITH LC/MS/MS

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Keywords: dNTP/NTP, dinucleotide, LC/MS/MS, isomeric separation

Introduction:
The determination of nucleoside triphosphates and cyclic dinucleotides in cells is of interest for various issues: The cyclic dinucleotides adenosine monophosphate-guanosine monophosphate (cGAMP) and diGMP could be a tumor marker in human fibroblasts. The quantification of deoxynucleoside triphosphates (dNTPs) and the corresponding ribonucleotide triphosphates (NTPs) can be used to measure enzyme activity in knockout cells.

Methods:
ATP and dGTP are isomers, CTP, UTP and dTTP have very similar masses. Therefore a complete chromatographic separation was an aim of the study. An HPLC-Method with a reversed phase column and a mobile phase gradient were applied to separate all analytes in less than 10 min. Two transitions per substance were used in multiple reaction monitor (MRM) mode of the tandem mass spectrometer.

Results:
An 8-point external calibration curve in buffer at the range from 7.8 – 1000 ng/mL for 10 nucleoside triphosphates and cyclic dinucleotides was prepared and measured with samples of the assay. Quantification was performed by peak area method. A weighted (1/x) regression first order was performed to determine the concentration of the analytes. The calibration curves were accurate and precise over the range from 7.8 – 1000 ng/mL and the correlation coefficient (r) exceeded 0.996 in all cases. The concentrations 7.8 and 1000 ng/mL are the lower and the upper limit of quantification (LLOQ and ULOQ). Coefficients of variation and accuracy were calculated for each standard and are supposed to vary by less than 15%. All tested real samples contained nucleotide. To estimate the matrix effect, the samples were additionally analyzed by the standard addition method.

Conclusions
A simple and sensitive LC / MS / MS method was developed to separate different, partially isomeric nucleotides. The method provides a convenient tool for measuring intracellular dNTP / NTP levels that can be used to measure the molecular phenotype of mouse cells. In addition, cyclic dinucleotide concentrations in human cells were determined to be potential tumor markers.

Novel Aspect:
The novel aspect of the study is that with only one measuring method different nucleotides in different matrices can be determined simultaneously for different questions.
860 - BIOPHYSICAL CHARACTERIZATION OF APTAMER-METABOLITE INTERACTIONS USING ION MOBILITY MASS SPECTROMETRY

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Intro (400 Characters w/out spaces)
Aptamers are Nucleic Acids (NAs) capable to bind selectively to a ligand, or to a family of molecules. In aptamer-based technology (e.g., drug delivery or sensors), it is crucial to understand how binding is occurring, to quantify affinities, and how they can be tuned. We interrogated several RNA and DNA aptamers by native MS and ion mobility, to understand how they fold upon binding and which quantifications limits are attainable.

Methods (400 Characters w/out spaces)
All NAs are analysed in MS-compatible solutions such as TMAA or NH4OAc with Mg2+ and K+(when needed) to mimic bio-relevant conditions. An Agilent 6560 ESI-IMS-Q-TOF in negative mode is used for most experiments and CCS were determined in helium. A Waters SynaptG2 HDMSis used for IMS comparisons on cocaine aptamers. A MicroCal ITC 200 is used as an independent measurement of KDs. UV-melting and CD in solution is used to get information about NAs folding state in solution.

Results (900 Characters w/out spaces)
Among the different systems tested, only DNA Cocaine aptamers MN4, MN19 and OR8 [1] and RNA Tetracycline aptamer [2] are giving significant conformational change upon binding by IM-MS. Although for tetracycline complex and free-aptamer two distinct conformational ensembles are present, cocaine aptamers are showing a transition between two conformational ensembles only for the free-aptamer ion. For cocaine aptamers CIU on charge state 7- is best revealing their relative stabilities in gas phase in function of length. Unfortunately, UV-Tm and CD do not allow to predict the conformational space in gas phase for the NAs tested. But we had recently evidence that collapsing can occur on duplex structures in gas phase [3]. Besides their shape, aptamer-ligand affinities are determined by MS-titration using a dT6 internal standard [4], and we defined the lower and higher KD attainable. ITC and MS KD values are comparable, both in TMAA and NH4OAc. For Malachite green and Cocaine aptamer MS data were precious to clarify the number of sites on ITC.

Conclusions (400 Characters w/out spaces)
Hairpin patterns in Aptamers are prone to compact and lose the in solution structure “memory” during ESI, if the charge states are too low. Here we show for the first time two cases, Cocaine (bound-form) and Tetracycline aptamers, where bound and unbound-form are giving distinct IMS signature at some charge states. We also suggest native MS as an independent quantification technique and to reconcile puzzling data from ITC and NMR.

Novel Aspect (150 Characters w/out spaces)
We discuss the advantages and limitations of native MS and ion mobility of DNA and RNA aptamers and their ligand complexes.
References
1 - Neves et al., ACS Sens. 2017, 2, 1539
4 - Gabelica et al., Anal Chem. 2009, 81, 6708.
This work is funded by the EU H2020 (Marie Curie ITN “MetaRNA”)

Keywords (5 max):
NativeMS, Aptamers, Nucleic Acids, CIU, Ion Mobility
Introduction:
Synergistic tandem mass spectrometry and computational chemistry approaches enable questions related to the structure, energetics and reactivity of nucleic acids and their building blocks to be investigated. Here the effects of modification and the local environment on nucleoside conformation and glycosidic bond stability and the reactivity of nucleic acids with potential drug candidates (amino glycoside, peptide and metal-ligand complexes) are presented.

Methods:
Several types of tandem mass spectrometers (GIBMS, QITMS, and FT-ICR MS) and approaches including: threshold, energy-resolved, and sustained off-resonance collision-induced dissociation (TCID, ER-CID, and SORI-CID) and wavelength-resolved infrared multiple photon dissociation (IRMPD) action spectroscopy, complemented by electronic structure calculations of the stable structures, energetics, IR spectra, and reactions mechanisms are employed.

Results:
Combined IRMPD action spectroscopy, TCID, ER-CID, and computational chemistry studies of the protonated and sodium cationized forms of the canonical DNA and RNA nucleosides as well as a variety of naturally occurring and synthetically modified nucleosides have been performed. The preferred sites of protonation or sodium cationization, nucleobase orientations, sugar puckering, conformers populated by electrospray ionization, and glycosidic bond stabilities are determined. The reactivity and preferred sites of binding of potential drug candidates with pre-micro and ribosomal RNA hairpins are elucidated using top-down sequencing and SORI-CID approaches. The RNAs investigated include: Helix 69, a hairpin RNA structure containing 19 nucleotides in the large subunit of the ribosome, and TAR, the HIV trans-activation response element, a hairpin RNA that acts as the binding site for the Tat protein. Drug candidates examined include: cisplatin and a series of amino acid-linked cisplatin derivatives, amino glycosides, and peptides.

Conclusions
The structures and glycosidic bond stabilities of protonated and sodium cationized canonical and modified nucleosides provide insight into structure-function of these genetics building blocks, evolutionary aspects of roles of modification, and impact of unnatural modifications. The binding and reactivity of drugs to RNAs provide insight into structure-function of functional RNAs, and sample preparation lessons for achieving quality mass spectral analyses.

Novel Aspect:
Effects of local environment/modifications on structures/stabilities of nucleosides are elucidated. Reactivity/binding sites of drug candidates to RNAs are determined.
Semi-Quantitative Determination of Oligonucleotide Drug Impurities: Main and Co-Eluting Species

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Title
Semi-Quantitative Determination of Oligonucleotide Drug Impurities: Main and Co-Eluting Species

Authors
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Introduction
An advanced Ion Pair Reverse Phase (IP RP) LC MS characterization method for impurities co-eluting with the main component is currently available and extensively used in our labs for release and stability testing of oligonucleotide therapeutics. However, the method requires individual extraction of each impurity with integration of the resulting ion chromatogram. For certain applications, e.g. optimization of synthesis conditions, purification development or evaluation of reagents, the number of samples requiring analysis is often large, but a semi-quantitative determination comparing impurity levels between the samples is sufficient. In this work, a process is described for using the average mass spectrum of the main product LCMS peak to automatically determine the amounts (%area) of the co-eluting impurities.

Methods
Ion Pair-Reversed Phase (IP-RP) HPLC chromatography coupled to low- and high-resolving power mass spectrometers has been used for the experiments using Agilent instrumentation. For an oligonucleotide drug component a list of chemical formulas of known impurities is generated using an established template. An algorithm was developed to automatically determine the isotopic distribution of each formula and its most abundant mass (MABM). For each chemical formula, a list of MABMs is determined corresponding to the charge states of the impurity in the ESI spectrum. An average mass spectrum is obtained by summation of the signal across the main LC peak incorporating the trace impurities. The relative amounts of the co-eluting impurities are determined by integration of the peak abundances of each species determined in the spectra.

Preliminary Data
The method has been extensively applied for the automated comparison of co-eluting oligonucleotide impurity levels in samples. For five different oligonucleotides, results have also been compared to those obtained by the quantitative conventional method, and were in very good agreement. The average absolute difference between the two approaches was 0.3%, with the best agreements achieved when the square root of the measured ESI abundance was used for the calculations. The repeatability of the method was evaluated, indicating an average standard deviation of 0.04% (calculated from all detected species). Application of the method for monitoring impurity changes in different lots of drug product readily identified the specific impurities statistically deviating from established values. Principal component analysis (PCA) was used to determine outliers. The loadings of PCA analysis were used to classify a set of 10 oligonucleotides in classification groups according to similarities in the formation of the same type of impurities. A plot of the PCA scores outlined the impurity types primarily contributing to the classification. For some impurities, multiple linear regression revealed a linear dependence of the total amounts detected to the number and type of a combination of base residues in the oligo. The current approach due to its rapidity and simplicity can be readily applied to determine relationships between the impurities detected and the experimental process development and manufacturing conditions potentially affecting them.

Novel Aspect
Development and application of an automated approach for the determination of impurity species co-eluting with the main oligonucleotide drug component.
330 - INTERACTIONS OF OXALIPLATIN AND CARBOPLATIN WITH DNA BUILDING BLOCKS AS STUDIED IN THE GAS PHASE: A COMBINED EXPERIMENTAL AND THEORETICAL STUDY

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Keywords: Pt-based anti-tumor agents, IRMPD spectroscopy, DFT calculations, nucleic acid building blocks

Introduction
In spite clinical successes for treating different types of tumors, cisplatin \( \text{[cis-Pt(NH}_3\text{)}_2(\text{Cl}_2)] \) displays severe toxic side effects and therapy resistance. This has motivated searches for structurally and/or functionally analogous alternatives, leading to carboplatin and oxaliplatin as second and third-generation platinum drug. The aim of this work is to study the interactions occurring in the gas phase between these two drugs and a set of DNA building blocks.

Methods
10-4M water solutions of either carboplatin or oxaliplatin and analyte (5’-dGMP) or (5’-dAMP) were mixed together and stored at room temperature for 24 h, to allow the formation of the different complexes. Complexes were generated in the gas phase by electrospray ionization. Their structure was probed by combining mass spectrometry and IRMPD spectroscopy to DFT calculations at the B3LYP/6-311G(d,p) level.

Results
This combination of approaches allows operating in the gas phase, which makes it possible to overcome any solvent or counter-ion effect, and thus allows describing the intrinsic reactivity of the metal cations. Presently, the complexes generated in the gas phase with adenine(A) and guanine (G) nucleobases are of the type \( \text{[(platinum drug)H(nucleobase)]}^+ \). Our DFT survey indicates that protonation preferentially occurs on one of the carbonyl group of both oxaliplatin and carboplatin. This is consistent with mechanisms proposed in previous studies.[1,2]

Comparison between IRMPD spectra and DFT calculations suggests that both oxaliplatin and carboplatin specifically interact with the N7 position of guanine. This interaction mode is therefore similar to what was observed with cisplatin.[3] Data obtained with adenine are more delicate to interpret. IRMPD spectroscopy points to a mixture of structures of comparable relative energies with both platinated agents, corresponding to different coordination schemes.

Conclusions
The present study shows that the interactions occurring between these two platinum drugs are similar to those observed with cisplatin. Concerning guanine, the interaction specifically occurs at the N7 position, in agreement with the remarkable stability of the corresponding structures. As for adenine, interaction with either N1, N3 or N7 sites results in structures which are close in energy, in agreement with the mixture of forms observed experimentally.

Novel Aspect
To our knowledge, this work is the first gas-phase experimental study of the interactions taking place between carboplatin and oxaliplatin, with A and G.
References

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474 - DEVELOPMENT OF THE LC-MS METHOD FOR THE ANALYSIS AND CHARACTERIZATION CHEMICALLY MODIFIED RNA

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Keywords: RNA, thiophosphate, modified nucleotide, isotope labeling, quantitative analysis

Introduction
Messenger RNA (mRNA) is a promising therapeutic agent with potential uses in cancer therapy, gene therapy, and cell reprogramming. The stability of mRNA largely depends on the 3'-end and 5'-end structures, which are targets for enzymatic degradation. Chemical modifications in those regions can be introduced into in vitro transcribed mRNAs in order to confer on them resistance to degradation and increase their translation efficiency, thereby increasing their therapeutic potential.

Methods
We developed an LC-MS method for quantitative assessment of incorporation of ATP analogs modified at the alpha-phosphate (ATPαS, ATPαBH3) into transcripts by RNA polymerases. Modified RNA was enzymatically degraded to single nucleotides, which were then resolved and quantified by ion pair chromatography coupled with ESI-QQQ. In order to improve the quantification, we synthesized RNA degradation products labelled with heavy oxygen (18O) within the phosphate and used them as internal standards.

Results
We obtained chemically modified RNA by in vitro transcription, wherein standard ATP was replaced or mixed at different ratios with an appropriate chemically modified ATP analog (ATPαX) containing an O-to-X substitution (X=S, BH3) at the alpha phosphate and differing in stereochemistry (SP versus RP). The assessment of efficiency and frequency of incorporation of the modified NTP versus natural NTP by SP6 and T7 RNA polymerase was analyzed by LC-MS. First, RNA was subjected to complete degradation into single 5'-nucleotides by SVPDE enzyme. The concentrations of AMP and phosphate-modified AMP analog (AMPX) in the resulting mixture were quantified by LC-MS method employing 18O-labelled standards. The analysis of AMP:AMPX ratio in the analyzed transcript as a function of ATP:ATPαS ratio in the transcription reaction provided insights into the structure of RNA as well as substrate stereoselectivity of RNA polymerases.

Conclusions
Proposed LC-MS quantitative method enabled determination of frequency of incorporation of different modified nucleotides into in vitro transcribed RNA, thereby providing a tool for establishing mRNA structure-biological properties relationship.

Novel Aspect
The proposed procedure could contribute to popularization of the application of low resolution equipment in the analysis of large molecules such as nucleic acids.

Acknowledgement: This work is supported by the National Science Centre, Preludium (UMO-2016/23/N/ST4/03186)
Keywords: miR-1587; G-quadruplex; dimerization; jatrorrhizine derivatives; ESI-MS

Introduction:
G-quadruplexes widely exist in genome and transcriptome [1], which have been considered as promising therapeutic targets [2]. However, reports about RNA G-quadruplexes are relatively fewer [3,4] and factors that influence high-order RNA G-quadruplex formation are still under investigation. In this study, ESI-MS was utilized to study the formation and properties of a RNA G-quadruplex formed by miR-1587.

Methods:
Most of the ESI-MS experiments were performed on a Finnigan LCQ Deca XP Plus ion-trap mass spectrometer in the negative ion mode. The high resolution ESI-MS spectra were collected on a Bruker SolariX XR FT-ICR mass spectrometer with a 9.4T magnet. All the CD experiments were carried out on a JASCOJ-815 CD spectrometer equipped with a Peltier junction temperature controlled cell holder and a 1.0 cm cell.

Results:
A guanine-rich human mature microRNA, miR-1587, was discovered to form stable intramolecular G-quadruplexes in the presence of K+, Na+ and low concentration of NH4+ (25 mM) by electrospray ionization mass spectrometry (ESI-MS) combined with circular dichroism (CD) spectroscopy. Furthermore, under high concentration of NH4+ (100 mM) or molecular crowding environment, miR-1587 formed a dimeric G-quadruplex through 3’-to-3’ stacking of two monomeric G-quadruplex subunits with one ammonium ion sandwiched between the interfaces. In contrast, DNA-1587 and dU-DNA-1587 sequences also formed three-layer G-quadruplexes, but was less stable and could not form dimeric G-quadruplex structures. Specifically, two synthesized jatrorrhizine derivatives with terminal amine groups could also induce the dimerization of miR-1587 G-quadruplex and formed 1:1 and 2:1 complexes with the dimeric G-quadruplex. In contrast, jatrorrhizine could bind with the dimeric miR-1587 G-quadruplex, but could not induce dimerization of miR-1587 G-quadruplex.

Conclusions:
In this study, the formation and property of intramolecular miR-1587 G-quadruplex were investigated. High concentration of NH4+, molecular crowding environment and two jatrorrhizine derivatives were discovered to be capable of inducing the formation of dimeric miR-1587 G-quadruplex. The results expanded our knowledge of RNA G-quadruplexes and provided new insights into the targeting of higher-order structures of miRNAs to regulate their functions.

Novel Aspect:
A dimeric miRNA G-quadruplex was first found by ESI-MS under high concentration of NH4+ or molecular crowding environments.

References
Keywords: Cross-linking, XPlex, Adipic Acid, Multiplex

Introduction:
Traditionally, chemical cross-linking is performed using NHS esters that react with nucleophilic groups. However, they are easily hydrolysable and have low solubility (sulfonated versions are very expensive). Reaction time, temperature and coverage are also suboptimal. Here, we describe a new cross-linking chemistry (Acid XPlex) overcoming these limitations using cheap, easily available reagents.

Methods:
Myoglobin and cytochrome C were subjected to cross-linking reaction using XPlex activated adipic acid at 5, 15 and 27 Celsius degrees, followed by tryptic digestion. Samples were subjected to MS and MSMS analysis a Q-TOF and an Orbitrap Fusion Lumos. Data were interpreted using Waters MassLynx and SIM-XL software. Cross-linking reactions using DSS were also performed for comparative reasons.

Results:
After condition optimization steps, reaction of myoglobin using adipic acid at 27 Celsius degrees showed 9 cross-linker molecules incorporation per protein compared to 6 of DSS. This difference is even more pronounced at lower temperatures where at 5 degrees only 2 DSS incorporations were observed, compared to 8 of adipic acid. The same results were obtained when using cytochrome C as the target protein. The method also allowed for a reduced reaction time (from 2 hours using DSS to 30 minutes using adipic acid). Another advantage of XPlex chemistry is the broader reactivity as cross-links are observed between Lys/Lys, Lys/Ser, Ser/Ser, Lys/Glu, Lys/Asp, Ser/Glu and Ser/Asp residues, improving the protein surface coverage and number of distance constraints. Special algorithms have been implemented in SIM-XL software to efficiently identify these multiplex cross-links.

Conclusions:
The Acid XPlex method presents several advantages over the use of NHS based cross-linkers, such as higher solubility, reduced reaction time, lower reaction temperatures, higher incorporation rates and, more importantly, broader reactivity and protein surface coverage, showing a potential to expand the use of XL-MS to proteins unstable in solution at higher temperatures or for prolonged periods.

Novel Aspect:
Novel chemical cross-linking chemistry with improved incorporation rates and reactivity at low temperatures.

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Introduction:
Intact antibody sequencing is one of the goals in current mass spectrometry. Electron capture dissociation (ECD) provides unique features on top-down sequencing of proteins, which can be an ideal tool to analyze intact antibodies. We have developed a small and high throughput ECD device based on an RF ion trap [1]. This technology was applied to an antibody. We also implement an online disulfide bond reduction technique [2].

Methods:
The ECD cell [1] was installed between Q1 and Q2 in a research grade quadrupole-TOF system. Typical electron beam irradiation time was 10 ms, and the electron beam intensity was tuned to obtain appropriate dissociation efficiency. The mass resolution of the TOF-MS is 35,000-47,000, which resolved isotope patterns of fragments up to Z~30+. A desalting LC column (Waters) was used for desalting, online reduction [2], and LC separation.

Results:
Humanized monoclonal IgG (NIST-mAb) was obtained from NIST. This mAb was analyzed by the LC-ECD-TOF mass spectrometer. De novo sequencing on the intact ECD spectrum obtained by a single LC run indicated three sequences, and two of them were matched to N terminal partial sequences of the variable parts in a light chain and a heavy chain appeared in the human genome. The intact ECD spectrum was further analyzed in top-down manner using the suggested full sequences (the full sequence is provided by NIST), where the data covered the variable parts of the light chain and the heavy chain in the mAb. ECD at 3 eV did not cleave the disulfide-bonded rings in the protein. To obtain nearly complete sequence coverages, IdeS digestion (Genovis) was applied. For the online reduction of disulfide bonds (ref. 2), DTT was injected to the intact mAb and the IdeS digest trapped on the desalting column for 1 min.

Conclusions:
By the reduction, we obtained sequence coverages of 84.7 % for the light chain, 78.3 % for the variable part of the heavy chain (Fd'), and 84.7% for the fixed part of the heavy chain (scFc). Further, ECD indicated the glycosylation site and its mass in scFc and CID informed the glycan composition.

Novel Aspect:
High throughput antibody analysis using LC-ECD TOF mass spectrometer combined with online disulfide bond reduction technique

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For research use only. Document number [RUO-MKT-10-7804]

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INTACT MASS ANALYSIS OF MACROMOLECULAR PEG-FAB CONJUGATE USING MULTI-MODE NATIVE LC COUPLED TO ULTRA-HIGH MASS RANGE NATIVE MS

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Keywords: Pegylation, PEG dendrimer, ion exchange, size exclusion, antibody

Introduction:
Monoclonal antibody Fab domain fragments can be utilized therapeutically to bind to and sequester specific antigens to decrease activity of disease targets in the cells of patients. Large multi-pronged polyethylene glycol (PEG)-based structures can be utilized to ‘bundle’ together multiple Fab domains to yield a drug with highly concentrated therapeutic activity and increased bioavailability (1). PEG-Fab conjugates pose enormous potential for sample heterogeneity, and thus present a significant analytical challenge in upstream characterization. Native MS and other charge reduction strategies yield increased spectral separation to allow the accurate intact mass analysis of such highly complex spectra. We present a semi-automated workflow for analyzing high mass PEG-protein conjugates utilizing native ion exchange coupled directly to an ultra-high mass range Orbitrap MS system.

Methods:
PEG-Fab conjugate was analyzed intact in native MS conditions using nanospray infusion, size exclusion chromatography (SEC), or ion exchange (IEC) chromatography. PEG-Fab conjugate was analyzed by SEC (Thermo Scientific MabPac SEC-1) or IEC (Thermo Scientific ProPac WCX) using isocratic conditions and pH gradient conditions, respectively. For SEC we used a mobile phase of 50 mM ammonium acetate pH 6.6. For IEC we used a gradient of pH 6.6 to 10.2 in a background of 50 mM ammonium acetate at a flow rate of 200 uL/min. LC-MS was performed using a Vanquish H-Class UHPLC with single wavelength UV detection coupled directly to an Orbitrap MS instrument capable of ultra-high mass range detection up to 80,000 m/z.

Results:
A major aspect of characterizing PEG-Fab conjugates is determining the weighted average of Fab domains which are attached to an individual PEG core structure. In order to assess the degree of Fab-conjugation, PEG-Fab conjugate was analyzed by static nano-spray infusion using an ultra-high mass range Orbitrap MS system operated with nitrogen or xenon as the HCD trapping gas for cooling ions during transmission. PEG-Fab conjugate in native conditions resulted in a complex spectrum which did not yield sufficient definition in order to confidently determine accurate masses to measure the average ratio of Fab-to-PEG. Addition of the charge reducing reagent TMGN produced a more simplified spectrum spanning a range of 20,000 to 50,000 m/z which enabled deconvolution for accurate mass determination. These results showed an average load of approximately 7 Fab domains, which is consistent with a low resolution result attained by MALDI-TOF MS data. We coupled IEC directly to our Orbitrap MS system to allow charge-based separation and on-line native intact mass analysis. We found PEG-Fab conjugate isoforms elute as individual peaks in increasing order of Fab load. IEC-MS spectra became further simplified compared to direct nano-spray infusion. Isoform identity and abundance could be further confirmed and relatively compared by leveraging both chromatographic and mass spectral data for intact mass analysis.

Conclusions
Native SEC and native IEC coupled directly to UV and Orbitrap MS with ultra-high mass range detection provides a flexible platform for analyzing large (500 kDa) compounds which exists in very complex mixtures such as PEGylated...
species. SEC and IEC have unique strengths in chromatographically resolving complexity which allow the Orbitrap MS system to obtain increasingly detailed information of molecular structure.

**Novel Aspect:**
Intact mass analysis of a complex PEG-protein conjugate using native ion exchange chromatography ultra-high mass range Orbitrap MS

**References**


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Introduction: Typically, native mass spectrometry (MS) requires costly and labor-intensive protein purification. Formerly we presented a novel approach for native MS analysis of overexpressed proteins directly from crude bacterial lysates [1]. Here we expand the method to the analysis of secreted recombinant antibodies directly from the growth medium of secreting HEK293 cells.

Methods: Growth media from adherent HEK293 cells grown in DMEM, or suspension-grown HEK293 cells grown in Freestyle medium were transfected with plasmids encoding light and heavy chains of various antibodies. Growth media containing the secreted antibodies were collected and buffer exchanged into MS compatible buffers. Low-expressing samples were concentrated. The antibodies were analyzed using either an Orbitrap [2] or a Synapt G2 HDMS QTOF-based platform [3].

Results: Initially, we tested an antibody secreted from adherent HEK239 cells. The growth medium was 2x buffer exchanged into 1M ammonium acetate and concentrated 10x before analysis. The antibody produced a well-resolved charge state series which corresponded in mass to the glycosylated form. PNGaseF treatment directly in the medium efficiently removed the N-linked glycosylations. We next examined growth media from transfected suspension-cultured cells and showed that the secreted antibodies could be easily measured directly from the growth medium without any concentration. Antibodies could also be reduced and deglycosylated in the growth medium, for detailed structural analysis. For deeper characterization we compared wild-type and engineered antibodies, designed for increased affinity and stability, and demonstrated that the engineered antibodies were expressed to much higher levels and showed higher stability. CCS analysis demonstrated that the wild-type and designed antibodies exhibited identical collision cross-sections.

Conclusions: We showed that direct native MS can be efficiently used for the structural analysis of secreted antibodies from crude growth media. Analysis of the antibodies directly from the growth medium allowed us to rapidly determine the identity, expression level, modification level and overall structure of the antibodies. Moreover, we showed that our method is not dependent on a specific MS platform but rather it is a general approach that can be applied on various instruments.

Novel Aspect: Direct native MS can be applied to the structural analysis of secreted antibodies from crude growth media, and can overcome the need for purification, saving time and effort.

References:


929 - CAN WE USE HDX-MS TO MODEL PROTEIN CONFORMATIONS?

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Keywords: Hydrogen deuterium exchange, protein structure determination

Introduction: Protein structures are commonly utilized to interpret hydrogen deuterium exchange mass spectrometry (HDX-MS) data not vice versa. But the ability to model structures by HDX-MS would exploit the many advantages of the technique particularly with regard to speed and sensitivity. The modelling of protein folds and homo/hetero protein assemblies by HDX-MS is described and the utility and limitations of the approach for different protein systems is discussed.

Methods: HDX-MS data of proteins and their assemblies were obtained by continuous labelling HDX-MS. Computational approaches were used to simulate HDX-MS data directly from protein structures or docking outputs. A range of structural analysis/evaluation techniques were employed to quantify the efficacy of the approach to model protein folds and assemblies including hierarchical clustering and binary structural classification.

Results: The HDX-MS patterns of proteins simulated directly from their atomic structures are compared to experimental data obtained in-house. Utilizing a simple expression to estimate HDX protection factors we show that it is possible to simulate protein HDX-MS patterns to sufficient accuracy to permit the modelling of protein conformations. Protein folds and hetero-assemblies can be modelled to high accuracy but the modelling of homo-assemblies is significantly more challenging due to the inability to prepare difference plots for these systems. Surprisingly, even poorly simulated HDX-MS data is shown to be efficient at identifying native conformations from a high background of decoy states. And in the case of homo-proteins the diagnostic ability of the HDX-MS simulations is shown to scale with the number of subunit interactions in the protein complex. Taken together these data add to our understanding of the use of HDX-MS for structural evaluation and provide an important foundation on which future developments in the area can be built.

Conclusions: HDX-MS has potential in protein modelling with applications in areas such as ab initio protein folding and protein-protein docking. [1] HDX-MS data can be simulated directly from protein structures significantly enhancing the throughput and applicability for quantitative ranking of modelling outputs. The present work sets a new precedent for the analysis of HDX-MS outputs and establishes greater synergy of the method with molecular modelling techniques.

Novel Aspect: The ability to utilize HDX-MS data to model protein structures represents a new way of working with the technique.

Title: Coupling online separations to native MS for the characterization of designed protein complexes.

Authors: Florian Busch, Mengxuan Jia, Zachary VanAernum, Aniruddha Sahasrabuddhe, Zibo Chen, Scott Boyken, David Baker, Vicki H. Wysocki

Introduction:
De novo designed protein complexes have significant potential to serve as future drugs and nanomaterials. Whereas it is possible to design, express, and purify a great number of proteins and protein complexes in a short time, methods for their subsequent structural characterization on a large scale are missing. Here, we demonstrate how online separation coupled to native mass spectrometry can be used to rapidly assess key properties of designed protein complexes.

Methods:
Protein samples were designed, expressed, and purified in the Baker lab. Gel filtration and ion exchange chromatography were performed with an Ultimate3000 HPLC system (Dionex) coupled to a modified QExactive EMR Orbitrap instrument (Thermo). UniDec[1] and Intact Mass (Protein Metrics) [2] were used for data deconvolution and analysis.

Results:
We determined the oligomeric states and ligand-binding properties of several hundred designed protein complexes in TBS buffer by online gel filtration coupled to high-resolution mass spectrometry. Hetero-dimeric coiled-coil structures, with characteristics matching those intended by design were further evaluated for interaction partner specificity based on their tendency to exchange/form off-target binding during a denaturation-refolding procedure. To deal with the complexity of such mixtures, we used ion exchange chromatography coupled to mass spectrometry for analysis and processed the data with Intact Mass. All-ion fragmentation was applied to confirm the protein complex identification. With several small mixing experiments including 6-16 heterodimers in each set, we filtered out 20 orthogonal heterodimers. For this set of 20 heterodimers, we found negligible subunit exchange during a denaturation-refolding procedure, consistent with the high specificity of the underlying designed protein-protein interactions.

Conclusions:
Gel filtration coupled to mass spectrometry can be used to rapidly perform the initial identification of protein designs suitable for further structural analysis. Complicated mixtures can be readily separated by ion exchange chromatography coupled to MS and identified by fragmentation. In all cases, deconvolution software is highly beneficial to determine the identity and oligomeric state of the designed protein complexes with high-throughput.

Novel Aspect:
On-line separation methods for native MS were developed to help with the determination of structural properties of designed proteins at high speed with minimal sample preparation requirements.

INTRODUCTION:
Fast Photochemical Oxidation of Proteins (FPOP) is a covalent labelling technique that utilises UV radiation to produce hydroxyl radicals which covalently label solvent-accessible side-chains. Following proteolysis, LC-MS/MS is used to localise and quantify the oxidation particular residues. Here, we assess the utility of FPOP and HDX as parallel techniques to probe protein structure and dynamics on ΔN6, an aggregation-prone variant of β2–microglobulin.

METHODS:
HDX-MS experiments were carried out using the LEAP Technologies automated system in-line with an M class ACQUITY LC and HDX manager followed by a Synapt G2Si MS/MS operating in HDMSE mode. FPOP was performed employing a 248 nm CompexPro Excimer UV laser to generate hydroxyl radicals used to oxidise solvent accessible side-chains. This was followed by protein proteolysis and LC-MS/MS using a Q ExactivePlusOrbitrap.

RESULTS:
>95% sequence coverage was obtained for both proteins using either HDX or FPOP. In the regions directly surrounding the N-terminal truncation, HDX indicates significantly higher deuterium uptake for ΔN6 compared with wild-type protein at the earliest labelling time-point (30 seconds). Key differences in labelling by FPOP were similarly clustered around the N-terminal truncation of the protein.

Examination of FPOP LC-MS/MS data showed certain modified residues eluting at multiple retention times, corresponding to the various positional isomers expected from hydroxyl radical attack on aromatic side-chains. Quantifying these peaks separately, changes in the labelling of positional isomers between the two proteins were observed (consistent with published NMR structures), and considerable differences in labelling trends between individual positional isomers and the combined residue level data were evaluated.

CONCLUSIONS:
The comparison of HDX and FPOP shows that they serve as informative parallel techniques to probe protein structure and dynamics. Moreover, FPOP can be used to interrogate structural changes at sub-residue level resolution. As protein aggregation is often caused by subtle structural changes including the exposure of hydrophobic side-chains (both of which are very sensitive to oxidative labelling), FPOP has proved invaluable to the study of protein aggregation.

NOVEL ASPECT:
A comparison between two different probes of protein solvent accessibility and dynamics offers greater understanding and sub-residue resolution.
Keywords: native MS, crude cell lysates, coupling energies, inter-protein hydrogen bonds

Introduction:
A powerful method to determine the energetic coupling between amino acids is double-mutant cycle analysis (DMC) [1]. Here, we show that double-mutant cycle analysis by native mass spectrometry [2] can be carried out for interactions in crude Escherichia coli cell extracts, thereby providing estimates for coupling energies under crowding conditions and obviating the need for protein purification and generating binding isotherms.

Methods:
The applicability of this native MS-based approach was tested for three different double-mutant cycles using the 1:1 complex between E2 colicin endonuclease (colE) and the Im2 immunity protein (Im). All proteins comprising the cycle, the two wild-type proteins and the two mutant proteins were co-expressed in E. coli using the pRSFDuet expression plasmid. Native MS experiments were performed using an Orbitrap EMR platform.

Results:
The charge series of the individual complexes forming each of the cycles could be well resolved in the crude lysates due to the overproduction of these proteins in comparison to the endogenous bacterial proteins and because of reduced binding of adducts. The relative concentrations in a cell lysate of the four co-existing complexes comprising each cycle were determined simultaneously from a single mass spectrum. Determining the concentrations of the complexes (without the need to define the concentrations of the individual proteins) allowed us to calculate the coupling energies under crowding conditions. We also determined the coupling energies using purified proteins in a buffer solution as a control. The values of the coupling energies determined for all three pairwise interactions in lysates are found to be very similar to the one determined for these interactions using purified proteins in buffer, thereby indicating that crowding has little effect on the strength of inter-molecular hydrogen bonds.

Conclusions:
Double-mutant cycle analysis by native mass spectrometry can be carried out for interactions in crude Escherichia coli cell extracts, thereby providing estimates for coupling energies under crowding conditions and obviating the need for protein purification and generating binding isotherms. Our results indicate that inter-molecular hydrogen bond strengths are not affected by crowding conditions.

Novel Aspect:
Our approach enables to characterize pairwise interactions by native MS of crude cell lysates obviating the need for any protein purification.

References:
1015 - ADVANCES IN ORBITRAP™ INSTRUMENTATION FOR NATIVE TOP-DOWN ANALYSIS OF NON-COVALENT PROTEIN COMPLEXES

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Keywords
Native MS, native top-down, pseudo-MS3, Orbitrap, Ultra-High Mass Range

Introduction
Native top-down studies of intact protein complexes have been reported since the early 1990’s, but their characterization using MS3 have only recently been reported and most work has been done on homomeric assemblies. In this work we explore new ways for extending native top-down performance to allow interrogation of heteromeric protein assemblies like proteasome by top-down pseudo-MS3.

Methods
Experiments were performed using a modified Thermo Scientific™ Q Exactive™ Plus MS in which the transmission of high m/z ions and the ability to perform pseudo-MS3 scans for native top-down analysis were improved by implementing several hardware and software modifications (see the Results section). Pyruvate Kinase, GroEL, rabbit 20S proteasome, LmrP membrane protein and AmtB membrane protein complex were used as model systems.

Results
Advances towards native MS and native top-down analysis of heteromeric protein complexes became possible only after addressing several major technical challenges. First, implementation of ‘in-source trapping’ capability addressed the insufficient or poorly controllable desolvation issue and allowed significantly improved fragmentation into subunits and stripped complexes in the inject flatapole region. Second, the reduction of RF frequencies on all RF guides and the mass filter was aimed at increasing the transmission of high m/z ions. Third, the adjustment of the voltage ramp rate on the central Orbitrap electrode facilitated successfully transmitting the high m/z ions from the C-trap into the Orbitrap analyzer.
The improvements in performance afforded by these modifications were demonstrated in a series of pseudo-MS3 experiments performed for several homomeric and heteromeric intact soluble protein complexes and membrane protein complexes.

Novel Aspect
Improved native top-down analysis of non-covalent protein complexes
ARCHITECTURE OF EUKARYOTIC MRNA 3’-END PROCESSING MACHINERY - THE ROLE OF CROSS-LINKING AND MASS SPECTROMETRY

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Keywords: XL-MS, Cryo-EM, pre-mRNA processing, structural proteomics

Introduction
Eukaryotic pre-mRNAs are processed at their 3’ ends by the Cleavage and Polyadenylation Factor (CPF). CPF cleaves pre-mRNAs, adds a poly(A) tail and triggers transcription termination. It is unclear how the complex is assembled and coordinated. A Cryo-EM investigation of CPF aided by nanoESI-MS and cross-linking-MS (XL-MS) shines new light on its assembly and activity providing an example of how XL-MS complements other structural approaches such as Cryo-EM.1-4

Methods
XL-MS experiments were performed on purified CPF complex, which is composed of four or five proteins: Cft1, Yth1, Fip1, Pfs2, with and without Pap1. The isotopically-coded NHS ester BS3 was used as cross-linker. Trypsin digests were fractionated at the peptide-level by SEC and fractions analysed by nano-scale LC-MS/MS on an Orbitrap Velos. Cross-links were identified using xQuest and spectra were manually validated.

Results
NanoESI-MS of CPF complex revealed an interaction network organized into three modules: nuclease, phosphatase and polymerase. The Poly(A) polymerase module of CPF was expressed in insect cells. The ~200-kDa recombinant four-subunit complex (Cft1, Pfs2, Yth1, Fip1) was imaged using cryo-EM at a resolution of 3.5 Å. Cryo-EM maps display only three ordered subunits (Cft1, Pfs2 and Yth1). AXL-MS investigation was performed to validate the structural model and to determine where Pap1 and Fip1 bind. Cross-links map agree with the atomic models, and the crystal structure of Pap1. Fip1 cross-links to the C-terminal part of Yth1 and the polymerase domain of Pap1. Pap1 also cross-links the C-terminal helical domain of Cft1, ZnF1 of Yth1, and the C-terminal region of Pfs2. Together, these data suggest that the flexible C-terminal half of Yth1 binds the intrinsically disordered protein Fip1, which in turn flexibly tethers Pap1 to the complex, allowing conformational freedom to add long poly(A) tails onto diverse RNA substrates.

Conclusions
Nuclease, Poly(A) polymerase and phosphatase activities of yeast CPF are organized into three modules. A partial cryo-EM structure of polymerase module was solved. XL-MS provided insights on the interactions of the whole polymerase module. These data, combined with in-vitro reconstitution experiments, show that the polymerase module brings together factors required for specific and efficient polyadenylation, to help coordinate mRNA 3’-end processing.

Novel Aspect
Combination of nanoESI-MS, cryo-EM, XL-MS and in-vitro reconstruction experiments described the assembly and activity of the eukaryotic mRNA 3’-end processing machinery.

References
INTRODUCTION

ABC importers are membrane proteins that couple the hydrolysis of ATP to the translocation of different molecules across cellular membranes. Depending on the fold of the transmembrane domains, they are classified as type I or type II importers [1]. To clarify the mechanism of these proteins, some questions need to be answered, such as the influence of substrate and ATP on the interactions between the transporters and their cognate substrate-binding protein.

METHODS

We selected two very well characterised ABC importers: the molybdate transporter ModBC-A and B12 transporter BtuCD as representatives of type I and type II importer families, respectively. We employed native mass spectrometry-based methods (MS), enabling us the study of membrane proteins and their ligand-binding properties [2].

RESULTS

Following purification of BtuCD and BtuF, we optimised native mass spectrometry conditions and obtained mass spectra of the two proteins in detergent solubilised form. When a solution of BtuF was added to BtuCD, the recorded mass spectrum showed the formation of the full BtuCD-F complex. After addition of vitamin B12, the spectrum still displayed the presence of the entire BtuCD-F complex but also revealed an increase in the intensity of the population of the uncomplexed BtuCD. Conversely, the addition of molybdate led to ModBC-A complex formation, while no complex was observed in the absence of substrate.

Mass spectra of BtuCD-F recorded after ATP addition showed the presence of the full complex bound to two ADP molecules. Interestingly, we detected an increase in the intensity of ADP-bound BtuCD. As for ModBC-A, following the addition of ATP we observed ADP-bound species, but we did not detect any additional charge states corresponding to ModBC.

CONCLUSIONS

Our MS data indicate that both vitamin B12 and ATP disrupt the interactions between BtuCD and BtuF. In contrast, molybdate is needed to let ModA dock onto ModBC and ATP does not affect ModBC-A complex formation. These observations provide critical insights into the mechanism of transport of these importers and allow us to propose new transport models that complement the previous ones and will serve as a prototype for more complicated systems.

NEW ASPECT

For the first time, native mass spectrometry has been applied to unravel the mechanism of action of transporters and provides further insights into their transport mechanism.

REFERENCES

Chemico-structural and functional characterization of filgrastim originator and three of its biosimilar drugs.

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Keywords (maximum 5 keywords defining the topics of the abstract): Originator, biosimilar, MALDI TOF/TOF mass spectrometry, liquid chromatography, zebrafish.

Introduction: (Limit of 400 characters)
The approval authorization of a biosimilar is based on strong comparability studies with its biological references product due to the complexity of the structure and of the nature of manufacturing process of biological drugs. The proof of similarities needs both the development of analytical workflow, to characterize the protein chemically, and development of clinical trials to evaluate the efficacy and the safety of drug.

Methods: (Limit of 400 characters)
Qualitative and quantitative analyses were performed by tandem time of flight matrix-assisted laser desorption/ionization mass spectrometry (MALDI-TOF/TOF-MS) and reverse phase liquid chromatography coupled with an UV-detector (RP-HPLC/UV). The evaluation of the biological activity of the biodrugs, in particular, their effect on immune system cell proliferation, was studied in zebrafish embryo, as animal model, using an innovative functional assay.

Results: (Limit 900 characters)
The comparison studies between the recombinant filgrastim and its biosimilars were performed RP-HPLC and MALDI-TOF-MS both on intact proteins and on digested proteins. In particular, analyses were carried out to evaluate the integrity of proteins, the absence of drug-related impurities, the similarity of amino acid sequence and the protein concentration. While qualitative analyses allowed us to establish the molecular weight of the biodrugs and to verify the identity of primary amino acid sequence, quantitative analyses confirmed the protein concentration in the pharmaceutical formulations. After chemical characterization of the biodrugs, the biosimilarity was assessed by in-vivo study, using zebrafish embryos as model, to verify the biological activity. Functional studies showed that all drug had similar capacity to activate the innate immune response in zebrafish. Overall, the obtained results allowed us to demonstrate both the structural and biological similarity of the biosimilar drugs in comparison with their originator.

Conclusions (Limit of 400 characters)
The analytical analyses performed in this study highlighted the structural similarity of biosimilar drugs Nivestim™, Tevagrastim® and Zarzio® and the originator Granulokine®. This result was reinforced by a similar in vivo biological activity.

References
Gianoncelli et al, 2018. Submitted

Novel Aspect: (Limit of 150 characters)
This work is focused on the comparison of the originator of filgrastim with its three biosimilars, to evaluate the structural similarity and biological activity.
Introduction: Human α-synuclein (αSyn) is an intrinsically disordered protein, whose aggregation is associated with the pathogenesis of several neurodegenerative diseases (e.g., Parkinson's disease). Although αSyn aggregation process in the patients' brain is not fully understood, dyshomeostasis of Ca²⁺ was suggested to induce the aggregation. In this study, we examined the detailed structural change of αSyn and proposed the mechanism of Ca²⁺-mediated αSyn aggregation[1].

Methods: Ion mobility-mass spectrometry (IM-MS) and solution small-angle X-ray scattering (SAXS) were utilized for structural characterization of monomeric αSyn in the presence of Ca²⁺. Molecular dynamics (MD) simulations were performed to generate structures that match the experimental data. Multiple biophysical techniques were also utilized for revealing aggregation kinetics and morphological properties of aggregates.

Results: First, we observed that Ca²⁺ accelerated the αSyn fibrillation. To understand the role of Ca²⁺, we investigated the structure of monomeric αSyn in the presence of Ca²⁺ using solution SAXS and IM-MS. Our IM-MS and SAXS results imply that population of extended conformers increases when Ca²⁺ ions are bound to αSyn. Based on the representative structures generated using MD simulation and ensemble optimization method (EOM), we suggested that the 1) Ca²⁺ induces αSyn to form extended conformer by electrostatic interaction, and 2) the extended conformers are prone to aggregation because hydrophobic NAC domain is more exposed to water than compact conformers. In addition, we observed that fibrils attached to each other in the presence of Ca²⁺. Our detailed examination showed that Ca²⁺ binds to acidic-rich C-terminal domains originated from different fibrils, provoking interfibrillar aggregation.

Conclusions: In this study, we characterized the Ca²⁺-mediated aggregation pathway of αSyn. Our results showed that binding of multiple Ca²⁺ ions induces the structural change of monomeric αSyn to expose NAC domain which is crucial for fibrillation, and provokes interfibrillar aggregation via electrostatic interaction.

Novel Aspect: Because of the biological importance of Ca²⁺ in αSyn aggregation, our study would be valuable for understanding pathology of αSyn-related neurodegenerative diseases.

References

For information please contact: scientific@imsc2018.it
923 - CHARACTERIZATION OF INTACT MONOCLONAL ANTIBODIES UNDER NATIVE AND REVERSE PHASE CONDITIONS USING HIGH RESOLUTION MASS SPECTROMETRY

Chris Nortcliffe (1) - Ryo Yokoyama (2) - Sibylle Heidelberger (1) - Kerstin Pohl (3) - Joerg Dojahn (3) - Ferran Sanchez (4) - Annu Uppal (5)

SCIEX, Biologics, Warrington (1) - SCIEX, Biologics, Tokyo (2) - SCIEX, Biologics, Darmstadt (3) - SCIEX, Biologics, Barcelona (4) - SCIEX, Biologics, Haryana (5)

Keywords: Native, NIST, Trastuzumab, TripleTOF 6600

Introduction:
A major requirement in the development of biopharmaceuticals is extensive characterization of the molecule at the intact level to ensure safety and efficacy. More recently characterization of monoclonal antibodies under native conditions is gaining importance over reverse phase conditions as it helps preserves the noncovalent interactions and retain the folded structure.

Methods:
Two monoclonal antibodies were studied using SCIEXTripleTOF® 6600 coupled to high-flow liquid chromatography (LC). Reverse phase chromatography was performed with water and acetonitrile containing 0.1% formic acid gradient. For native analysis, isocratic gradient of 50mM ammonium acetate buffer was used. MS detection was carried out using high resolution TOFMS mode.

Results:
Two antibodies Trastuzumab, and NIST standard were analyzed under native and reverse phase conditions. The TripleTOF® 6600 system yielded high resolution accurate mass data with excellent sensitivity and reproducibility for both the molecules analyzed. The traditional reversed phase chromatography involves separation based on organic solvent gradient resulting in denaturing conditions and a broad distribution of highly charged species at lower m/z ranges. Relatively lower charge states are observed with narrow distributions and higher m/z under native conditions thus resulting in higher spatial resolution and improved baseline resolved peaks.

Conclusions
The high quality spectra obtained with TripleTOF® 6600 system along with the deconvolution using BioPharmaViewTM software enabled the accurate molecular weight determination of all the possible glycoforms with excellent reproducibility.

Novel Aspect:
Evaluation of the TripleTOF® 6600 systems for the Intact Mass analysis of mAb’s under native conditions.

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Introduction:
Native mass spectrometry has emerged as an important tool in structural biology and is routinely used to analyze soluble proteins. However, the investigation of membrane proteins and their complexes remains challenging; until the beginning of 2017, only about 30 membrane proteins have been investigated, mostly using nESI-MS. In this area LILBID-MS has recently shown great potential, which is further examined here in a comparison of the capabilities of these Methods:

Methods:
We compared the performance of the complimentary methods nESI and LILBID in the analysis of the soluble protein Avidin (tetramer) and the dimeric membrane protein EmrE. In addition, we performed a comparative screening of different buffers, concentrations and additives to explore the current limitations of both MS techniques. The nESI measurements were performed on a Waters Synapt G2-S, while the LILBID spectra were recorded on an in-house built reTOF spectrometer.

Results:
LILBID and nESI show different dissociation pathways. Avidin tetramer dissociates via dimers in LILBID, while in nESI, a highly charged monomer exits the complex. For EmrE, the native dimeric state can be detected almost exclusively using LILBID, whereas in nESI the dimer appears only as a minor species. While with nESI, in case of most membrane proteins, it wasn’t possible to avoid a certain amount of dissociation, we were able to demonstrate a gentle analyte release via IR-laser desorption in LILBID-MS, preserving almost the entire intact protein complex.
LILBID demonstrates a slightly higher tolerance towards non-volatile buffers TRIS-HCl and HEPES. High buffer concentrations require higher energy input for desolvation, leading to partial dissociation of the protein complexes. Both methods show a significant peak broadening with increasing salt concentration, the detected complex mass also increases.[1]

Conclusions:
Soluble proteins and protein complexes can be analyzed convincingly using both Methods:
If more desolvation of the analyte is needed, as for non-volatile buffers and additives or detergents used to stabilize membrane proteins, LILBID has benefits due to its different ion release mechanism.
When the native state of membrane protein complexes is of interest, LILBID can reveal this routinely, whereas nESI has its advantages in areas, where a high resolution is needed.

Novel Aspect: This is the first study comparing the capabilities of the two complimentary native MS methods nESI and LILBID in the analysis of soluble and membrane protein complexes.

References
1304 - CATION EXCHANGE CHROMATOGRAPHY IN DISPLACEMENT MODE FOR ONLINE ANALYSIS OF INTACT PROTEINS BY MASS SPECTROMETRY

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University Medical Center Hamburg Eppendorf, Institute for Clinical Chemistry and Laboratory Medicine, Mass Spectrometric Proteomics, Hamburg, Germany (1)

Keywords: Displacement Chromatography, Top Down Proteomics, Intact Proteins

Introduction:
Combination of mass spectrometry with chromatography is the key to successful mass spectrometric top down protein analysis [1]. Ion exchange chromatography in its classical form is incompatible with MS due to high salt concentrations in the eluent [2]. Displacement chromatography offers opportunity to perform cation-exchange chromatography (CEX) without using salt. In this study we tested if proteins eluting on-line from a CEX column into an ESI-MS can be detected.

Methods:
An equimolar protein mixture (Lysozyme, Ribonuclease A, Cytochrome C and Myoglobin) was loaded (1.5 mg) onto a CEX column until it was saturated, recognized by the detection of the breakthrough of the proteins. Adsorbed proteins were eluted by pulsed injection of the displacer spermine. First 90 pulses contained 10 mM spermine, next 50 pulses 25 mM. The HPLC system was run with 0.1% formic acid as solvent and eluting proteins were detected by an orbitrap mass spectrometer.

Results:
Lysozyme and Ribonuclease A were eluted from the column by first 90 pulses of spermine (10 mM). Three different charge states from each protein were chosen and monitored in the corresponding spectra. Over the pulses, lysozyme and ribonuclease A eluted with constant concentration. A separation of these two proteins was not observed. After the injection of 140 pulses of spermine (25mM) Cytochrome C co-eluted with both previous proteins. Only Myoglobin stayed on the column and was not displaced by spermine. It eluted by washing the column with 1M ammonium acetate.

Conclusions:
By application of displacement chromatography mode an online coupling of ion-exchange columns with MS detection for intact proteins is possible. However, the affinity of spermine obviously was not strong enough for displacing myoglobin. Thus, finding a suitable displacer with higher affinity for displacing proteins from CEX is still needed. Once found, it should be possible to even separate individual proteoforms of proteins.

Novel Aspect:
Separation and detection of intact proteins by online coupling of ion-exchange columns with MS detection by application of displacement mode chromatography.

References:
A CROSS-LINKING/MASS SPECTROMETRY WORKFLOW BASED ON MS-CLEAVABLE CROSS-LINKERS AND THE MEROX SOFTWARE FOR MAPPING PROTEIN-PROTEIN INTERACTIONS

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Keywords: Cross-linking/Mass Spectrometry, Structural proteomics, Protein Interaction, Software, Collisional Activation

Introduction:
Mass spectrometry (MS)-cleavable cross-linkers are moving into the focus of the cross-linking/MS approach for studying 3D-structure of proteins and protein assemblies. They undergospecific fragmentations under collisional activation conditions generating characteristic product ion patterns. They have an enormous potential for a fast and reliable identification of cross-linked peptides even from highly complex samples, e.g. whole cells and organisms.

Methods:
We developed a robust and widely applicable workflow that allows a facile identification of cross-links for deriving spatial constraints from proteins and protein complexes. Our protocol combines the synthesis of novel cross-linkers, protein cross-linking, bottom-up proteomics analysis, and data analysis based on the in-house MeroX software [1]. Two orbitrap mass spectrometers, Orbitrap Fusion Tribrid and Q-Exactive Plus, were employed.

Results:
We designed and synthesized four novel MS-cleavable (photo)-cross-linkers. 2,2'-Azobis(2-methylpropanimidate) is an innovative cross-linking principle that after collisional activation induces free radical-initiated sequencing (FRIPS) of connected peptides in positive ion mode [2,3]. 1,1-carbonyldiimidazole is the first zero-length MS-cleavable linker for nucleophilic groups (amines and hydroxyls) in proteins [4]. 3,4-Diallylurea is a novel photo-activatable, MS-cleavable reagent to selectively target cysteines [5]. We unveiled the true reactivity of diazirine-base cross-linkers, which are in fact photo-activatable and MS-cleavable reagents to target carboxylic acids in proteins [6]. All cross-linkers were investigated to study several protein systems, such as the tumor suppressor p53, which is an intrinsically disordered protein, and the whole E. coli ribosome. We are currently extending our cross-linking/MS workflow to the in-vivo analysis of protein-protein interactions, exemplified for Drosophila embryos.

Conclusions
Our integrated cross-linking workflow allows to map protein 3D-structures and protein-protein interactions in-vitro and in-vivo. We have synthesized innovative, MS-cleavable cross-linking principles that target different functional groups in proteins, such as amines, hydroxyls, thiols or react non-specifically with all 20 proteinogenic amino acids. The MeroX software is able to analyze cross-linked products in an automated fashion.

Novel Aspect:
We present an integrated workflow based on novel MS-cleavable cross-linking principles and a fully automated analysis of cross-linked products by the MeroX software.

References
Evaluation of an Isotope-Labeled MS/MS-Cleavable Cross-Linker for Protein Structure Analysis

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Introduction:
Previous studies have shown the benefits of the amine-reactive, CID-MS/MS-cleavable cross-linker DSBU (disuccinimidyl dibutyl urea) for structural proteomics studies based on cross-linking/MS[1, 2]. To further facilitate the automation of cross-linking/MS experiments we now introduce a D12-stable isotope labeled version of the DSBU-linker combining the advantages of MS/MS-cleavable linkers and isotope labeling.

Methods:
Non-labeled DSBU and 1:1-mixtures of DSBU-D0 and -D12 were used to cross-link bovine serum albumine as well as the E. coli ribosome. Cross-linked proteins were proteolyzed and analyzed by nano-HPLC/nano-ESI-Orbitrap MS/MS (higher energy collision-induced dissociation, HCD) on Orbitrap Fusion and Q-Exactive Plus mass spectrometers using mass difference-triggered MS/MS experiments. MS and MS/MS data were automatically analyzed and annotated with MeroX [3].

Results:
The basis for a reliable, targeted selection of cross-linked peptides for MS/MS experiments is a mass difference between two isotopic species, which is large enough (D12) to create non-overlapping isotope patterns of a single species as well as overlapping elution profiles of the D0 and D12 species during LC/MS analysis. We could show that the D12-labeled DSBU-linker meets both requirements. The observed average retention time differences between D0 and D12 species were only ca. 10 seconds when employing 90-minute LC gradients. This enabled the efficient selection of cross-linked products for fragmentation by HCD, compensating the dilution effect caused by the application of two different cross-linker species. Employing mixtures of deuterated and non-deuterated cross-linkers combined with mass difference-based, targeted fragmentation seems to be advantageous compared to standard data dependent experiments using only one cross-linker species (156 versus 116 cross-linking sites for BSA).

Conclusion:
The application of mixtures of deuterated and non-deuterated DSBU-linker is advantageous for structural proteomics studies based on cross-linking/MS. In combination with the dedicated software tool MeroX [3], a basis for automated, robust, and reliable automated cross-linking/MS studies is provided.

Novel aspect:
A D12-stable isotope labeled version of the CID-MS/MS cleavable DSBU-linker allows for an efficient mass-difference based, targeted analysis of cross-linked products.

Keywords:
Cross-linking mass spectrometry, MS/MS cleavable cross-linker, isotope-labeled cross-linker

References:

Title: Deep biosimilarity assessment by monitoring multiple critical quality attributes of an intact monoclonal antibody drug using native IEC and SEC coupled to native Orbitrap MS

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Keywords: antibody, ion exchange, charge variant, native MS, size

Introduction: Manufacturing of innovator biologics can be successfully mimicked to produce generic “biosimilar” drug products. In order to satisfy safety and efficacy requirements, a biosimilar drug must be reasonably comparable to an innovator. Comparability is directly assessed by measuring a panel of critical quality attributes (CQAs). Recently, a multi-attribute method has been demonstrated to measure several CQAs simultaneously using LC-MS peptide mapping data. Additional CQAs, such as charge variants or size variants are conventionally monitored at the intact level using ion exchange (IEC) or size exclusion chromatography (SEC), respectively, coupled to UV detection. Native LC-MS approaches, such as SEC-MS and IEC-MS, can be inclusive with UV detection to combine the traditional gold standard chromatographic assays with accurate mass measurement to allow isoform-specific monitoring of multiple CQAs of intact therapeutic proteins.

Methods: Intact trastuzumab was separated by SEC or IEC directly coupled to MS. Mobile phases consisted of aqueous 50 mM ammonium acetate. SEC was performed using isocratic pH 6.6 mobile phase. IEC was performed using a pH gradient from 6.8 to 10.1. LC-MS was accomplished using a Vanquish H-Class UHPLC system with a variable wavelength detector directly connected to a Thermo Scientific™ Q Exactive™ HF-X Orbitrap™ mass spectrometer. Native LC-MS raw data were analyzed using a time-resolved deconvolution approach utilizing Sliding Window and ReSpect algorithms in Thermo Scientific™ BioPharma Finder™ software. Thermo Scientific Chromeleon software was used to integrate XIC peak areas and relatively compare CQAs of innovator and biosimilar drug samples.

Results: Native IEC-MS analysis of intact trastuzumab resulted in a chromatographic profile consistent with previously published data. Time-resolved deconvolution analysis of the innovator drug resulted in identification of several
specific isoforms comprised of differential N-glycosylation in combination with a variety of low level charge-impacting PTMs, such as deamidation, C-terminal lysine removal, or sialylation of N-glycans. We confirmed that the main peak consisted of several isoforms corresponding to variable N-glycosylations of the trastuzumab amino acid sequence. When comparing the innovator and biosimilar products, we found marked differences in glycoform profile measured at the main peak. Furthermore we found measurable differences in several charge variant isoforms when comparing innovator and biosimilar. These differences observed were consistent with a comparison of these same two samples via MAM HRAM peptide mapping analysis. Native SEC-MS of intact trastuzumab innovator and biosimilar samples showed marked differences in the levels of fragmentation as well as aggregation in response to a forced degradation treatment.

Conclusions
Biosimilarity of antibody therapeutic drug products can be accessed at the intact level to assess size variants and charge variants using either SEC or IEC coupled to UV and MS detection. This method was effective and reproducible for determining detailed molecular differences between an innovator drug, Herceptin, and a biosimilar.

Novel Aspect:
Method for monitoring multiple CQAs of an intact biotherapeutic compound to allow direct comparison of innovator and biosimilar products in a convenient and deeply insightful. Both SEC and IEC coupled to UV and native MS can be used

References

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Introduction: (Limit of 400 characters)
Vascular endothelial growth factor (VEGF) is recognized as an essential regulator of normal and abnormal blood vessel growth.\cite{1} The humanized mAb bevacizumab (Avastin) targets VEGF and blocks angiogenesis, resulting in suppressed tumour growth.\cite{2,3} In this study, native MS, IM-MS, and global, as well as bottom-up HDX experiments were conducted to determine similarities in the higher-order structures of various lots of biosimilars to its innovator drug.

Methods: (Limit of 400 characters)
The main isoform of human VEGF (165) was purchased from R&D Systems. Avastin and biosimilars were exchanged into 250 mM C2H7NO2 (pH 7.4) for all native MS, IM-MS and global HDX experiments. VEGF and antibodies were exchanged into 150 mM C2H7NO2(pH 6.7) and HDX was performed on the VEGF/Antibody complex and VEGF only to determine the epitope sites. Time-resolved global and bottom-up HDX was conducted using an in-house rapid mixing device and microfluidic platform.

Results: (Limit 900 characters)
Native MS revealed that all six biosimilar lots had a more heterogenous pattern and an overall greater level of glycosylation compared to Avastin. IMS-MS revealed identical drift times for Avastin and the biosimilars, consistent with gas-phase structures having the same collision cross-section. In the global HDX analysis, no differences in deuterium uptake were observed between Avastin and the biosimilars between 0.046 – 3.77 seconds. To localize subtle conformational changes and elucidate epitope sites, local HDX was conducted on VEGF only and the VEGF/antibody complex. At 8 and 4 seconds, the significant decreases in uptake identified for VEGF+Avastin were located at a.a. 27-43, 91-103 and 105-119 containing most of its known epitopes. Similar decreases were observed for VEGF+Biosimilar at a.a. 91-103 and 106-122. Interestingly, at lower time points, VEGF+Biosimilar displayed significant increases in deuterium uptake at a.a. 19-22, 91-103 and 154-162, while VEGF+Avastin continued to see decreases at the epitope, albeit to a lower extent.

Conclusions (Limit of 400 characters)
Although there were differences observed in the glycosylation patterns, this did not seem to have an effect on the overall solution-phase dynamics of the antibodies. Although the binding site is the same for Avastin and the biosimilar, differences in allosteric perturbations could be observed at earlier time points which may be the result of how the antibodies engage VEGF. These increases could be attributed to differences in the thermodynamics of binding.

Novel Aspect: (Limit of 150 characters)
Using native structural mass spectrometry techniques in tandem with time-resolved HDX for biosimilar characterization and epitope mapping.

References
Introduction:
C-Met is a glycosylated receptor tyrosine kinase of the hepatocyte growth factor. Upon ligand binding C-Met transmits intercellular signals by its unique multi-substrate docking site. However, pathophysiological activation of the C-Met pathway leads to tumorigenesis, schizophrenia and cardiomyocytes death [1].
Here we report a study of the C-Met interaction with two DNA aptamers that bind the target protein (C-Met) with high affinity and specificity

Methods:
The two C-Met aptamers used are CLN0003 and CLN0004 where produced using SELEX procedure [2]. For epitope identification, the aptamers where immobilized on sepharose beads. The binding epitopes were identified by proteolytic extraction and excizon-MS [3]. C-Met was digested with two enzymes. The unbound peptides where washed and the elution was done by slight acidification. Affinity determinations were carried out with a SPR biosensor.

Results:
According to the SPR affinity data the aptamers are interacting with the protein with two different binding events. The KDs where calculated with tree different methods to validate the results.
In the case of CLN0003 interacting with the protein we obtained the following KDs: KD1= 10-6 M and KD2 10-8 M. For CLN0004 the Kds are as follows: KD1= 10-7 M and KD2 10-8 M.
Epitope analysis showed that the protein has indeed two different binding sites for the CLN003 aptamer. The two regions for the binding sites of the C-Met protein to the CLN0003 aptamer are (524-543) and (557-568). (SEECLSGTWTQQICLPAIYK and LTICGWDFGFRR).
In the analysis of the epitope for the CLN0004 aptamer the results show that two different binding sites occur as well, one in the region of (251-257) with the corresponding sequence: HTRIIRF and one in the region of (381-388) with the sequence: NSSGCEAR.

Conclusions:
Because the C-Met protein has been linked to so many types of cancers it is important to determine the inhibiting mechanisms [4]. The work presented here can be used both for biomarker discovery, by using the CLN0003 aptamer or for delaying the cancer development. Furthermore, we present the discovery of the exact extracellular region necessary for inhibiting the interaction between the natural receptor and C-Met.

Novel Aspect:
By proteolytic-MS we found for the first time the interaction sites of the C-Met protein with two different C-Met aptamers.

References:
LILBID-MS REVEALS INFLUENCES OF THT AND COOMASSIE ON AGGREGATION KINETICS OF AMYLOID-BETA (1-42) AND OSYNULEIN

Janosch Martin (1) - Rene Zangl (1) - Tobias Lieblein (1) - Nina Morgner (1)
Goethe University, Institute of Physical and Theoretical Chemistry, Frankfurt (1)

Keywords: LILBID-MS, Thioflavin T, Coomassie, amyloid-beta, α-Synuclein

Introduction:
Many proteins can spontaneously self-assemble into β-sheet-rich fibrillar aggregates, so called amyloids. Two examples are amyloid-beta (1-42) (Aβ42) and α-Synuclein (α-Syn), the proteins of interest regarding Parkinson’s disease (PD) and Alzheimer’s disease (AD), respectively.[1] To analyze aggregation of amyloids, a commonly used method is the Thioflavin T (ThT) assay. Coomassie - used for staining proteins - is another important dye for protein analysis.

Methods:
In contrast to a ThT-assay laser-induced-liquid-bead-ion-desorption mass spectrometry (LILBID-MS) enables to take a detailed look at small oligomers. Furthermore, there is no need to add analysis required substances. Due to this LILBID can be used to study and compare not only the oligomerization kinetic by time-resolved measurements, but also to reveal the effect commonly used dyes like ThT and Coomassie have on this aggregation kinetic.

Results:
LILBID was employed to analyze and compare the aggregation kinetics of α-Syn and Aβ42. Using LILBID it is possible to detect a range of α-Syn and Aβ42 oligomers. Time-resolved measurements demonstrate faster aggregation of Aβ42 compared to α-Syn.

The fluorescence dye ThT which tags β-sheet structures of a protein is still under discussion due to the potential to affect the aggregation of some proteins, e.g. Amyloid-beta (1-40).[2] Therefore the influence of ThT on the aggregation behavior of α-Syn and Aβ42 was investigated. It can be shown that ThT neither promotes nor inhibits the oligomerization of these proteins, as long as it is purified.

Recent studies also indicate an influence of the protein staining dye Coomassie on α-Syn aggregation.[3] Due to this, LILBID was also used to study how Coomassie affects the amyloidogenic oligomerization of α-Syn and Aβ42. We could show that Coomassie promotes the oligomerization in case of α-Syn, whereas Aβ42 aggregation is not influenced.

Conclusions:
Time-resolved experiments were performed to analyze the influence of ThT and Coomassie on α-Syn and Aβ42. It could be shown that ThT does not influence the aggregation behavior of these proteins. In contrast, Coomassie promotes formation of off-pathway oligomers in case of α-Syn, whereas Aβ42 aggregation is not affected. These are important factors, relevant for interpretation of results obtained with the respective analysis compounds.

Novel Aspect:
Requiring no additional substances, LILBID enables to analyze the potential influence of fluorescence dyes commonly used for aggregation analysis (see ThT-Assay).

References:
278 - CHARGE-INDEPENDENT MASS SPECTROMETRY OF SINGLE VIRUS CAPSIDS ABOVE 100MDA WITH NANOMECHANICAL RESONATORS

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Keywords: Nanomechanical resonators, Native MS, instrumentation, polymer, virus.

Introduction:
Nanomechanical resonators can measure the mass of individual particles accreting on their surface [1]. As their frequency-to-mass relationship scales inversely with the resonator’s dimension, research groups have engaged in a race to measure the smallest detectable mass with increasingly small resonators [2]. However, nanomechanical resonators have best performances in the MDa to GDa mass range, where most technologies, including MS, struggle to measure masses.

Methods:
We developed a novel architecture for nanoresonator-based MS of high-mass biological nano-particles combining solution nebulization by surface acoustic wave nebulization, efficient transfer and focusing by an aerodynamic lens without relying on electromagnetic fields, and mass measurement using an array of frequency-addressed nanomechanical resonators.

Results:
The system’s particle transfer and focusing performances were characterized using NIST standard 45 nm polystyrene nanoparticles. Particle transfer from solution to the detector chamber reached ~7.5%, and the particle beam could be collimated to a 1.5 mm diameter with divergence better than 1°. Mass measurements of NIST nano-particles were in agreement with expectations based on polystyrene density and specified size distribution. The system was then employed for the measurement of empty and filled bacteriophage viral capsids with theoretical masses up to 105.4 MDa demonstrating its bio-analytical capabilities. Measured masses were within ~1% of their theoretical values for both empty and filled capsids. The measurement of the mass of bacteriophage T5 capsids containing the viral genome is, to the best of our knowledge, the largest defined molecular mass ever measured, and with a resolution of 75, it is also the highest reported resolution for single particle measurements above 10 MDa.

Conclusions
We have successfully developed a new nanoresonator-based MS system for high mass measurement. This system determined the mass distribution of ~30 MDa polystyrene nanoparticles with a detection efficiency 6 orders of magnitude higher than previous nanomechanical-MS systems with ion guides, and successfully performed the highest molecular mass measurement to date with less than 1 picomole of bacteriophage T5 105 MDa viral capsids.

Novel Aspect:
An efficient nanoresonator-based MS system architecture allowed individual particle mass measurement without ionization in the MDa to GDa mass range.

References
Native mass spectrometry (MS) has a strong potential for its application towards the systems where other biophysical techniques are futile (1). In yeast, the dedicated guided entry of tail-anchored proteins (GET) pathway targets and inserts tail-anchored (TA) proteins into the endoplasmic reticulum (ER) membrane. A complex of two membrane proteins, Get1 and Get2, captures the TA protein from the Get3 cytoplasmic chaperone and mediates its membrane insertion.

Methods
We applied high resolution native MS based methods to recombinently purified Get complex proteins: Get1 and Get2 membrane proteins and Get3 soluble protein. The experiments were performed on a Thermo QExactive-Plus instrument that has been modified for transmission of intact protein ions under native conditions and for trapping the ion in the source region under optional elevated activation energy (2).

Results
Overexpression and purification of Get1-Get2 membrane proteins was optimised and a full detergent screen was performed to achieve correct stoichiometry of the complex. We show that Get1-Get2 forms a dimer of dimers in surfine, providing the first direct evidence for their stoichiometry. With the help of conventional lipidomics approach we showed that the complex specifically interacts with a molecule of phosphatidylinositol and is destabilised by detergents that disrupt this interaction, indicating lipid-protein interactions are important within the Get1-Get2 complex. A separately purified Get3 protein was then mixed with Get1-Get2 complex and we demonstrate that Get1-Get2-Get3 interacts with a 2:2:4 stoichiometry and that each pair of identical membrane proteins bind one Get3 dimer, providing new insights into the structure and function of the TA protein targeting complex at the ER membrane.

Conclusions
Our native MS and denaturing lipidomics data explain the role of detergents and lipids in the stability and correct stoichiometry of membrane protein translocation pathway complex. These observations for the first time explain insights into the mechanism of TA proteins translocation to ER membranes and provide a platform for further biophysical characterisation of the complex by X-ray crystallography or electron microscopy.

Novel Aspect
MS potential for understanding the fundamental biological processes, in this case, Get complex is revealed and the study opens a door for membrane protein stoichiometry related questions.

References
507 - QUANTIFICATION OF INHIBITOR BINDING TO WILD TYPE AND MUTANT ISOCITRATE DEHYDROGENASE (IDH1) BY NON-DENATURING MASS SPECTROMETRY

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Keywords: native MS, drug binding affinities

Introduction
A single point R132H mutation of human isocitrate dehydrogenase 1 (IDH1) is responsible for inhibition of epigenetic regulators and is associated with a variety of cancers including over 70% of gliomas and 30% of myeloid leukemias. Inhibitors of the R132H IDH1 isoform are currently in preclinical and clinical trials. We present ‘native’ (non-denaturing) MS data on the binding of several of these inhibitors to IDH1 and we compare the results with data from other Methods:

Methods
An IDH1 / inhibitor solution in ammonium acetate buffer, at different enzyme/inhibitor ratios, was analysed using a Nanomate nESI source (Advion) coupled to a Synapt qTOF MS (Waters). Inhibitors tested included Novartis 224 and GSK 864, and AGI-5198, AG 120 and enasidenib (Agios). The binding stoichiometries for the wild type (wt) and R132H IDH1 and the dissociation constant KD were defined and native MS data were compared with the data from UV-absorbance, ITC and NMR.

Results
Mass spectra were consistent with the dimeric state of IDH1, single-site binding for AGI-5198 and AG-120, and two binding sites for GSK864 and Novartis 224. Enasidenib showed almost no binding to R132H IDH1 and single-site binding to wild type IDH1. KD measurements for the enzyme/inhibitor complex ranged from the low µM for AG-120 and GSK 864 to high µM values for AGI-5198/wt IDH1. For AG-120, GSK864 and Novartis 224, the difference between affinities to the wild type and R132H mutant enzyme was small. A large difference in KD was found for AGI-5198 binding to wt and R132H IDH1: 580 µM and 80 µM, respectively. KD values for AG-120 and GSK864 were in the same low µM range as the KD values from our NMR measurements. KD values for AGI-5198 and Novartis 224 were much higher than those from NMR. This may be indicative of a difference between the inhibitors in the binding sites. Native mass spectra of the complexes between the two forms of IDH1 and their respective substrates were also recorded. Studies on the effect of inhibitors on these complexes are currently underway.

Conclusions
We have demonstrated the utility of ‘native’ mass spectrometry in quantification of drug binding to a human enzyme involved in the development of brain and other cancers. We have compared binding stoichiometries and affinities of various inhibitors, and now are evaluating the inhibitor effect on the enzyme/substrate binding. Most of the data corresponds well with the results from other methods, the rest can be rationalised in terms of different binding mechanisms.

Novel Aspect
Stoichiometry of binding of various inhibitors to the IDH1 mutant and wild type enzyme that is involved in brain cancer, and quantitification of binding affinities.
AN OPTIMIZED ENRICHMENT STRATEGY FOR IMPROVED MASS SPECTROMETRY ANALYSIS OF CHEMICALLY CROSS-LINKED PEPTIDES

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Keywords: Cross-linking, MS-cleavable crosslinkers, enrichment, protein-protein interactions

Introduction:
Chemical cross-linking in combination with mass spectrometry is a powerful method to determine protein-protein interactions. However, this method suffers from low identification rates without enrichment/fractionation. In this study, we assessed azido MS-cleavable crosslinkers. Specifically, we evaluated different probes, capture resins, elution protocols and fractionation for increased cross-linked peptide identification by mass spectrometry.

Methods:
Amine-reactive, MS-cleavable crosslinkers including DSSO and Azido-A-DSBSSO[1] were used to crosslink standard proteins and cell lysates. For azido-containing crosslinkers, samples were labeled with different biotin probes before digestion and enrichment. Samples were analyzed by LC/MS on the Thermo Scientific™ Orbitrap™ Lumos™ mass spectrometer. Thermo Scientific™ Proteome Discoverer™2.2 software and XlinkX node were used for data analysis.

Results:
A major challenge of crosslinking mass spectrometry is the relatively low abundance of crosslinked peptides after protein digestion. This results in very few or no crosslinked peptide identifications by MS if the sample complexity is greater than a few hundred proteins. Therefore, enrichment of crosslinked peptides is necessary to identify them in complex samples. To specifically enrich modified peptides, affinity groups such as biotin have been incorporated into the crosslinker structure. However, biotin-enrichment of crosslinked peptides is also challenging due to highly abundant mono-linked peptides, contamination by endogenous biotinylated proteins and/or biotin in elution buffers, and poor recovery from avidin/streptavidin resins. In this work we optimized protocol for an enrichable crosslinker, Azido-A-DSBSSO. The total time of sample prep was reduced 3 times. To further separate monolinks from fully crosslinked peptides, we also developed a workflow using biotin enrichment followed by SCX fractionation.

Conclusions
This new XL-MS workflow allows effective protein cross-linking in vivo to capture authentic protein interactions, selective enrichment of cross-linked proteins and peptides to improve their detection. Our optimized enrichment crosslinked peptide workflow with fractionation increased our identification of crosslinked peptides by 20% compared to enrichment alone for BSA samples and 30% for complex cell lysate samples.

Novel Aspect:
Optimized sample preparation and enrichment of MS-cleavable crosslinked peptides for mass spec analysis.

References
TOWARDS DEFINING OF THE MOLECULAR MATURATION PATHWAY OF THE MITOCHONDRIAL SULFHYDRYL OXIDASE ERV1

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Keywords: Enzyme maturation, protein oligomerisation, cofactor binding, Erv1, activated IM-MS

Introduction:
Erv1 (Essential for Respiration and Vegetative growth) is an FAD-dependent sulfhydryl oxidase which catalyses disulfide bond formation and is required for the import and folding of small cysteine-containing proteins in the mitochondrial intermembrane space (IMS) [1,2]. Our aim is to use hybrid MS to define the maturation mechanism: oxidative folding, cofactor-binding and oligomerisation; of this essential enzyme to benefit the design of better biocatalysts.

Methods:
Experiments were performed on full length Erv1 and its isolated compact C-terminal domain. Native MS and collisional activation prior to ion mobility, (aIM-MS) were applied to provide insights into Erv1 oligomerisation and FAD-binding (Waters SynaptG2S). FAD was titrated into apoErv1 to study the assembly pathway. Charge reducing agents were used to mimic the solution like oligomeric order of Erv1.

Results:
Native MS experiments have shown full length Erv1 (purified in presence of FAD) to exist in tetrameric, dimeric and monomeric state. Both, tetrameric and dimeric Erv1 display over bimodal charge state distribution, characteristic for ordered-disordered systems. Still, Erv1 displays unusually broad charge state distribution even for a disordered system [3]. Addition of charge reducing agent to the buffer resulted in shift of the oligomerisation equilibrium to narrow dimeric and tetrameric states and mimicked solution measurements. Highly conserved C-terminal domain of Erv1 displayed a narrow charge state envelope characteristic of folded proteins. aIM-MS shows how intrinsically unfolded Erv1 species are more prone to release of FAD prior to the subunit dissociation. The energy barrier for subunit dissociation in compact multimers is lower than the cofactor release barrier. Titration experiments of FAD into apoErv1, present mainly as apo monomer and somewhat as apo dimer, show the assembly into a fully occupied tetrameric Erv1 upon binding of the cofactor.

Conclusions:
Findings from MS provide new insights to define the maturation mechanism of Erv1. Erv1 exits in several multimeric states in the presence and absence of FAD, with a preference towards monomeric species in absence of the cofactor. FAD binding is essential for coordination of monomeric and dimeric Erv1 into tetramers.

Novel Aspect:
Application of native MS and top-down IM-MS to study enzyme maturation process, along with use of charge reducing agent to mimic solution conditions.

References
ENRICHMENT OF ELECTROCHEMICALLY CLEAVED PEPTIDES FOR MIDDLE-DOWN PROTEOMICS APPLICATIONS

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Keywords: electrochemistry, protein digestion, spirolactone, biotinylation

Introduction
Enzymatic digestion of proteins is standard procedure in bottom-up proteomics. Alternatively, digestion can be achieved by electrochemical (EC) oxidation of proteins, leading to specific cleavage at the C-terminal side of Tyr and Trp residues. EC oxidation generates a mixture of cleavage and non-cleavage products. Cleaved peptides have a unique spirolactone at the C-terminus, which can be employed for their enrichment [1,2].

Methods
Model peptides and small proteins were oxidized at 1-2 V in an electrochemical flow-through cell with a carbon or boron-doped diamond electrode. The cleavage products were specifically derivatized with amine-containing reagents, including amine-biotin, at different pH values in the presence of metal ions. Derivatized peptides were affinity-enriched and analyzed by LC-MS/MS.

Results
Model tripeptides including LWL were oxidized to optimize the cleavage conditions and the reaction conditions with amine-biotin. At basic pH the peptide-spirolactone reactivity is highest, and a hydrolysis side reaction can be prevented by stabilization with Cu(II) ions. Other metal ions do not confer stability. The optimized conditions were then applied to small proteins, including lysozyme, leading to extensive cleavage at most Tyr and Trp residues. Enrichment of the cleaved, labelled peptides with streptavidin beads allowed the identification of the proteins in the digest. The amine-biotin tag readily fragments giving rise to a peak at m/z 375 which was used to verify the presence of enriched peptides. Further improvement of the enrichment using direct coupling of peptide-spirolactones to activated resins is expected to enable analysis of more complex EC-cleaved protein mixtures.

Conclusions
Cleavage of proteins with multiple Tyr and Trp residues and efficient derivatization with amine-biotin in the presence of Cu(II) was achieved. Biotinylated peptides were enriched with affinity capture, and LC-MS/MS analysis was used to identify the protein. Specific biotin fragments enable verification of biotinylation.

Novel Aspect
An electrochemical protein digestion method was optimized for protein identification using peptide derivatization and enrichment.

References
Introduction:
Synaptic vesicles (SVs) are small storage organelles for neurotransmitters. They are densely packed with proteins and pass through a trafficking cycle including fusion with the presynaptic membrane [1]. Membrane fusion depends on formation of the SNARE complex which connects the two membranes [2]. We combine chemical cross-linking and native mass spectrometry (MS) to unravel protein assemblies in SVs as well as the interactions formed during membrane fusion.

Methods:
SVs were purified from rat brain. Individual proteins were expressed in E. coli and purified via a His-tag. Proteins were cross-linked using BS3 cross-linker. After tryptic digestion, cross-linked peptides were enriched by gel filtration, analysed by LC-MS/MS on a Q Exactive Plus mass spectrometer and identified using pLink software [3]. Assembled protein complexes were further studied by native MS using a modified Q-ToF Ultima mass spectrometer [4].

Results:
SVs were purified from rat brain and major proteins were identified. Cross-linking then delivered first interaction networks in intact SVs showing that Syb plays a central role in complex formation presumably due to its high flexibility. Using botulinum toxin to cleave the soluble segment of Syb and thus removed it from the protein networks allowing us to identify additional protein interactions. To study Syb in detail, we purified different variants, including full-length Syb, the soluble domain and a shorter segment. Using cross-linking and native MS we found that all variants formed oligomers in solution; the shortest variant formed higher order oligomers suggesting unspecific aggregation. By incubating the soluble segments of SNARE proteins or the full-length proteins incorporated into liposomes (i.e. membrane mimics) we studied the intermediates of SNARE complex formation. Cross-linking and native MS revealed protein interactions and stoichiometries in these assemblies.

Conclusions:
Our results provide novel insights into the heterogeneous protein interaction networks in SVs which are key to our understanding of signal transduction in the neuronal synapse. In addition, by employing individually purified proteins and variants thereof, we provide insights into the interactions formed in solution or when the proteins are incorporated into membrane mimics. We are thus able to monitor SNARE complex formation and uncover intermediate states.

Novel Aspect:
We provide first insights into protein interactions in SVs and pave the way to study membrane fusion by combining complementary MS techniques.

References:
Keywords: Native MS, Protein Complexes, Antibodies, Crude Proteins Samples, Protein-Ligand interactions

Introduction:
Key processes in a cell are carried out by protein complexes whose functions require tight regulation. The classical, high-resolution methods used for understanding protein regulation are often insufficient when attempting to study large protein assemblies, due to their size, heterogeneity and dynamic composition – thus, new methodologies are essential. Native MS is one such method.

Methods:
I will present our studies involving different developments and applications of native MS approaches with examples focusing on protein-protein interactions, protein-ligand systems and antibodies. I will also show a direct MS method that enables characterization of recombinant proteins directly from crude samples. Involving proteins produced in bacteria, yeast, insect and human cells.

Results:
The direct-MS approach can be carried out for proteins produced in prokaryotic and eukaryotic expression systems. The method provides immediate information on the expression, identity, solubility, oligomeric state, post-translational modification, ligand binding, overall structure and stability of overproduced proteins. I will show that the expression profile, post-translational modifications, overall structure and stability of engineered therapeutic antibodies, as well as glycosylated proteoforms of a modified protein can be immediately assessed by analysis of the crude samples. Moreover, I will demonstrate that the method is applicable for throughput characterization of protein variants, as mutants and engineered constructs. Our method is general and can be applied on various mass spectrometry platforms.

Conclusions
The major advantage of the method is that it considerably shortens the time gap between protein production and characterization while saving the burden associated with purifying inactive variants. The approach is particularly suitable for selecting the ideal protein expression host system, optimizing codon usage, expression levels and quality and employing high throughput investigations of lead candidates.

Novel Aspect:
Given the broad use of recombinant proteins in biochemical investigations and in industrial and therapeutic applications the method is expected to be widely utilized.

References
Keywords: Structural MS, Calmodulin, Aluminum hydroxide, Adjuvant adsorption, Hydroxyl radical footprinting.

Introduction:
Possible conformational modifications of antigens during aluminum hydroxide adjuvant adsorption are discussed in literature for formulated vaccine for its potential impact on antigen immunogenicity [1-4]. The development of a mass spectrometry (MS) based technique called hydroxyl radicals footprinting (HRF) for the direct characterization of adjuvant-adsorbed protein is discussed in this poster showing a case study on calmodulin (CaM).
CaM is a small and well characterized protein that is part of calcium signaling pathways [5]. CaM is an acidic protein with two globular domains connected by a central linker. Each domain contains two EF-hand sites that bind intracellular Ca2+ ions. Upon Ca2+ binding the EF-hand domains undergo to a conformational rearrangement, exposing hydrophobic protein domains that can bind a wide range of target proteins. Hydroxyl radical footprinting technique (HRF) [6] coupled to MS was chosen to detect conformational changes of CaM, if any, when absorbed on aluminum hydroxide and then exposed to Ca2+ ions. Hydroxyl radicals are highly reactive species that chemically modify the surface accessible amino acid side chains with a chemical susceptibility to oxidation (+ 16 Da mass shift, or +32 or +14 depending on the residue), enabling monitoring of a wide number of target sites and consequently providing structural information.

Methods:
Sample treatment protocol was developed ex novo to be compatible with aluminum hydroxide adjuvant. Fenton chemistry catalytic reaction [6] was used to generate hydroxyl radicals in solution and regenerate the Fe(II) species with sodium ascorbate. The three Fenton chemistry reagents (hydrogen peroxide, Fe(II)-EDTA, and sodium ascorbate) were added together with the sample. Hydroxyl radicals reaction with CaM was tested at 4 different time points (0, 2, 5, and 10 minutes) then reaction was quenched with thiourea. Subsequently protein were desorbed from adjuvant to allow the enzymatic digestion. Protein desorption was obtained by overnight incubation of the samples with phosphate buffer. Tryptic peptides are then analyzed on Q-Exactive Plus mass spectrometer (Thermo Fisher Scientific) by LC-MS/MS DDA top5 experiment. Data were processed to enable relative quantification of the oxidized methionine-residue. The 9 methionine residues distributed along the protein sequence of CaM were selected as probes for structural modification probing. Protein conformational characterization was correlated to the different solvent exposure of the methionine residues calculated on their oxidation amount linear regression slopes at different time points.

Results and Conclusions:
Nine methionine residues (belonging to the protein sequence) where monitored on 4 different conditions, considering or not adjuvant and Ca2+ ions:
1) In-solution CaM;
2) In-solution CaM 10mM CaCl2;
3) Adjuvant-adsorbed CaM;
4) Adjuvant-adsorbed CaM 10mM CaCl2.
Significant change in methionine oxidation rate slopes was observed in both cases of sample 2) and 4) respect to their no-Ca2+ reference samples 1), and 3).
This study demonstrated that HRF was able to detect induced conformational changes of CaM when exposed to Ca2+ ions in aluminum hydroxide-adsorbed samples.

Novel Aspect:
HRF is a new application for interface-adsorbed protein structural characterization by MS. Implementation of this technique could provide an valuable analytical tool for adjuvanted vaccine efficacy and quality assessment.

References:
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Keywords: native mass spectrometry, crude proteins samples, recombinant proteins, proteins analysis, ion mobility mass spectrometry

Introduction:
Characterization of over-expressed proteins is usually coupled to costly and time-consuming purification steps. We previously presented a native MS approach for protein analysis directly from crude bacteriallysates without purification1. Although prokaryotes expression systems are very efficient, they lack the machinery required for PTMs, highlighting the need for eukaryotic hosts. Here we present a method for characterization of proteins directly from crude samples from such systems.

Methods:
As model systems we used different eukaryotic hosts that secrete recombinant proteins, the yeast P. pastoris expressing the cellulose binding module (CBM) from C.thermocellum and human serum albumin (HSA), and Sf9 insect cells expressing Transferrin receptor 1 (Tfr-1). Growth media from cells were collected and buffer exchanged into a MS compatible buffer. The different samples were analyzed using either an Orbitrap or a Synapt G2 HDMS QTOF-based platform2.

Results:
Initially we analyzed by native MS samples directly from P.pastoris and Sf9 growth mediaovercoming the need for purification procedure. Examination of the different growth conditions allowed us to monitor expression already after 24h of induction, from cultures, grown in 100µl medium in 96 well plates. We were able to monitor co-factor binding and validate that Ca2+ is associated with the CBM binding pocket using incubation of media with EGTA and calcium acetate. Furthermore, IM-MS spectra were measured for CBM directly from P. pastoris growth media. Using arrival time distribution, the CCS (Ω) was calculated and found to be highly correlated to theoretical CCS. Next, we examined the Tfr1 protein, secreted from Sf9 cells. Analysis of the crude growth medium generated highly resolved spectra, which revealed four different glycosylation states of the protein. We validated this by releasing theN-linked oligosaccharides by PNGase-F.

Conclusions:
Here we show that direct native MS can be efficiently applied to different eukaryotic secretion systems. By examining the culture media of different expression systems we demonstrate that it is possible to rapidly determine the identity, solubility, oligomeric state, ligand binding, modification level and overall structure of secreted proteins. Moreover, we show that our method is not dependent on a specific MS platform but rather it is a general approach that can be applied on various instruments.

Novel Aspect:
Here, we show that direct-MS can dramatically shorten the time gap between protein production and characterization.

References:
Keywords: Native MS, crosslinking, top-down, pseudo MS3

Introduction: The structural determination of protein complexes plays an important role in the fundamental understanding of their catalytic function. Multiple analytical methods, including hybrid approaches such as cryoelectron microscopy, cross-linking, and native mass spectrometry, are usually required for such type of analysis[1]. In this study we combined multiple mass spec based structural proteomics techniques to characterize the rabbit 20S proteasome complex.

Methods: Rabbit 20S proteasome complex was obtained from Boston Biochem. LC/MS bottom up, crosslinking and intact/top down analysis were performed using a Thermo Scientific™ UltiMate™ 3000 RSLCnano system and a Thermo Scientific™ Orbitrap™ Lumos™ mass spectrometer. Native MS experiments were performed using a modified Thermo Scientific™ Q Exactive™ Plus MS[2]. Data were analyzed with Thermo Scientific™ BioPharma Finder3.0, Proteome Discoverer™ 2.3, and ProSightPC.TM

Results: The 20S proteasome complex contains 28 subunits arranged into four stacked rings: seven alpha non catalytic subunits and seven beta-subunits[3]. In the first series of experiments we performed bottom up, intact and top down analysis using databases of proteasome proteins from rabbit, human, and all species in UniProt. We identified 224 proteins and 17 different types of proteasome subunits and used the results to verify or correct the originally homology-based rabbit sequences. We then used this curated rabbit 20S proteasome database for all other experiments. For structural characterization, we performed native MS intact and top down experiments using a modified Q Exactive Plus mass spectrometer. The measured mass of the 28 subunits complex was consistent with expected value of 716.4 kDa and pseudo-MS3 experiments enabled identification of alpha 2 and beta 6 subunits. Crosslinking experiments using DSSO are combined with restraints from native and top-down MS to validate the structure obtained through electron microscopy.

Conclusions: Combining results from different MS methods, including novel native top-down -pseudo MS3 approach, we were able to perform comprehensive analysis of the rabbit 20S proteasome.

Novel Aspect: Complete workflow for structural characterization of protein complex

References:
Introduction:
The F1FO ATP synthase of Acetobacterium woodii and the heterodimeric ABC transport complex TmrAB from Thermus thermophilus are responsible for ATP synthesis/hydrolysis and ATP-dependent membrane transport mechanism, respectively. We are interested in the assembly of subunits and investigate requirements for the different assembly steps. For TmrAB we apply ion mobility measurements to analyze conformational changes in dependency of different ligands.

Methods:
In LILBID-MS a droplet generator generates droplets of an aqueous sample. These are transferred into vacuum and irradiated with an IR laser ($\lambda = 2.94 \, \mu m$) which leads to an explosive expansion of the droplets, ionization and finally a detection by a time-of-flight analyzer [1]. The Synapt G2-S HDMS offers ESI-mass spectrometry combined with ion mobility (IM) measurements.

Results:
First we focused on the soluble part F1 of the F1FO ATP synthase of Acetobacterium woodii. The plasmids pKV003 (7036 bp), pKV004 (6928 bp) and pKV019 (6822 bp) (which include either the atpA- (encodes $\alpha$), the atpD- (encodes $\beta$) or atpG-atpC (encodes $\gamma \varepsilon$) genes) were transformed in competent E. coli BL21gold(DE3) cells. The $\alpha$, $\beta$-subunit and the $\gamma \varepsilon$-complex were successfully purified by tandem affinity (Ni-chelating and Strep-Tactin) chromatography and can be characterized by LILBID-MS. Recently we are able to show the assembly efficiency of $\alpha$, $\beta$-subunits and also with the central stalk $\gamma \varepsilon$-complex of the ATP synthase in dependency of ATP and Mg2+. Furthermore the steps of assembly were investigated by varying the laser energy.

A second project investigates three different ion mobility experiments we monitored conformational changes (inward/outward facing structure) of the heterodimeric ABC transport complex TmrAB related to ligand transport over the transmembrane by adding ATP/ADP at permissive conditions [2][3].

Conclusions:
At soft LILBID conditions we could show that the assembly efficiency in the soluble part F1 can be improved in an ATP/Mg2+ environment. Unfortunately the assembly is not yet efficient as we expected. We will vary the position for the purification tags to improve the assembly with the central stalk $\gamma \varepsilon$.
With ion mobility experiments we are now able to monitor conformational changes of the ABC transport complex in dependence of substrate binding and addition of nucleotides.

Novel Aspect:
Investigation of the assembly of a protein complex, via native MS studies. Analyzing conformational changes of membrane proteins with ion mobility.

References:

836 - GAS-PHASE ELECTROPHORESIS (NES GEMMA) OF VIRUS-LIKE PARTICLES: RELATING PARTICLE SURFACE-DRY SIZE AND MOLECULAR WEIGHT

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Keywords: Gas-phase electrophoresis, Virus-like particle, VLP, nES GEMMA, native MS

Introduction:
Gas-phase electrophoresis (on a nES GEMMAaka nES DMA) [1] separates surface dry particles according to their electrophoretic mobility diameter (EMD) which corresponds to the size of spherical nanoparticles. As shown for proteins and viruses [2,3], the EMD also relates to the molecular weight (MW) enabling to calculate an analyte MW based on its EMD. We now focus on virus-like particles (VLPs) and show the deviation of their EMD / MW correlation from other analyte cases.

Methods:
VLP samples were desalted and transferred to ammonium acetate solution prior to native ESI MS and nES GEMMA analysis. Data for the following VLPs were combined / obtained: Human Rhinovirus Serotype 2 (HRV-A2) empty subviral particles [3], Hepatitis B Virus VLPs with T3 and T4 symmetry [4] and several norovirus VLP strains.

Results:
VLPs of sufficient purity and concentration can be analyzed via native ESI MS and nES GEMMA. Comparing EMD values from nES GEMMA and MW values from native ESI MS experiments yields an EMD / MW correlation for VLPs. The particle MW is related to the EMD via a function to the power of approx. 3.5. Therefore, the EMD / MW correlation for VLPs was found to be significantly different from a similar correlation obtained for viruses (power of approx. 3.7) [3] and proteins (power of approx. 2.9) [5]. Hence, in order to be able to calculate reasonable MW values based on EMD data, the substance class (protein, intact virus, VLP) of the analyte in question needs to be known. If this requirement is met, nES GEMMA based calculation of a particle MW represents an interesting alternative at atmospheric pressure to the native MS approach.

Conclusions:
We demonstrate that an EMD / MW correlation for VLPs can be obtained. Based on this data and EMD values obtained via nES GEMMA, MW values for VLPs of unknown MW can be calculated. In addition, we demonstrate the deviation of EMD / MW correlations for proteins and virus particles from the VLP case. A larger VLP dataset will further improve our EMD / MW correlation for VLPs.

Novel Aspect:
We (i) present an EMD / MW correlation for VLPs and (ii) demonstrate the deviation of this VLP correlation from the protein and intact virus case.

References:
966 - NATIVE MASS SPECTROMETRY TO IDENTIFY STOICHIOMETRIES AND STABILITY OF CRISPR-CASCADE COMPLEXES

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Keywords: Native mass spectrometry, proteinstoichiometry, CRISPR-Cas complexes

Introduction:
The CRISPR-Cas system of bacteria or archaea is an adaptive immune system against foreign DNA. The type I systemuses a large ribonucleoprotein surveillancecomplex called ‘Cascade’ in which the crRNA targets the foreignDNA by base-pairing and thus triggers DNA degradation. We used native mass spectrometry (MS) to determine the protein stoichiometries of CRISPR-Cascade complexes containing longer or shorter crRNAs than the wild-type complex.

Methods:
Streptococcus thermophilus (St) CRISPR-Cascade was purified as described [1]. The purification buffer was exchanged against 200 mM ammonium acetate prior to native MS. Intact St-CRISPR-Cascade complexes were then analyzed on a Q-ToF Ultima mass spectrometer modified for transmission of high mass complexes [2] using gold-coated capillaries prepared in-house [3]. Mass spectra were processed and analyzed using MassLynxx and Massign software [4].

Results:
St-CRISPR-cascade complex was purified and cascade subunits were identified by LC-MS/MS and database searching. All five subunits (i.e. Cse1, Cse2, Cas5, Cas6 and Cas7) were identified. Next, we used native MS to define the stoichiometric of the wild-type CRISPR-cascade complex containing crRNA, Cse1, Cse2, Cas5, Cas6 and Cas7 subunits. The native MS spectrum revealed a Cse11(Cse2)2(Cas7)6Cas51Cas61crRNA1 complex. The native MS spectrum also showed subcomplexes which lost Cse1 and Cse2 subunits as well as some copies of Cas7.

We further analyzed the impact of shorter or longer crRNA on the stoichiometry of the cascade complex. For this, we employed crRNAs which are 12 nucleotides longer or shorter. The native MS spectrum of the complex containing longer crRNA showed one abundant CRISPR-cascade complex with a stoichiometry of Cse11(Cse2)3(Cas7)8Cas51Cas61crRNA1. In contrast, the mass spectrum of complex containing shorter crRNA revealed several complexes; the most abundant complex was Cse11(Cse2)1(Cas7)4Cas51Cas61crRNA1.

Conclusions:
We conclude that an increase/decrease of 12 nucleotides in the crRNA results in addition or loss of two Cas7 and one Cse2 subunits. The CRISPR-cascade complexes containing longer crRNA appear to be more stable when compared with the shorter crRNA containing complexes and form mostly one abundant complex. Currently, we analyse Cascade complexes with variable crRNA length to gain insights into their stoichiometries and its impact on complex stability.

Novel Aspect:
We studied the impact of crRNA length on protein stoichiometry and complex stability of CRISPR-Cascade complexes.

References:
CHARACTERIZATION OF DEUTERATED ALPHA-CRYSTALLINE USING BENCH-TOP MALDI-TOFMS.

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Keywords: MALDI-TOFMS, deuterated protein, crystalline, cross-linker

Introduction:
Deuteration of protein is a one of the important technique in structural biology by using NMR or neutron scattering method. Incorporation of deuterated subunit protein into the hydrogenated one brings a new aspect of molecular dynamics in formation of multimer. As recent techniques for the deuteration have been more precise and more cost-effective, a quick and facile method to characterize deuterated proteins has been anticipated. In this report, we applied newly developed bench-top MALDI-TOFMS to analysis of deuterated αB-crystalline to study substitution of hydrogen to deuterium in three different ways, molecular weight analysis, in-source decay, and tryptic peptide analysis. Furthermore, we will report multimer formation of the protein by using high mass detector and cross-linking chemistry.

Methods:
Human recombinant hydrogenated/deuterated αB-crystalline was prepared with overexpression in E.Coli. with/without deuterated culture medium, as described[1]. Three samples, deuteration ratios of which were roughly estimated as 0%, 73%, and 98% (denoted as D0, D73, and D98, respectively), were subject to MALDI-TOFMS. Concentrations of them were 3 to 15 mg/mL. Bench-top MALDI-TOFMS (MALDI-8020, Shimadzu Corp., Japan) was applied to perform molecular weight measurement of whole proteins, in-source decay, and tryptic digest analysis in linear mode. Sinapinic acid, 1,5-DAN, and CHCA were used in those experiments. A cross-linker reagent was obtained from Covalx (Switzerland). All samples were reacted with the linker following an instruction provided from the company. After the reaction, desalted samples were mixed with sinapinic acid, then applied onto a stainless MALDI plate. MALDI-TOFMS (AXIMA-Performance, Shimadzu Corp., Japan) equipped with HM3 high mass detection system (Covalx, Switzerland) was applied to detection of cross-linked multimer in positive linear mode. The TOF analyser was calibrated using BSA and its multimer

Results:
Even though the newly developed bench-top MALD-TOFMS has only linear mode, its performance was sufficient to estimate the deuteration ratio of the protein. Whole protein analysis of three samples was conducted with sinapinic acid, and mass shifts against D0% (hydrogenated) protein were estimated. While theoretical shift for 100% deuterium substituted protein was 1107.5, an observed shift of D98% was 1100, which indicates 99% deuteration ratio as almost expected. As well, an observed mass shift of D73% was 762.8, indicating 69% deuteration ratio, which was a little lower than expected. In-source decay using 1,5-DAN was conducted to examine an internal sequence of the proteins. Despite lower mass resolution caused by higher laser irradiation, a series of c-ions, observed up to 67 residues, were well-assigned to deuterated amino acids within the modest error, suggesting almost no significant bias of the deuterium substitution at any amino acid. Then, three proteins were digested with trypsin and isotopical distributions of each digested peptides were investigated. By simulation of isotopical distribution, deuteration ration of the peptides were estimated as 99%, which was consistent with the value obtained with whole protein analysis. Additionally, hydrogenated αB-crystalline was subject to cross-linking MS with high mass detector to observe multimer formation. As preliminary result, widely disperse multimers were detected at the mass range from m/z 200k to 500k. Interestingly, whereas an intense monomer signal and a weak dimer signal were also observed, there
was no intermediate formation of multimer, which is consistent with the proposed concept of “traveling subunits” that could be a transiently liberated in subunit exchange[1].

Conclusions:
Deuteration of protein was investigated with a newly developed bench-top MALDI-TOFMS. Molecular weight analysis, digested peptide analysis, and ISD enabled to estimate a deuterated ratio, which is critical factor in study of a dynamics of protein multimer with small angle neutron scattering. The accessible bench-top instrument could contribute to structural biology in prior use for the neutron scattering or NMR. Furthermore, the multimer of the crystalline was stabilized with cross-linking chemistry, and observed with high mass detector, through-put of which could be higher than native MS using ESI in analysis of multiple subjects.

Novel Aspect:
A deuterated ratio of B crystalline was estimated with a newly developed bench-top MALDI-TOFMS, and multimer of the protein was observed with cross-linking chemistry and high mass detector successfully.

References:
Takata T, et al., Scientific Reports, 6, 29208 (2016)
Introduction:
Liquid extraction surface analysis (LESA) is a powerful method for measuring proteins from surfaces. In this work we present development and initial testing of a cryo platform for LESA MS. We explore the use of native sampling solutions for probing proteins directly from frozen surfaces.

Methods:
An Advion Triversa Nanomate nano-electrospray source coupled with a Waters Synapt G2Si mass spectrometer was used for LESA MS. Modifications to the sample recess were made to incorporate water cooled copper bars and a thermoelectric peltier cooling system. Protein and LESA sampling solutions were prepared using either 100 mM ammonium acetate or 1:1 water/methanol with 0.1% formic acid. Experiments were conducted at room temperature and using the frozen stage.

Results:
Preliminary results were obtained for ubiquitin. In ammonium acetate (20°C), native LESA sampling yielded ions with a narrow charge state distribution (5+ and 6+). However, H2O/MeOH with 0.1% FA as the solvent resulted in a broad charge state distribution of ubiquitin ions (5+ to 13+) and slower average ion-mobility, indicating protein unfolding. For ubiquitin dissolved in H2O/MeOH with 0.1% FA, denatured sampling also produced ions with a broad charge distribution. However, surprisingly, the mass spectrum acquired by native sampling of this denatured protein is dominated again by only 5+ and 6+ ions, which could be the evidence of protein re-folding. This was further supported by faster average mobility. The results using cryo sampling show very similar trend at room temperature, though the relative abundancies of +5 and +6 ions differed for native LESA of denatured vs native proteins (1:2 for the former; 2:1 for the latter). The ion mobility was slightly slower than that of the original folded protein ion.

Conclusions
Protein unfolding or re-folding was observed to occur in LESA mass spectrometry study. The charge state distributions in the acquired mass spectra can not necessarily be used to judge whether the original structure of the studied protein is folded or unfolded. By employing cryo sampling method, the extent of protein unfolding or re-folding during LESA process can be largely minimized, therefore making truly native surface sampling of protein promising.

Novel Aspect:
A cryo-stage was developed for LESA-MS. With this protein re-folding and unfolding can be minimized during sampling so truly native surface analysis could be fulfilled.
Native mass spectrometry analysis of norovirus glycan interaction

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Introduction:
Human noroviruses (HuNoV) are non-enveloped RNA viruses belonging to the Caliciviridae family and the main cause of viral gastroenteritis. From the crystallographic structure, it is apparent that the norovirus capsid protein protruding (P) domain binds to glycans on the cell to mediate cell attachment. A detailed investigation of P domain-glycan interaction by native mass spectrometry (MS) is used to shed light on the mechanism behind viral cell entry.

Experiments:
Native MS is applied to investigate glycan binding to different HuNoV strains. Blood group antigen B tetrasaccharide was used to characterize glycan binding affinity and build titration curves. Other groups of carbohydrates such as gangliosides, human milk oligomers and globosides have been measured. We also tested multivalent glycan mimetics with different spacing and number(s) of glycan of interest for binding to P dimers to resemble glycan-binding in the natural situation. The number of binding events per type of natural saccharides and multivalent ligands will be compared and investigated between norovirus genotypesto elucidate binding profiles of glycans.

Results:
The results elucidate the dissociation constants (KD) of natural glycans and glycan mimetics in norovirus cell attachment. Blood group antigen B revealed highest binding affinity among tested glycan candidates with a range of 100µM KD for the first binding event. A similar value was obtained by Saturation transfer difference nuclear magnetic resonance (STD-NMR). Another galactose containing natural glycan however showed similar binding affinity in native MS, while NMR data shows that single galactose cannot bind. The glycan mimetics binding affinity increases with increasing number of galactoses. The fucose containing mimetics showed good affinity but no avidity yet indicating that the spacing or rigidity requires further optimization. Another set of glycan mimetics based on these results are currently under investigation.

Keywords:
Native Mass Spectrometry, Noroviruses, Glycan, P domain, Binding.

References:
Introduction:
Human saliva, which is often found at the scene of the crime, must be distinguished from other fluids in order to be recognized as legal evidence. However, conventional methods to identify saliva have lack of specificity due to cross-reactivity with other fluids. Here, we have explored saliva glycosylation using nanoLC/MS to examine saliva-specific signatures which can differentiate human saliva from other biological fluids.

Methods:
Whole saliva was collected from 30 healthy donors. N-glycans were directly released from saliva (50 μL) by PNGase F and then enriched by PGC-SPE. Saliva N-glycans were comprehensively analyzed by nanoLC-PGC chip/Q-TOF MS and tandem MS. To determine human saliva-specific glycans, human saliva N-glycans were compared with human fluids and animal saliva. Individual and environmental variations of saliva glycosylation were also assessed.

Results:
Approximately 100 N-glycans were determined through human saliva samples. Interestingly, most glycans found in saliva were decorated with more than one fucose. In particular, highly fucosylated N-glycans containing at least 4 fucose residues were observed in abundance (up to 14%). Interestingly, sialylated N-glycans were highly abundant (>70%) in human serum. Acidic glycans with O-acetylation on a sialic acid were found in rat saliva as major components at a high rate while sulfation on outer HexNAc was solely observed in bovine saliva. Conclusively, highly fucosylated N-glycans (n=19) and significant fucosylation levels (>50%) were determined as saliva-specific signatures for identifying human saliva from other vital fluids. These significant glycans were further investigated to validate individual variation and sample stability. Human saliva-specific glycans were consistently observed regardless of the collection date while they were gradually decreased during storage time of saliva (1, 2, 3, and 7 days) at room temperature.

Conclusions:
This study demonstrated the feasibility of MS-based glycomic approach for distinguishing human saliva from other body fluids. Nineteen highly fucosylated N-glycans were exclusively present in human saliva and fucosylated N-glycans account for more than 50% of total glycans found in saliva. Saliva-specific glycans and their amounts can be used as a bio-signature to differentiate human saliva from other biological fluids in forensic science.

Novel Aspect:
For the first time human saliva-specific glycans were identified by mass spectrometry-based glycomic approach.

References:
29 - SINGLE-INJECTION SCREENING OF 664 FORENSIC TOXICOLOGY COMPOUNDS USING AN INNOVATIVE BENCHTOP HIGH RESOLUTION MASS SPECTROMETER

Janna Anichina (1) - Oscar Cabrices (2) - Xiang He (2) - Holly McCall (2) - Laura Baker (2) - Alexandre Wang (2) - Adrian Taylor (3)

SCIEX, Product Applications Laboratory, Concord (1) - SCIEX, Applications, Redwood City (2) - SCIEX, Product Management, Concord (3)

Keywords: Quadrupole Time-of-flight mass spectrometry, drug screening, single injection

Introduction:
Quadrupole Time-of-flight mass spectrometry (QTOF-MS) provides high-resolution, accurate-mass data for full-scan information of both precursor and product ions making it ideal for forensic screening where unknown compounds in biological samples must be identified. In this presentation, a single-injection method for screening for the enhanced identification of 664 most up-to-date forensic compounds using an innovative benchtop QTOF-MS system is described.

Methods:
Urine and whole blood samples were spiked with stock standard mixtures and used to determine the retention times of the 664 compounds. Samples were extracted and further diluted with mobile phase for chromatographic separation. Mobile Phase was ammonium formate in water and formic acid in methanol, 600 µL/min flow rate. The QTOF-MS was operated in positive electrospray mode with information dependent acquisition MS/MS methods for accurate compound identification.

Results:
Separation performance was evaluated with different mobile phases (acidic and neutral), gradient conditions, and column types. Results indicate that most of isomeric compounds were fully resolved with a neutral buffer and a 10-min linear gradient. Analyte retention time (RT) was a critical element for accurate identification all 664 compounds using this screening method. Reproducibility tests indicated that the RTs obtained from the optimized LC conditions were consistent and reproducible. RTs measured have %CVs of less than 5% for all compounds. RT inter-day reproducibility resulted in %CVs less than 5% over 3 days. RT variability in human whole blood and urine samples indicated that the %CV for 3 individual lots is less than 5%. The optimized LC conditions combined with MS/MS spectra, enabled accurate compound identification with this workflow. Retrospective analysis was also performed on the acquired data sets to screen for new compounds without having to re-inject samples, allowing to re-process data sets as new targets were discovered.

Conclusions
We have developed a QTOF-MS screening method for 664 forensic compounds, the optimized LC conditions combined with MS/MS spectra, enabled accurate compound identification. In addition, because the data was acquired in a non-targeted approach the processing method designed here for screening targeted compounds can be quickly adjusted and used for unknown compound identification using a non-targeted data processing, without having to re-inject samples.

Novel Aspect:
High resolution Mass Spectrometry Analysis of 664 forensic compounds in a single injection using an innovative benchtop QTOF-MS system.
30 - ULTRA-SENSITIVE FORENSIC ANALYSIS WORKFLOW OF COCAINE AND METABOLITES IN HAIR SAMPLES USING LC-MS/MS

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Keywords: triple quadrupole linear ion trap mass spectrometry, Cocaine metabolites, hair analysis

Introduction:
Forensic analysis of cocaine in hair requires a sensitive and reliable analytical workflow. There are two major challenges for the detection of this compound and its metabolites in hair samples: Low concentration and matrix interferences. In this presentation, an analysis workflow combining the use of triple quadrupole linear ion trap mass spectrometry with solid phase extraction (SPE) for the picogram detection of Cocaine and Metabolites in hair is described.

Methods:
Hair samples were washed, dried and cut into ~ 2 mm segments. The analytes were extracted via sonication for four hours. Supernatants were subsequently passed through SPE cartridges, dried and reconstituted in mobile phase for HILIC separation. Mobile Phase was ammonium formate in water and acetonitrile, 400 µL/min flow rate. A SCIEX QTRAP® 6500+ LC-MS/MS system operating in positive electrospray mode was used for detection, with two MRM transitions for all analytes.

Results:
One of the main challenges encountered throughout the study was to obtain clean hair extracts for the reliable detection and quantitation of cocaine and metabolites from hair due to the presence of complex matrix contents. To remove these interferences, Phenomenex Strata-X SPE cartridges were successfully used to selectively isolate the drug and its metabolites. Analyte extraction recoveries were demonstrated to be greater than 80 % enabling the analytical workflow to obtain sub pg/mg Lower Limits of Quantitation (LLOQ) in hair matrix. The workflow showed excellent accuracy (>95%) and precision (< 15%), with excellent linearity resulting in R2 values of 0.9990 for all analytes. In addition to quantitation, the SCIEX QTRAP® 6500+ LC-MS/MS system enabled simultaneous identification and confirmation of illicit drugs and their metabolites through utilization of Enhanced Product Ion Scan (EPI) by acquiring full scan MS/MS data. Forensic drug identification and confirmation was achieved using automated MS/MS library searching.

Conclusions
A quantitative SPE-LC/MS/MS analysis workflow for simultaneous determination of Cocaine and metabolites at picogram levels was developed and evaluated in hair matrix. The method was demonstrated to be sensitive, precise and accurate. Utilization of the SCIEX QTRAP® 6500+ LC-MS/MS system was demonstrated to provide unique advantages in the ability to maximize selectivity when confirming and quantifying low level metabolites in hair.

Novel Aspect:
An efficient analysis workflow designed with triple quadrupole linear ion trap mass spectrometry allows the picogram detection of the cocaine and metabolite in hair samples.
Introduction:
Identification of latent fingerprint composition has been carried out by several techniques. The technological development of recent years has boosted the increasingly visible use of mass spectrometry. However, so far, the chemical / biochemical fingerprint analysis has been performed by conventional mass spectrometry Methods:

Methods:
In the present work we characterized the lipidic profile of fingerprints on a non-porous surface by direct analysis using ESI-FT-ICR mass spectrometry and demonstrate the applicability of ESI-FT-ICR-MS with Electrospray Ionization (ESI) in the detection and characterization of lipids from human fingerprints.

Results:
In a single analysis, thousands of chemical species of different origins can be identified, thus generating extremely valuable information that will lead to a chemical signature of a person at a given time. Concerning lipids, 140 lipids were identified, corresponding to 13 FA sub-classes, 6 PR sub-classes, 17 ST sub-classes, 12 PK sub-classes, 2 SP sub-classes and 1 GL sub-class, making a total of 51 sub-classes.

Conclusions:
This technique uncovered a high number of lipid subclasses, both of endogenous and exogenous origin, like what will be obtained in real cases. This characterization will allow the identification of individualizing and differentiating elements to generate forensic evidence in criminal investigations.

Novel Aspect:
FT-ICR-MS analysis of human fingerprint.
A QUALITATIVE/QUANTITATIVE LC-QTOF-MS ASSAY FOR FORENSIC DRUG SCREENING IN URINE – FEASIBILITY STUDY AND BASIC METHOD VALIDATION

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Keywords: HRMS, LC-QTOF-MS, qual/quant, screening, retrospective

Introduction:
For forensic analysis, screening methods using LC-QTOF-MS are valuable tools due to the possibility of qualitative/quantitative and retrospective data evaluation in a single run. Here, a workflow was validated for qualitative and quantitative analysis of drugs and drugs of abuse in human urine and to assess its limitations and to prove that cut-off values are met, a validation including LOD, LOQ, linearity, accuracy, selectivity, and precision was carried out.

Methods:
93 substances of forensic relevance were spiked into ten different urine samples at concentrations 1 - 50 ng/mL. Samples with internal standards were precipitated with acetonitrile. The dried residues were reconstituted and analyzed on three LC-MS systems (Bruker Elute UHPLC with impact II QTOF) in ESI pos. mode with full scan and broadband CID spectra. Data evaluation was performed with TASQ using a database with MS and chromatographic information on 2184 compounds.

Results:
Identification and semi-quantification at the lowest concentration (1.0 ng/mL) was achieved for 60 % of the compounds; four compounds (paracetamol, norclomipramine, piritramid, and levetiracetam) were not detected, probably due to matrix effects and/or low ionization yields. LOD was set to the concentration at which a substance was detected in 95% of all measurements according to the following identification criteria: retention time ± 0.3 min, signal to noise ratio > 3:1 for all ions, [M+nH]n+ and [M+nH+1]n+ detected (MS), and at least two qualifier ions with minimum one being a true fragment of the molecular ion (bbCID). The precision ranged from 8 % to 30 %. Overall accuracy was good. Noteworthy, designer benzodiazepines and fentanyl derivatives were detected with extraordinarily high sensitivity. Typical ‘date rape drugs’ like flunitrazepam, doxylamine, and diphenhydramine showed LODs sufficient for detection of a recent uptake of these drugs.

Conclusions:
All substances with legal cut-offs according to German regulations were detected below the respective concentrations (factor 5).
Extrapolating the presented urine analysis results, application to blood and hair samples seems promising and is to be evaluated/validated. Given the high frequency of new psychoactive substances emerging on web-based drug markets, this is of great interest to the forensic field due to the possibility of retrospective data evaluation.

Novel Aspect:
Fully retrospective, highly sensitive qualitative/quantitative HRMS screening assay featuring semi-automated data acquisition and evaluation.
Introduction:
Cases of suspected driving under the influence of drugs (DUID) according to § 24a (2) of the German road traffic act (RTA) usually pre-screened by immunoassay (IA) and positive results are confirmed by LC-MS/MS since neither the qualitative nor the quantitative information from IAs is admissible in court. We developed a fast and automated LC-MSn method, combining the ease-of-use of IAs with unambiguous compound identification of an MS analysis.

Methods:
Step one of this project covers a general feasibility study including two different data acquisition modes (AutoMSn and sMRM) for qualitative and/or quantitative analysis. It contains a sample preparation of 0.5 ml serum using LLE with chloroform/isopropanol, applicable for all relevant analytes except THC. Analysis is carried out on an Acclaim C18 column using an UltiMate3000 LC-system coupled to an amaZon speed ion trap within 5 minutes runtime.

Results:
During this study two data acquisition methods were evaluated and implemented: The Auto-MSn-mode facilitates fast and easy qualitative analysis with automated result reporting whereas the sMRM-mode offers a first quantitative estimate of the analyte concentration, simplifying subsequent confirmation analysis. Both methods include the § 24a (2) RTA relevant compounds: amphetamine, methamphetamine, ecstasy, heroin, morphine, cocaine and benzoylecgonine as well as codeine and 6-acetylmorphine and have been validated according to the GTFCh guidelines. Selectivity and LODs were verified for both Methods: For the sMRM-method linearity, matrix effects, stability and accuracy were also determined. Measurement of 60 real case samples showed that neither the AutoMSn- nor the sMRM-method generated false-positive or false-negative results.

Conclusions:
Although sample preparation is still carried out manually at this point, the developed LC-MSn approach would be a suitable replacement for IA testing in DUID cases according to § 24a (2) RTA. As a next step of this project, an online SPE is currently evaluated and will be implemented to enhance the degree of automation of this approach.

Novel Aspect:
Detection of § 24a (2) RTA relevant compounds using a fast and robust LC-MSn method to avoid immunoassay pre-screening in routine DUID analysis.
Introduction:
In current forensic sciences, the method of Analysis, Comparison, Evaluation and Verification (ACE-V) is used as an attempt to identify people who have been at a crime scene through latent fingerprints [1]. Due to the limitations of this technique, other analysis may be necessary for criminal prosecution, such as the skin microbiome that degradate the fingermarks, which is stable over time and can delimit the list of suspects [2], [3].

Methods:
Skin microbiome samples were collected from the distal phalanges of the right hand of 6 volunteers, grown in a blood agar plate at 37°C for 24 hours, followed by the isolation in a liquid medium cultivated in the same conditions. Then, an extraction protocol to MALDI-TOF-MS was performed, applying 1μl of each supernatant in MSP96 plate. The microorganism identification was conducted using the MALDI Biotyper software. Ethical protocol for research under number CAAE 42845114.0.0000.5650.

Results:
Preliminary results identified the phyla Firmicutes and Actinobacteria as the most prevalent ones. The genera Staphilococcus, Micrococcus and Bacillus were identified with scores between 2,000 and 3,000 by Maldi Biotyper, presenting a safe result of genus and species. The species S. epidermis, S. capitis, S. warneri and M. luteus were the most abundant in comparison to S. carnos, S. conhii, S. hominis and B. licheniformis species. Fresher microbial cultures present higher scoring results in MALDI MS with Biotyper. It was also identified diversity among genders, with higher diversity in female samples versus male samples. Also, it was found different diversity degrees between omnivorous and vegetarian diets within female gender, especially in the genera Micrococcus and Bacillus.

Conclusions:
In the human skin the microbial richness and diversity on the surface are affected by several factors, such as cosmetics, diet, diseases, age, sweat, hormone production, among others [4] - [9]. Due to these interferences, the microbial "impression" would be individual [2]. Mass spectrometry by MALDI-TOF offers fast and accurate results [10] - [12] and its use in the forensic microbial analysis will provide an analytical reproducible scientific evidence for forensic experts.

Novel Aspect:
The use of mass spectrometry by MALDI-TOF and Biotyper to characterize individual and common microbiome for application in Forensic Identification.

References:


Introduction

Roses are the most important Dutch cut flowers and are grown by companies under a global GAP (Good Agricultural Practice) certificate that is used by e.g. European supermarket organizations. Certified companies have strict requirements for safe, sustainably grown, high-quality and traceable products. Next to the Netherlands, Kenya and Ecuador are the main exporting countries for roses. The question rises whether roses of unknown origin, which do not meet these strict requirements, or roses from areas with a phytosanitary export ban due to occurrence of certain plant diseases, are exported illegally under this certificate to the EU. Therefore, UvA, Naktuinbouw and De Ruiter BV develop isotope analysis technology for provenance determination of roses. The stable isotopic composition of H, C, N and O from plant materials depends on regional geological and climatic patterns, and therefore provides a regional fingerprint of the plant.

Methods

In this study, we focus on development of methods for isotope ratio and gas chromatography (GC) mass spectrometry analysis of stems and water from roses that were harvested monthly throughout the 9-month season. Elemental analysis (EA) coupled to isotope ratio mass spectrometry (IRMS) was applied to investigate the use of δ13C, δ15N and total C and N profiles in the stems as a fingerprint for the country of origin. Furthermore, the δ18O isotopic composition of the xylem water in the rose stems, extracted via freeze-drying, was linked to δ18O of irrigation water and the GNIP (Global Network of Isotopes in Precipitation) IAEA database, to determine differences between roses from different geographical origins. A third method used was quantitative GC-MS analysis and compound specific GC-IRMS analysis of leaf wax alkanes, extracted via solid phase extraction.

Results

The best separation of the groups was obtained from the water analysis using a combination of the δ2H and δ18O values of the rose and irrigation source water. The optimal time of freeze drying was at least 22 hours. δ2H and δ18O values of source water do not vary much over the season and the variation within one batch is low, furthermore they correspond well to the global mean water line (GMWL). The results show a correlation between the source water and the local long-term GNIP averages (Pearson’s R = 0.9168 for δ2H and 0.9029 for δ18O) and between the stem- and source water (Pearson’s R = 0.9959 for δ2H and 0.9921 for δ18O), with constant isotope fractionation between the source and the rose water data of about -4. δ13C and δ15N values of bulk rose stem analysis with EA-IRMS can also be used to distinguish roses from different origins, especially Dutch roses which were clearly separated from Ecuadorian and Kenyan samples. The measured δ15N isotope values also correspond well to the reported literature values [1], even though other plants were used in this study. GC-MS analysis of the relative concentrations of C27, C29, C31 and C33 in leave wax extracts showed clustering according to the country of origin. In a previous study, similar results were obtained for the same alkanes in bell pepper [2, 3].

Conclusions

Three analytical strategies were investigated, based on IRMS analysis of δ2H and δ18O of rose and irrigation source water, δ13C and δ15N of rose stems, and on quantitative GC-MS analysis of leaf wax-alkanes. All strategies were...
able to discriminate between Dutch, Ecuadorian and Kenyan roses that were monitored monthly throughout the season. The source water and long-term regional GNIP data showed good correlation with the rose water data, with constant isotope fractionation of about -4. These multi-isotopic methods are therefore suited as a tool to verify the geographical origin of roses in order to prevent fraud and phytosanitary problems.

Novel Aspect
This is the first time a combined strategy of multi-isotopic EA-IRMS and quantitative GC-MS methods has been developed as a tool to verify the geographical origin of roses.

References
THE DEATH OF THOMAS CHATTERTON - AN LC-MSN INVESTIGATION.

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Keywords: morphine, degradation, forensics, LC-MS/MS

Introduction: (Limit of 400 characters without spaces)
Thomas Chatterton (1752 – 1770) was a literary genius who tragically died in unexplained circumstances [1]. At the time, the Coroner claimed that he “swallowed arsenic in water and died, in consequence thereof” despite no arsenic being found at the scene. In his belongs was a memorandum book, which had a dark stain running through its pages. A police forensics report in 1947 stated that the stain “gives a positive reaction for opium alkaloids” but gives no further details.

Methods: (Limit of 400 characters without spaces)
Herein, is a study to try to shed some light on Chatterton’s death using high resolution LC-MSn on an Orbitrap Elite instrument. Preliminary tests were performed with codeine (an opium alkaloid) to test if it was possible to recover it from paper after being deposited. This test proved successful, so then a sample of a stained page from the memorandum book was extracted using methanol and dichloromethane and analysed by the same Methods:

Results: (Limit 900 characters without spaces)
The preliminary study showed the recovery of codeine from office paper. The extracts also contained unidentifiable components probably from the paper itself, as well as the common phthalate and siloxane contaminants. Codeine was recovered intact as well some possible degradation products, but these were not investigated further. In the main study, a 2mm2 sample of a stained page from the memorandum book was extracted and analysed by both direct infusion ESI-MS and HPLC-MSn. As expected, from a sample of this age, the extract was very complex, and no signal was observed for any intact opium alkaloids. The LC-MS analysis, however, showed a cluster of at least 15 peaks with molecular weights in the 200-300 m/z range. 8 of these have been confirmed by accurate mass MS/MS analysis to be known degradation products of morphine [2]. A further 7 components have also been identified as being possible unpublished degradation products of morphine, codeine or thebaine.

Conclusions (Limit of 400 characters without spaces)
This study demonstrates the extraction and identification of at least 8 known morphine degradation products from a 250-year-old book by high-resolution HPLC-MSn. The presence of a further 7 opium alkaloid degradation products is also proposed. These results indicate that the stain originally contained opium alkaloids, most probably in the form of laudanum – a popularly prescribed medical treatment of the day.

Novel Aspect: (Limit of 150 characters without spaces)
The demonstration of modern analytical methods to the study of a ‘mystery’ stain in a 250-year-old book found at the scene of an unexplained death in 1770.

References

VALIDATION OF BLOOD PROTEOMIC SIGNATURES FOR THE FORENSIC DETERMINATION OF BLOOD AND ITS PROVENANCE

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Keywords: Blood, Forensics, Proteomics, MALDI

Introduction
Analysis of blood at a crime scene can inform an investigation but current enhancement techniques can provide false positives. Bottom up proteomic MS can detect blood and indicate its origin [1]. Blood specific proteins can be visualised directly on the ridges of a fingermark [2]. In this study, human and animal blood, biofluids, and substances that elicit a positive response to presumptive testshave been examined-pre- and post-enhancement. Blood identification was based on proteotypic peptides. Manual and automated PMF methods were trialled to identify the samples.

Methods
Bloodstains and fingermarks on glass slides were received from Elite Forensics©. Stains were digested with 250µg/ml trypsin after swabbing and extraction, followed by spot analysis via MALDI MS Profiling. Fingermarks were digested in situ using a SunCollect auto-spraying system, incubated for 1 hour and then sprayed with matrix (αCHCA) using the same system. MALDI analyses were carried out on a Synapt G2 HDMS. Images were processed using Waters HDI software.

Results
Preliminary results show a 100% correct identification rate when human blood samples were analysed blindly in enhanced and non-enhanced form. This classification rate is based on 1/3 of the samples analysed so far. The prior use of blood enhancement techniques has not interfered with the retrieval of proteotypic peptides. Other biofluids and substances such as egg yolk were correctly classified as non-blood. Manual PMF is based on specific criteria including ion signal exclusion from the mass lists. Whilst manual PMF is highly successful in sample classification, the use of the automatic PMF software correctly attributes human blood with a low probability score. Animal blood presents additional difficulties. Prior investigations have correctly classified animal blood. In this study, some animal blood samples were classified as non-blood, likely to be as a result of blood sampled from meat packets rather than venous blood, which has led to the acquisition of spectra with low signal to noise impacting on the PMF, both in manual and automatic mode.

Conclusions
This first stage validation study has developed methods to correctly classify human blood and non-blood samples. Whilst correct classification of animal blood is possible, determination of provenance has been compromised. Further research into improvement of MS acquisition methods as well as of the treatment of the data is in progress. Venous animal blood (instead of from meat packets) will also be trialled and results compared to assess robustness of the Methods:

Novel Aspect
Proteomics MALDI MS methods were validated for use in police casework. Robustness of PMF software using a peptide library was assessed against manual PMF of proteotypic peptides.

References

For information please contact: scientific@imsc2018.it
Keywords: Drugs of Abuse, LC-MS, Data Independent Analysis, SWATH, Differential Ion Mobility Spectrometry, ExD

Introduction:
Screening of Drugs of Abuse (DoA) in biological matrices is mainly based on triple quadrupole or high resolution mass spectrometers using data dependent acquisition workflows (DDA). The development of data independent acquisition such as SWATH, differential ion mobility (DMS) and electron induced dissociation (ExD) opens new possibilities for comprehensive and multidimensional for simultaneous qualitative and quantitative analysis.

Methods:
Data were acquired on either a triple quadrupole linear ion trap (QTRAP5500, Sciex) equipped with DMS (Selexion, Sciex) or a quadrupole time of flight equipped with DMS and a prototype chimeric collision cell allowing to perform collision induced dissociation (CID) or electron induced dissociation (EID). Human urine samples were analyzed using reversed phase liquid chromatography.

Results:
Various workflows have been development for the direct analysis of DoA on tissues or in biological fluids such as urine using multidimensional MS approaches. For direct MS analysis using ambient ionization or for LC-MS modifier assisted DMS offers an additional separation dimension. Contrarily to pharmaceutical investigation toxicological screening cannot rely and the origin/nature of the drug neither on the intake time nor the frequency. Comprehensive LC-SWATH/MS allows to collect in the same analysis precursor ions as well as fragment ions. Information can be obtained either hypothesis driven or with the use of multivariate analysis post-acquisition. This provides a powerful tool for the screening of DoA as well as the monitoring of the exposome or to monitor the change in endogenous metabolites in human urine samples. In addition to CID electron induced dissociation of singly charged precursor ions (protonated or adducts) generates additional information for structural identification of metabolites.

Conclusions
The complexity of biological samples and the problematic of DoA call for improved methods for screening and quantification. Novel development in mass spectrometry such as DIA, ion mobility and EID with or without chromatography separation can be used to extend the toxicological knowledge as the intake of a DoA can also be monitored based on their contaminants.

Novel Aspect:
Integration of data independent acquisition, ion mobility and electron induced dissociation in drug abuse screening.

References
Keywords: Testosterone esters, doping analysis, gas-chromatography, mass spectrometry.

Introduction:
The detection of pseudoendogenous steroid doping is currently based on the evaluation of the urinary steroid profile as described in WADA Technical Document TD2016EAAS [1]. Despite this some physiological and/or pathological conditions could compromise the application of these criteria [2-3]. For this reason the direct detection of intact synthetic esters of testosterone in plasma/serum gives unequivocal proof of exogenous testosterone administration.

Methods:
After liquid-liquid extraction at pH 9 (carbonate buffer 20% w/V) with n-hexane/ethylacetate (70/30 V/V) [4] all plasma/serum samples were analyzed by gas-chromatography coupled to tandem mass spectrometry (GC-MS/MS) with electronic impact (EI) ionization and multiple reaction monitoring (MRM) acquisition mode. Testosterone esters were revealed as trimethylsilyl derivates.

Results:
In the first step of our work a qualitative validation was carried out for all testosterone esters studied (acetate, propionate, valerate, isocaproate, caproate, benzoate, hexahydrobenzoate, enanthate, cypionate, octanoate, decanoate, phenylpropionate, undecanoate, laurate). Considered parameters are specificity/selectivity and limit of detection. According to the WADA rules [5] were also investigated the mass spectrometric and the chromatographic criteria for the identification of analytes for doping control purposes. The criteria were satisfied for twelve compounds and the method is specific and selective for all of these.

In a second step a quantitative validation was carried out for five compounds present in the most marketed pharmaceutical preparations probably abused by athletes. Considered parameters are specificity/selectivity, limit of detection and quantification, recovery, linearity, repeatability and accuracy. The selected esters are identified and quantified in plasma/serum with an error less than 15 %.

Conclusions
The determination of testosterone esters in plasma/serum by GC-MS/MS should allow a confirmation of the administration of exogenous testosterone for doping purpose. The presented method was qualitative validated for twelve esters and quantitative validated for five esters and can be used to detect the presence in blood of those testosterone esters currently present in pharmaceutical preparations that can be abused by athletes.

Novel Aspect:
Number of esters detected in each sample and possibility to confirm the composition of seized pharmaceutical preparation by responsible authorities.
References
IDENTIFYING WOOD FROM PROTECTED SPECIES WITH DART FINGERPRINT SPECTRA AND THE SOFTWARE PLATFORM OMFF

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Keywords: DART-MS, wood identification, software, fingerprinting, ambient ionization

Introduction
According to the Food and Agricultural Organization (FAO), global production of major wood products increases at a 3-6% per year. Some commercially valuable wood treated products are illegally used from endangered tree species, that are not easily identified. DART-MS has been used to differentiate morphologically similar species with great results[1–3]. We want to build a software with a scalable reference database for forensic applications.

Methods
Reference spectra are generated from wood libraries at the Forensic Laboratory for Wildlife and Fish (US Forest Service) from different tree species and samples. An SQL reference database was constructed with 4,950 spectra from 301 different species. The Open Mass Fingerprinting Framework (OMFF) was designed as a webpage for easy distribution, with html and bootstrap at the front end and Django to improve security of the back end. Multivariate statistical analysis was implemented for data processing and identifying specific chemical markers.

Results
A software for tree identification based on the DART-MS chemical profile data was constructed (OMFF). DART-MS provides a fast analysis which requires minimal time and amount of sample making it ideal for processed wood products (guitars, furniture, etc.). The program is user-friendly, open source webpage that includes a reference database for identifying of wood samples. Specific chemical markers are obtained from DART-MS fingerprint reference spectra and compared with unknown samples. The results provide a list of species with a comparison probability score. The software produces statistically meaningful information. While the number of samples for some species in the reference database is small, the system can be expanded. The program was designed for non-expert users; therefore, it can be implemented at critical points such as customs and ports.

Conclusions
The implementation of the OMFF software will help diminish traffic of protected wood species. CITES provides tools for wood identification such as morphological, DNA and microscopy characterization. Using all the available tools will improve identification confidence levels for wood species.

Novel Aspect
OMFF provides a reference database and reliable classification mechanisms for non-expert users to identify morphologically similar wood samples.

References
Introduction:
In real cases the analysis of questioned documents is undertaken with mostly non-invasive methods of examination of paper base & covering materials. The methods applied should provide reliable and sufficient information to address the following questions like whether the document is genuine, if it has been altered (e.g. erased, bleached, faded) or reproduced, if the covering materials (toners, inks) appearing on each page of document have the common source [1].

Methods:
ICP Mass Spectrometer with Time-of-Flight Analyzer coupled with Laser Ablation system was applied for inorganic chemical elements characterization of different inks. In this study 13 random, promotional pens were collected and used to leave a straight few cm lines on a white piece of paper. The LA-ICP-MS parameters were optimized in order to achieve satisfactory intensity of the ink signal for selected m/z, leaving the paper base almost untouched.

Results:
Inks are complex mixtures of different colorants, vehicles, and additives, which depending on the company, color and type are adjusted in composition to produce the desired writing characteristics. In order to avoid loss or destruction of a potential forensic evidence hardly destructive methods like LA-ICP-MS are desirable. This technique with high discrimination and classification power can be successfully applied to differentiate inks on the same document as well as to compare inks on other documents. In this work the mass spectra profiles for each randomly selected & commercially available on the market inks (blue and black) were recorded and compared. They all presented specific elemental features which made them distinctly different from each other. The gathered information about elemental composition differences was supported by the feedback from chemometric

Conclusions:
The gathered data originating from collected mass spectra coupled with multivariate statistical methods present a useful tool for discrimination of studied unknown inks with insubstantial sample destruction and without any methodological calibrations, which all being crucial from a forensic practice point of view. The same methodology can be applied to analyze real handwritten questioned documents since there is a justified need to use non-destructive

Novel Aspect:
There is a constant need to create method with high discrimination power which can deliver objective results offering the differentiation among brands & within the same brand.

References
Detection of ricin and abrin toxins in complex matrices by two novel galactose enrichment methods and high resolution LC-MS/MS

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Detection of ricin and abrin toxins in complex matrices by two novel galactose enrichment methods and high resolution LC-MS/MS

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Keywords: ricin, abrin, galactose affinity, LC-MS/MS

Introduction:
Ricin and abrin are highly poisonous plant toxins belonging to the type 2 ribosome-inactivating protein (RIP-II) family. These proteins are composed of A- and B-chains linked by a single disulphide bond. The A-chain confers cellular toxicity, while cellular entry is through the galactose binding properties of the B-chain, which can be utilized for isolation of these toxins. There is a demand for improving toxin detection capabilities due to the terrorism threat posed by them.

Methods:
Immobilized D-galactose gel (Thermo Scientific) in self-packed microspin columns and home-made galactose-coated 96-well microplates were utilized for enrichment of ricin and abrin from different kind of samples. Peptides from the trypsin digested protein samples were identified by high resolution mass spectrometry analysis (EASY nanoLC linked to Fusion Orbitrap, Thermo Scientific). Released adenine in the depurination activity assay was measured with LC-MS/MS (UPLC linked to Xevo TQD, Waters).

Results:
Two rapid galactose affinity enrichment methods were developed for ricin and abrin analysis from complex matrices. Miniaturized immobilized D-galactose gel affinity columns were shown to be suitable for trace enrichment and high sample throughput. This method can also be adapted for preparative purification of RIP-II toxins. Also the solid-phase enrichment by galactose array worked for trace enrichment and can be automated with microplate automation instruments.

Ricin and abrin are fairly resistant to proteolysis. Protein unfolding was done with an acid-labile surfactant - urea mixture before digestion with trypsin. Identities of the resulting peptides were unambiguously confirmed by accurate mass LC-MS/MS product ion scans of peptides unique to each of the toxins. Quantification was accomplished with isotope labeled AQUA peptides for both proteins and the achieved sensitivity and detection limits were comparable to those of immunocapture enrichment methods.

Conclusions:
Both galactosyl ligand methods were shown to have the capacity to enrich trace amounts of ricin and abrin from various matrix extracts. Together with functional assay results, the high resolution LC-MS/MS data provides
unambiguous identification of ricin and abrin in the samples. The solid-phase enrichment by galactose array can be adapted for high-throughput screening.

Novel Aspect:
High throughput galactose affinity methods for ricin and abrin with detection limits similar to the ones reported for other enrichment methods were developed.
432 - LC-HRMS VS NMR FOR THE IDENTIFICATION OF KETOCAINE IN SEIZED MATERIAL

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Keywords: Forensic analysis; HRMS; NMR; Ketocaine

Introduction: The timeliness of analytical assessments is fundamental for Police forces, initially relying on rapid drug-screening tests. Scientific Police Service of Italian National Police adopts Young’s reagent for the detection of cocaine; however, there are numerous examples of false positives that require attention to the interpretation of test result and, in some cases, confirmatory analysis must be performed. In this study an interesting case is presented.

Methods:
The analytical protocol adopted for the identification of the suspect compound involved both NMR and HMRS. The sample was divided into two aliquots: one was analysed by means of LC-HRMS with Orbitrap technology and the other one was subjected to analysis by NMR. The two techniques have not been used in a complementary but compositive way: namely the analysis have been performed independently.

This approach involved both the comparison of the molecular ion and the fragmentation pattern with the database spectra by differential analysis, providing stronger identification.

Standard 1H and 13C NMR spectra were coupled with two-dimensional experiments: 1H-1H TOCSY, 1H-1H COSY, 1H-13C HSQC and 1H-13C HMBC.

Results:
LC-HRMS identified Ketocaine by involved both the comparison of the molecular ion and the fragmentation pattern by differential analysis, from spectra database Compound Discoverer 2.0, using ChemSpider as source. Forensic identification require something more (more identification points), so the study of the molecule and its fragmentation was also performed. The match of theoretical fragmentation pattern with the experimental one confirmed the identification.

NMR experiments carried out in different acquisition modes (1H-1H TOCSY, 1H-1H COSY, 1H-13C HSQC and 1H-13C HMBC) led to a suitable amount of data for a correct identification of the unknown compound: the interpretation of spectra and the signals assignment led to identification of ketocaine.

Conclusions

In this case, both NMR and HRMS proved themselves as self-sufficient techniques in the resolution of the analytical-forensic problem: in particular, HRMS, with the advantage of the speed of analysis and interpretation of data thanks to dedicated software, proves of undoubted utility for forensic investigations.

Novel Aspect:
The suitability of HRMS as a self-sufficient technique in the resolution of the analytic-forensic problem was demonstrated for the identification of unknown compound.
INTRODUCTION: (Limit of 400 characters)
In the field of investigations in drugs of abuse, one of the objectives of the police forces is the characterization of the spread and the consumption of psychotropic substances: this help to trigger adequate monitoring and prevention measures. Through different techniques, it is possible to identify the components present in seized samples in order to characterize the illicit substances.

METHODS: (Limit of 400 characters)
A combination of targeted and untargeted analyses were performed on seized material of vegetal origin. Screening test and GC analyses showed an unusual combination of compounds. UHPLC-MS/MS analyses were performed in IDA and in MRM. LC-HRMS analyses followed by elaboration of raw data files by means of GNPS web platform and in-silico annotations in order to predict the structure of all illicit compounds.

RESULTS: (Limit 900 characters)
On the basis of the preliminary analyses there was the suspect of different tryptamine compounds in the seized samples so a semi-untargeted analysis by UHPLC-MS/MS were performed in IDA, showing different tryptamines that were confirmed in MRM (i.e. DMT, NMT, 2-MTHBC), together with some cannabinoids, including THC, in one sample. This issue stimulated further investigations.

With the aim to obtain a complete overview of all features, untargeted HPLC-HRMS analysis was performed. Molecular Network was generated to perform cluster of similar MS/MS spectra and to propagate spectral annotation of seized drug samples. Data shows that different compounds, belonging to tryptamines, were confirmed by spectral library search annotation on GNPS. Other 8 tryptamines were assigned comparing the results obtained with MRM analysis and similar pattern of fragmentation using in-silico prediction annotation.

CONCLUSIONS: (Limit of 400 characters)
Through different analytical techniques, it was possible to identify the main vegetable components present in samples. Combination of targeted/untargeted analysis and molecular network proves to be a useful tool for forensic investigations. Untargeted analysis and in-silico annotation allowed detecting 8 compounds undetected by MRM analysis and confirming the analytes detected in target analysis.

NOVEL ASPECT: (Limit of 150 characters)
Combination of targeted/untargeted analysis, with suitable data managing, resulted necessary to characterize seized sample for subsequent evaluations.

REFERENCES

Introduction:
Fingerprints are the most important form of evidence in the human identification [1]. Moreover, they may be composed of a number of different contaminants, for example psychoactive substances[2,3]. During the last few years, time-of-flight secondary ion mass spectrometry (TOF-SIMS) has become a very sensitive analytical technique in surface science [2,3]. This technique can be used in chemistry and forensic science for detection of various molecules (drugs).

Methods:
The mass spectra and images of the studied samples were recorded using secondary ion mass spectrometer TOF-SIMS IV (ION-TOF GmbH, Münster, Germany), which is equipped with a high mass resolution time-of-flight analyser of a reflectron type. During analysis, Bi3+ primary ion gun was used, working at 25 keV and an average ion current was 0.5 pA. The analysis areas were 100 × 100 μm² and 500 × 500 μm² for obtaining secondary ion mass spectra and images, respectively.

Results:
Time-of-flight secondary ion mass spectrometry (TOF-SIMS) was applied in the forensic research for detecting and imaging of cannabis in the fingerprints. The studied fingerprints were: 1. imprinted directly on the lift tape by a contaminated finger with cannabis; 2. left on a glass surface and developed with two types of latent fingerprints powders; 3. transferred using the lift tape.

In the positive and negative ion spectra of the studied samples many peaks derived from cannabis can be detected: cannabinol, cannabidiol, tetrahydrocannabinol, cannabivar, cannabidiolic acid. The ions characteristic of the powder, sweat and marijuana are also visible in the spectra of fingerprints revealed with latent fingerprints powder and transferred with lift tapes.

Conclusions:
The TOF-SIMS technique appears to be a very effective and useful tool in forensic investigations, which enables the identification of drug residues left on fingerprints imprinted on selected bases. Moreover, it has been shown that revealing fingerprints with the black fingerprint powder is not disadvantageous for the TOF-SIMS application in the fingerprints analysis.

Novel Aspect:
There is a possibility of detecting foreign substances on fingerprints, such as drugs, after revealing fingerprints with the latent fingerprint powder.

References

TOF-SIMS DETERMINATION OF DETECTION LIMIT OF AMPHETAMINE IN ‘STREET DRUGS’ AND POLLUTED FINGERPRINTS

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Keywords: Secondary ion mass spectrometry, fingerprints, drugs, forensic research, image technique

Introduction:
ToF-SIMS technique is a very effective and powerful method to examine the surface composition of different materials, including those of key importance in the forensic chemistry i.a. drugs [1-2]. Currently, drugs sold in the streets contain a variety of different ‘fillers’. It is important to develop a method which has discrimination power and will allow simultaneous identification of all substances present in the mixture, even in very small quantities.

Methods:
The aim of this study is focused on the evaluation of the TOF-SIMS technique as a potential tool applied in the forensic analysis of fingerprints. Studied fingerprints were contaminated with trace amounts of amphetamine and other substances which are typically used as ‘fillers’. These compounds do not occur naturally on fingertips and can present a significant value as evidence linking the suspect with the crime scene.

Results:
In this work, clean fingerprints as well as contaminated by drug were investigated. Moreover, in order to determine the limit of detection of amphetamine, the analysis of drug tablets containing various amounts of mixtures of amphetamine and caffeine (filler) in different proportions was performed. This assessed LOD was 0.625%. Also fragmentative ions derived from amphetamine and caffeine were examined, which undoubtedly indicted their potential origin. Additionally, the ToF-SIMS technique allowed the visualization of the fingerprint contaminated by a trace amount of powdered drug.

Conclusions
The results of this study has demonstrated that the ToF-SIMS technique is a powerful tool which enables the identification of drug traces left on a contaminated fingerprint. This method allows the determination of very small amounts of the drug. Moreover, ToF-SIMS technique enables not only the identification of the substance forming the fingerprint but also the visualization of very small areas of the fingerprint, including the course of the fingerprints.

Novel Aspect:
Novel aspects are: designing a method enabling the assessment of LOD of amphetamine in home made tablets and its identification and visualization in the fingerprints course.

References:
73 - MODAFIENDZ METABOLITES IDENTIFICATION BY MEANS OF LC-HRMS: FROM IN SILICO PREDICTION TO IN VITRO CONFIRMATION WITH RAT HEPATOCYTES.

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Keywords: NPS, Nootropics substances, Metabolites, LC-HRMS

Introduction:
New psychoactive substances (NPS) represent a tough challenge, since analytical standards are not available or included in libraries[1]. When metabolic pathway is unknown, determine target analytes is critical, so metabolites identification become fundamental for clinical and forensic investigations[2]. The aim of this work was to identify the metabolites of Modafiendz, a synthetic nootropic[3].

Methods:
In-silico experiments were carried out by means of MetaPrint2DReact program, used to predict possible metabolites and cultures of rat hepatocytes were used for in vitro experiments. In order to obtain information about chemical structure of metabolic pathway, LC-HRMS experiments were carried out by means of Orbitrap analyzer, while analytes separation was carried out using a PFP-C18 Column.

Results:
Once the smile code was introduced in the in silico program, a total of 15 metabolites were predicted: 7 of phase I and 8 of phase II. The incubation with hepatocytes allowed to mimic the physiological environment of the liver[4], in which the substances would be found, obtaining a realistic picture of the metabolic pathway for Modafiendz. The results obtained in silico allowed to hypothesize the metabolites structures and masses to be searched in HRMS. As a positive control of the real metabolic activity of the cells, Modafinil and its metabolites were used: Modafinil Acid and Modafinil Sulfone have been identified. In the samples obtained from in vitro experiments 6 metabolites were identified, 2 belonging to phase I and 4 belonging to phase II. In order to characterize the observed metabolites, analyzes were performed in PRM at different collision energies (NCE).

Conclusions:
In this work the chemical structures of the Modafiendz metabolites were identified using rat hepatocyte followed by UHPLC-HRMS analysis. The most produced metabolites are dehalogenated derivatives obtained from an initial oxidation reaction, as regards the phase I metabolites and the metabolites conjugated with glucuronic acid or methylate, deriving from the phase II reactions.

Novel Aspect:
For the first time metabolic pathway of Modafiendz was identified. This information is essential to develop methods to confirm the use of this NPS[5].

References
Introduction:
Novel psychoactive substances have introduced significant identification challenges to the forensic science community. Developing methods that can correctly categorize emerging drug analogs is a pressing need. This presentation describes a procedure for obtaining a class-identification map of an unknown compound. The resulting classification map can be used to compute the uncertainty of the class-identification.

Methods:
The hybrid search is a new library search method that considers fragment-ions along with neutral-losses when computing spectral similarity [1]. A query spectrum is first searched against a library to generate a preliminary hit-list. The hybrid dot-product calculation is used to generate a dissimilarity matrix from the spectra in the preliminary hit-list, which is then clustered using our novel multi-objective agglomerative-hierarchical clustering algorithm.

Results:
The result of this mass spectral library search and clustering procedure is a dendrogram from which the likely classification of the query compound can be proposed. This dendrogram can be used to compute the uncertainty of the proposed class identification. As a numerical test, twenty-two SWGDRUG Library [2] compounds were assembled into a sub-library. It contained spectra for five analogs each of JWH-167, fentanyl, MDMA, and cathinone, and two additional spectra of “unknown” compounds. A preliminary implementation of the multi-objective classification method was able to classify the first unknown as a fentanyl analog with a certainty of 56%, and as a fentanyl, JWH-167, or cathinone analog with 93% certainty. The second unknown was only classifiable as a fentanyl, MDMA, or cathinone analog, but with 99% certainty.

Conclusions:
This presentation describes a procedure for obtaining a class-identification for an unknown seized compound, using the new hybrid search to compute inter-spectral similarity, and a novel multi-objective scheme for agglomerative hierarchical clustering. The resulting classification tree can be used to compute the uncertainty of the class-identification.

Novel Aspect:
The hybrid dot-product for spectral similarity, a new multi-objective hierarchical clustering algorithm, and computation of uncertainty for class identification.

References
2. www.swgdrug.org/ms.htm

For information please contact: william.wallace@nist.gov
Keywords: Criminalistics, foreign substances analysis, TDP/DART-MS

Introduction:(400 /400 characters)
Evidentiary materials in criminal come in many forms such as fine segments of fiber or bits of resin. Adhesive collection sheets are often used, but even if a fine particle can be collected, the instrumental analysis is affected by the adhesive compound adhered to a fine particle slightly, so the difficulty of distinguishing task can also further increase. The purpose of this work is to identify fine plastic materials without confounding influence from the adhesive tape.

Methods: (397 /400 characters)
The samples of this study were “resins sticking to adhesive tape”. Thermal desorption and pyrolysis(TDP) combined with DART-MS was composed of Q-TOF mass spec equipped with DART ion source and ionRocket TDP device. The samples were cut to ~1 mm2 and put into the sample pot. TDP temperature condition was room temperature to 600 °C, a rate of 100 °C/min. To structurally and compositionally characterize samples, Kendrick Mass Defect(KMD) analysis was used.

Results: (874 /900 characters)
Nylon-6, polylactic acid(PLA) and polyethylene glycol(PEG) were used as fine plastic materials and scotch tape was used as the adhesive tape. In the mass spectra at 300–400 °C (the pyrolysis reaction starting temperature), the complicated spectra of the samples show that the fine plastics were mixed with scotch tape, making it difficult to distinguish between them. From the KMD plots, the difference between the scotch tape and fine plastics were clearly identified. For the nylon-6 fine plastic, a plot of 113 Da intervals derived from its repeating structure was plotted at a different position from that of the peaks originating from the scotch tape. In a similar manner, plots with a 72 Da interval for PLA and a 44 Da interval for PEG were observed at different positions from that of the scotch tape. By setting the Kendrick mass unit based on the mass of the repeating structure, compounds with common repeat units could be aligned in the horizontal direction on the KMD plot, whereas compounds with different structures were shifted.

Conclusions: (304 /400 characters)
TDP/DART-MS enables the direct and rapid analysis of plastic samples. A combination of TDP/DART-MS and KMD analysis enables the identification of fine plastics without the need to detach foreign matter collected from adhesive tape. Thus, the combination of TDP/DART-MS and KMD analysis can be used for material identification of evidence in criminal cases.

Novel Aspect: (98 /150 characters)
A combination of TDP/DART-MS and KMD analysis is useful for material identification of evidence in criminal cases.
Introduction:
Mass spectrometry-based proteomics is an indispensable tool for molecular, cellular biology and for the emerging field of clinical medicine. Especially, phosphoproteome is one of key signatures to understand the mode of action and mechanism of drug resistance of kinase inhibitors at the molecular level, pathway level and system level.

Methods:
Recently, we have developed a key technology on peptide separation for rapid and sensitive phosphoproteome analysis [1]. We also developed a sensitive method to identify phospho tyrosine proteome using an immune affinity enrichment method [2]. Combining these technologies, we performed temporal characterization of non-small-cell lung cancer cell lines treated with erlotinib.

Results:
We quantified over 12000 phosphorylation sites including 600 phosphorylation sites on tyrosine residue across six cell lines. Especially, phosphotyrosine-proteome can detect bypass kinases which were known to be related to the drug resistance, such as FGFR, AXL, MET. Furthermore, network analysis and kinase-substrate enrichment analysis predicted erlotinib-dependent activation of kinases and signal signatures. We extracted kinases and other enzymes which are up-regulated in resistant cells and selected 46 inhibitors for drug screening. 24 of 46 inhibitors inhibited cell growth of at least one resistant cell line.

Conclusions
Combination of phosphoproteome and phosphotyrosine-proteome profiling is useful to detect markers for drug-efficacy and target candidates for overcoming drug resistance.

Novel Aspect:
Novel markers for drug-efficacy and target candidates for overcoming drug resistance were identified by Phospho proteome analysis.

References
1293 - QUANTIFICATION OF MAJOR PEANUT PROTEIN ALLERGEN ISOFORM GROUPS FROM PEANUT PROTEIN EXTRACTS

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Keywords: Absolute peanut protein quantification LC/MS/MS

Introduction:
Peanut allergy is a potentially fatal condition that has seen a rise of 50% in the recent 15 years currently affecting 0.6-1.3% of the US population. Desensitization by exposure to peanut allergen doses is being evaluated in several clinical trials. Accurate quantification of the peanut allergens is key to successful desensitization. We here report on the development and qualification of a LC/MS based method for absolute quantification of peanut protein content.

Methods:
Discovery analysis of peanut protein extract PPE digests was carried out as nanoLC Orbitrap MS/MS analyses, and suitable peptides were selected for quantification. PPE digest allergen quantification was carried out on a triple quadrupole MS in MRM mode. Unlabeled and heavy isotope labeled peptides (internal standards) were used to generate calibration curves. Intact peanut protein standards were applied for accurate determination of total process recovery.

Results:
Highlights of the peanut proteome discovery efforts and quantification method development rationale, selection of representative peptides, development and qualification results for the analytical methods to quantify four major allergen proteins (Ara h1, h2, h3 and h6) including linearity, accuracy and reproducibility (triple replicate, triple inter day replicate, as well as reproducibility), will be presented together with the results from the analysis of several PPE research batches. The average recovery was determined to be 61% applying intact allergen standard protein. The intra- and inter-day CV% was below 6.6%, and accuracy is within +/- 10%. The stability data for freeze-thaw and 2-hour room temperature stability and 12 hour protein extract digest stability were all below 15%.

Conclusions
An LC/MS/MS based method for quantification of four major peanut allergens from peanut extracts has been developed and qualified. The method was found to have good sensitivity, reproducibility, and dynamic range and thus to be suitable for monitoring the concentrations of the major peanut allergen proteins in research batches obtained from peanut extracts. The method was designed to detect and quantify the isoforms of each of the 4 major peanut protein allergens.

Novel Aspect: Absolute quantification of PPE with multiple isoforms by LC/MS/MS. Application of intact proteins for accurate recovery determination
Introduction

Mass spectrometry (MS) is now routinely used to monitor post-translation modifications (PTMs) levels in biosimilar monoclonal antibody (mAb) characterization. Among them, methionine (Met) oxidation is accepted as one of the most commonly observed PTMs during development, manufacturing, formulation and storage processes. To ensure the safety and efficacy of the drugs, Met oxidation should be tightly controlled during mAb development. It may affect biological function, structure and stability of the protein. In the current study, one of the best-selling therapeutic monoclonal antibody and its biosimilar candidate were subjected to the chemical oxidation via different concentrations of hydrogen peroxide. Under forced oxidative stress, Met oxidation profiles as well as the binding kinetics of FcγRI for reference and biosimilar were compared.

Methods

To determine Met oxidation, UPLC-MS experiments were carried out on a Xevo® G2-XS QToF mass spectrometer (Waters, Milford, MA) equipped with electrospray ionization (ESI). Both biosimilar and reference samples were incubated in the dark with different concentrations of H2O2 (0.1%, 0.5%, 1.0%, 3.0%) for 24 h at room temperature. After applying oxidative stress, both biosimilar and reference antibodies were denatured, reduced, and alkylated before trypsin digestion at 37°C. The resulting peptide mixture were separated by reversed phase C18 column (Waters Acquity UPLC BEH C18, 100 x 2.1 mm, 1.7 μm, 300 Å) with a 90 minute UPLC run. The separated peptides were identified by online tandem mass spectrometry (MS/MS) Q-ToFMS system. Binding kinetics of 0.1% and 3.0% H2O2 treated biosimilar and reference antibodies were measured and compared by surface plasmon resonance (SPR) technique on a Biacore T200 (GE Healthcare).

Results

Both biosimilar and reference comprised of one Met on the light chain (LC-Met4) and four Met residues (HC-Met34, HC-Met83, HC-Met256, and HC-Met432) on the heavy chain. LC-MS/MS experiments were used to identify and quantify Met oxidation in the amino acid sequence. Oxidation of Met residue resulted in a +16 Da mass shift compared to the unoxidized counterpart. The oxidized peptides were confirmed through careful interpretation of their MS/MS spectra, i.e. 16 Da mass increase in b and/or y ions. Incubation of both products with 0.5% H2O2 for 24 h at room temperature leads to oxidation of Met256 and Met432 residues completely. Those residues are in the Fc region (CH2–CH3 interface) and exposed to the protein surface. It has been shown that they are more prone to oxidation compared to the other Met residues. On the other hand, Met34 is located on the complementarity-determining region (CDR) and it may impact antigen binding kinetics. Our data showed that increasing H2O2 concentration has nearly the same impact on Met34 oxidation for both reference and its biosimilar candidate. Moreover, Met4 and Met83 residues have shown nearly 20% oxidation under 3.0% H2O2 treatment for two products. In addition, the binding kinetics data will also be discussed for oxidized biosimilar and reference mAb.

Conclusions

The high-level similarities in oxidation profiles of Met4, Met34, Met83, Met256 and Met432 residues have shown that the biosimilar and reference mAb have a similar three-dimensional structure. Additionally, SPR analysis has demonstrated that Met34 oxidation has a similar impact on antigen-binding kinetics for both products.

Novel Aspect

Comparison of Met oxidation profiles of biosimilar and reference monoclonal antibodies under oxidative stress through LC-MS/MS and surface plasmon resonance.
583 - QUANTITATIVE EVALUATION OF ADENOSINE 5'-TETRAPHOSPHATE AND OTHER FIVE ANALYTES RELATED TO NICOTINAMIDE PHOSPHORIBOSYLTRANSFERASE BY LC-MS IN MELANOMA CELLS AND MOUSE PLASMA

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Keywords: Ap4, NAMPT, B16 cells, mouse plasma

Introduction:
Nicotinamide phosphoribosyltransferase (NAMPT) is the rate-limiting enzyme in nicotinamide adenine dinucleotide (NAD) synthesis[1]. Adenosine 5'-tetraphosphate (Ap4) is a nucleotide known to be a vasoactive purinergic mediator in mammals[2]. However, it has never been reported whether NAMPT can catalyze the synthesis of Ap4. So, the aim of the work was to develop abioanalytical LC-MS method to quantify Ap4 and NAMPT related analytes in biological samples.

Methods:
Three cells lines (B16 WT, B16 FLAG-NAMPT and ShNAMPTLow) and twenty-three plasma samples of healthy male C57BL/6 mice were analyzed. The bioanalytical method developed is a LC-MS method that consists in an isocratic elution on a Phenomenex Luna HILIC column. Each analyte (Ap4, adenosine 5'-diphosphate, adenosine 5'-triphosphate, nicotinamide, nicotinamide mononucleotide and NAD) was monitored in MS/MS, SRM or MRM mode.

Results:
Intracellular Ap4 levels were increased more than two times in cells over-expressing NAMPT (B16 FLAG-NAMPT: 1.17 ± 0.20 nmol/mg protein v. B16 WT: 0.56 ± 0.10 nmol/mg protein; p<0.05) and were significantly reduced in cells silenced for the enzyme (ShNAMPTLow: 0.31 ± 0.06 nmol/mg protein; p<0.05).
Moreover, metabolic perturbation conditions modulated Ap4 and nicotinamide mononucleotide synthesis in B16 WT cells. In particular, Ap4 and nicotinamide mononucleotide levels were significantly reduced in low glucose concentration and serum free conditions, respectively.
Finally, Ap4 mice plasma levels showed a good correlation with extracellular NAMPT levels, despite their inter- and intra-subject variability. Not surprisingly, Ap4 also correlated, to a greater extent, with ATP levels measured in parallel [3].

Conclusions:
A new LC-MS bioanalytical method was developed to quantify six analytes including Ap4 in B16 cells and in two different biological matrices. Murine cells and plasma with engineered or naturally fluctuating NAMPT levels showed matching Ap4 fluctuations.

Novel Aspect:
This study reports that NAMPT regulates in vivo Ap4 levels, conferring an unpredicted signaling role to this enzyme.

References
Introduction:
Exhaled breath contains thousands of Volatile Organic Compounds (VOCs) which are products of metabolic activity, and promising biomarkers for a range of diseases. Breath also includes VOCs produced during the metabolism of pharmaceuticals and other xenobiotics. Evaluating longitudinal changes in the breath VOC profile is relevant for a wide range of applications such as disease monitoring, measuring response to therapeutic interventions, assessing effects of environmental exposures, and studying pharmacokinetics. This study describes how the Breath Biopsy platform can be used to capture multiple breath samples over time, uncovering detailed changes in the concentration of volatile metabolites present in breath.

We present two main experiments: first we analyze VOCs in breath following ingestion of a peppermint capsule and show that Breath Biopsy can be used to observe the “washout curve” in for target compounds in a single individual using repeated, robust breath collection and analysis over a period of 8 hours. Secondly, we examine the biological variation in a longitudinal study where the washout experiment is repeated in the same individual multiple times over the course of 5 weeks.

Methods:
Breath samples were collected using the ReCIVA Breath Sampler and analyzed using the Breath Biopsy platform in the Breath Biopsy Clinical Laboratory (Owlstone Medical Ltd, UK). Samples were pre-purged to remove excess water and desorbed using a TD100-xr thermal desorption autosampler (Markes International) and transferred onto a VF-5ms column (60m x 0.25mm x 0.25 um; Agilent Technologies) using 1:2 split injection. Chromatographic separation was achieved via a programmed method (40-310˚C in 60 min. at 1.8 mL/min.) on a Trace 1310 GC oven (Thermo Fisher Scientific) and mass spectral data acquired using an electron ionization time-of-flight (i.e. EI-TOF) BenchTOF HD mass spectrometer (also Markes International). Raw Markes data files were converted from using TOF-DS (Markes International) and MassHunter Quant (Agilent Technologies) was used for further data processing.

Results:
Analysis of breath captured 30 minutes after consumption of the peppermint capsule shows a large increase in the VOCs α-pinene, β-pinene, limonene, eucalyptol and p-menthan-3-one compared to baseline pre-ingestion controls. Breath collections made every 30 minutes after this initial capture show a washout curve for all peppermint-related compounds, decreasing to baseline levels. A wide range of fold changes was observed across all peppermint-related compounds, with most abundant GC peaks not necessarily resulting in highest fold changes. In contrast, breath metabolites e.g. acetone that are not peppermint-related show little or no change compared to control. From the washout experiment, three time points were selected (pre-ingestion control, peak at 45 minutes and plateau at 3 hours) for inclusion in a longitudinal study where the washout experiment was repeated multiple times over 5 weeks.

In the longitudinal study, fold changes for all compounds are presented for the peak and plateau time points, relative to the corresponding pre-ingestion control. Breath metabolites acetone and isoprene show only small
differences between peak and plateau fold change as expected, however large fold changes are observed for the peppermint-related VOCs such as α-pinene and β-pinene, although some variation is observed for peak data points between washout experiments.

Conclusions
The study demonstrates that the Breath Biopsy platform can be used to study longitudinal changes of exhaled VOCs in a reliable and reproducible way. The peppermint washout experiment is a useful way to assess platform performance and future work will investigate the biological variability that exists for uptake and breakdown of substrate. The Breath Biopsy platform unlocks potential new use cases for breath analysis in metabolomics and pharmacokinetics studies.

Novel Aspect:
Breath-based platform allowing standardized sample collection and pre-concentration using the ReCIVA Breath Sampler, followed by analyte separation and data acquisition via a TD-GC-MS methodology.

References
4. L. M. Heaney, Bioanalysis, 8 (13), 1325-1336 (2016).

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Introduction:
To perform therapeutic drug monitoring (TDM) of Sunitinib (SUN) and its active metabolite N-desethyl SUN, a robust and sensitive LC-MS/MS quantification method is required. However, the geometric photoisomerization of SUN and its metabolite makes working in the dark highly recommended. Unfortunately, these conditions are unsuitable for clinical application and the aim of this work was to study the isomerism phenomenon in human plasma to overcome this issue.

Methods:
The geometric isomerism of SUN and N-desethyl SUN has been studied in human plasma. A LC-MS/MS method developed and validated for this study, has been applied for the analysis of these samples: the analytes were extracted from the matrix with a protein precipitation and separated on a Synergi Fusion-RP column (Phenomenex, USA) and the system consisted of a Prominence UFLC XR (Shimadzu, Japan) coupled with an API 4000 QTRAP mass spectrometer (SCIEX, USA).

Results:
When the SUN samples were exposed to light, the formation of E-isomer did not reach completion with nearly 55% formed after 30 min. An analogue equilibrium achievement was noticed for N-desethyl SUN, but its E-isomer reached a maximum of only 22%. Then, the E- to Z-isomer reconversion kinetics was investigated in dark conditions at different temperatures, different analyte concentrations and in presence of acids. The reconversion rate resulted increased by higher temperatures thus indicating a thermal reversion, not influenced by the analyte concentration. Low pH values seemed to stabilize the E-isomer thus slowing down the reconversion. To decrease the E- to Z-isomer reconversion time, samples were incubated in a heated-water bath at different temperatures (40, 50, 60, 70, 80 and 90°C) just before the analysis, reaching the maximum percentage of the Z-isomer for both SUN and N-desethylSUN in only 5 min with temperature equal or higher than 70°C. Moreover, no degradation has been observed for both the analytes after the heating step.

Conclusions:
The developed method does not require the protection from the light during the sample preparation but only a heating step with a heated water bath at 70°C for 5 min. The incubation of the samples allows the rapid reconversion of the E-isomer, formed during the sample preparation, to the desired Z-form. Despite the additional step of 5 min little extends the processing time, it strongly facilitates the handling of the samples making this method more feasible for TDM.

Novel Aspect:
For the first time, the study of the geometric isomerism of SUN and N-desethyl SUN in human plasma has led to the development of an analytical method to support TDM.

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Introduction:
In Anopheles gambiae, the most efficient vector of malaria parasite Plasmodium falciparum, 3-hydroxykynurenine (3-HK) represents a toxic metabolite. In the adult mosquitoes, the excess of this toxic metabolite is removed by a specific 3-HK transaminase (Ag-HKT) which converts the 3-HK into the more stable xanthurenic acid. Interfering with 3-HK metabolism in A. gambiae is a potential approach for the development of transmission-blocking drugs and insecticides.

Methods:
LC-MS method was optimized for the evaluation of the kinetic parameters of Ag-HKT and the kinetic characterization of inhibitors of the enzyme. An enzymatic assay was set up by using the recombinant Ag-HKT. The chromatographic separation of analytes was performed in a gradient mode on a Synergy column, using an acidic mobile phase. Mass spectrometric detection was achieved with an ion trap equipped with an ESI source, in positive ionization scan, operating in SRM mode.

Results:
The LC-MS method was used for the determination of kinetic constants values for Ag-HKT. The Km and Vmax calculated for Ag-HKT resulted in agreement with those reported in the literature [1]. Moreover, this selective method was applied for the evaluation of Ag-HKT inhibition by using a compound whose synthesis was previously described in the literature [2] and a new promising inhibitor (3-INI).

The Ki value of the known inhibitor obtained with the LC-MS method was in agreement with that reported in the literature [2]. On the basis of the results obtained in our experiments a competitive mechanism of inhibition was delineated verifying the accuracy and reliability of the LC-MS assay. The method was applied to the characterization of 3-iNI, a new synthetic compound. The determination of its mechanism of action through the analysis of the overlaid Lineaweaver-Burk reciprocal plot revealed a competitive mechanism of action. The Ki value was determined suggesting that the new synthetic compound is more potent than the reference compound.

Conclusions
The LC-MS method was developed and its applicability was confirmed by determination of the kinetics parameters of Ag-HKT and the determination of inhibitory potency of a known reference compound. Besides, a new synthetic compound was analyzed resulting in a more potent inhibitor with an improved Ki. New compounds will be synthesized starting from the structure of previously studied inhibitors with the aim of discovering more potent and selective Ag-HKT inhibitors.

Novel Aspect:
The novelty is the application of LC-MS method for the determination of mechanism of action and Ki values of new potential Ag-HKT inhibitors.

References
Introduction:
Drug detection and identification is a current analytical challenge requiring a robust detector which is sensitive, quantitative, and has sufficient separation power to resolve potential interferences. Drugs such as fentanyl are typically used in combination with common street drugs, however because fentanyl is hundred times more potent than heroin it therefore poses unique dangers. An overdose of fentanyl can be caused even by trace amounts.

Methods:
PTR-TOF-MSequipped with the a VOCUS reaction cell was tested for high sensitivity drug detection in real-time. The low vapor pressure of many street drugs requires coupling to a thermal desorption system in a swab like fashion. After instrumental calibration, simulated real samples containing few nanograms of fentanyl were tested. Collision Ion Dissociation was induced by varying the extraction voltage to confirm drug identification at structural level.

Results:
Fentanyl detection was achieved in seconds without any pre-separation or sample preparation even in complex mixtures. The soft ionization achieved in the Vocus reactor protonated fentanyl ions. Calculated and measured exact mass and isotopic distribution matches perfectly giving support to the molecular composition assignment determined by exact mass analysis. Confirmation of fentanyl detection was achieved by performing real-time Collision Induced Dissociation using VOCUS PTR-TOF. The collisional energy of protonated fentanyl is varied by changing the extraction voltage after the VOCUS reactor. Higher extraction voltages produce higher collisional energies which in turn increase ion fragmentation. The fragmentation pattern provides information about the molecule structure as in MS/MS techniques. At higher collisional energies the parent ion corresponding to protonated fentanyl (C22H28N2O·H+) is more fragmented into C13H18N+ and C8H9+ ions, matching fentanyl fragments from MS/MS databases.

Conclusions
Vocus PTR-TOF coupled to a thermal desorption system allowed real-time detection, identification, and quantification of fentanyl in a swab without any sample preparation or preconcentration step. Collision Induced Dissociation allowed fentanyl confirmation via matching with MS/MS databases. Single digit nanogram detection limits were achieved. Faster desorption rates for example from a swab desorber would result in even lower detection limits.

Novel Aspect:
Real-time drug identification and quantification in swabs was achieved by using PTR-TOF equipped with a VOCUS reaction cell and performing Collision Induced Dissociation.
Cannabis Pesticide Analysis: A Validated and Robust Analytical Method for Pesticides Measurement in Cannabis by Liquid Chromatography Tandem Mass Spectrometry

Keywords: Mass spectrometry, chromatography, sample preparation, method validation

Introduction
Quantitative analysis of pesticide residues in cannabis is of great importance to scientific researchers, scientific instruments manufacturers, cannabis industry stakeholders and government regulatory institutions. Besides the complexity of cannabis matrix, more stringent regulatory action limits for pesticides from certain states within the USA, have furthered analytical challenge to accurately quantify these pesticides. Nonetheless, the need for simple, robust and validated methods for pesticide analysis with good data reproducibility and accuracy are critical to all stakeholders in the cannabis industry. This work shows a validated method for analysis of 70 pesticides, including compounds typically analyzed by gas chromatography, in cannabis flower using LC-MS/MS with ESI source. Limits of quantitation were below all current regulatory action limits for pesticides in cannabis in USA. Good reproducibility and precision were obtained for all analytes with %RSD ≤ 23 for all pesticides in the cannabis matrix.

Method Summary
Acetonitrile cannabis extracts were spiked with 70 pesticides at different concentrations and internal standards added. Subsequently, spiked samples were diluted at different levels (1:1 and 1:5) with 0.1% formic acid in acetonitrile. All diluted and spiked extracts were directly injected into a PerkinElmer LC coupled with the QSight MS/MS system. Calibration standard curves were generated in both diluted standard solvents and the cannabis matrix. Method reproducibility and precision were determined with 7 replicates of spiked sample injections. Limits of quantitation at two different spike levels were established with at least 3 replicate injections. A 20 min LC-MS/MS was used for pesticides analysis in cannabis on a reverse phase C18 column.

Preliminary Results
Due to the complexity of the cannabis matrix, the reproducibility, precision and stability of the instrument are always of utmost importance for any routine analysis of pesticides in a laboratory. In addition, stakeholders are interested in high sample throughput with reliable and validated methods in order to augment their overall efficiency in the cannabis industry. Chromatographic analysis including column reconditioning for all 70 pesticides was achieved within 20 min. The instrument sensitivity ranged from 0.5 to 50 ppb for all pesticides, which was well below action limits set by California and Oregon. The limit of quantitation for the pesticides in the cannabis matrix was within 0.001–0.1 μg/g for 1:1 diluted spiked samples and 0.001 – 0.3 μg/g for 1:5 diluted spiked samples. The reproducibility (% RSD; n=7) for each pesticide in both diluted matrices was ≤ 23%. The calibration correlation coefficient for each analyte in the cannabis matrix was ≥ 0.98 with linear range over 3 orders of magnitude. Inter-day (n=7) variability of area response was ≤ 6% without the use of internal standards and < 2% with internal standards.

Conclusion
A high throughput quantitative analytical method for analysis of pesticides in cannabis was fully validated in accordance with the FDA guidelines for chemical analysis in food using liquid chromatography in combination with tandem mass spectrometry. The limit of quantitation for each pesticide was below the required action limits set by California and Oregon states in the USA.

Novelty
Validated liquid chromatography tandem mass spectrometry method for analysis of 70 pesticide residues, including compounds that are conventionally analyzed on gas chromatography platform, in cannabis with limit of quantitation below the action limits set by Oregon and California states.
Introduction:
Betamethasone (BET) is a glucocorticoid widely used in sports medicine for the treatment of asthma and acute injuries. BET is prohibited in sport competitions when administered by systemic routes (e.g., oral, intramuscular), and is allowed using local treatments. The aim of the present work was to examine the urinary profile of BET and its metabolites after different administration routes.

Methods:
A validated method was used to determine urinary levels of BET, 6β-hydroxy-BET, and fifteen additional metabolites. Urine samples were hydrolyzed with β-glucuronidase, extracted with ethyl acetate and analyzed by LC-MS/MS. The method was applied to urines collected in clinical studies where BET was administered by topical, intranasal, intra-articular, peri-articular, oral and intramuscular routes to male and female volunteers (n=40).

Results:
After topical (10 mg/day for 5 days) and intranasal (320 µg/day for 3 days) uses, maximal BET concentrations were 6 and 32 ng/mL, respectively. In most of the samples collected after intranasal treatment, BET concentrations were below 30 ng/mL.
Intra-articular and peri-articular single administrations led to maximal BET concentrations of 1420 and 981 ng/mL, respectively. All samples collected 72h after both administrations gave BET concentrations lower than 30 ng/mL.
After a single oral dose of 0.5 mg, the maximal BET concentration was 82 ng/mL, and all samples collected 24h after intake resulted in BET concentrations below 30 ng/mL. Single intramuscular doses of 6 and 12 mg gave maximal BET concentrations of 1446 ng/mL. In most of the samples collected 72h after intramuscular administration, BET concentrations were lower than 30 ng/mL. After both administrations, BET excretion rates reached maximal values during the first hours after intake and gradually decreased.
The excretion profiles of most of the metabolites were similar to those of BET.

Conclusions:
As expected, different excretion profiles were obtained depending on the administration route. It will be difficult to distinguish intra-articular and peri-articular uses from intramuscular treatments because excretion profiles obtained after these three administrations routes are similar. Topical and intranasal treatments can be differentiated from oral and intramuscular administrations, using a reporting level for BET.

Novel Aspect:
Since BET can be administered by different routes, this information could be useful for the anti-doping community to know how it behaves depending on the administration route.
Introduction:
Glycosylation is a critical post-translational modification in monoclonal antibodies. However, it is inherently heterogeneous and challenging to analyze, leading to a proliferation of analysis methods. An interlaboratory study was coordinated by NIST to determine variability in measuring N-glycans across laboratories and to derive consensus values for NISTmAb, an IgGk1 reference material. This work describes results from 103 reports worldwide [1].

Methods:
Participants performed glycosylation analysis of NISTmAb and mod-NISTmAb using their own method. Labs recorded, in a provided template, methods used and percent fractional abundances of glycans in both samples. A pilot study was conducted to polish the study’s protocol. Consensus values and ratios were derived for glycans reported by seven or more labs. Data was analyzed using robust techniques to assess measurement reproducibility and characterize distributions.

Results:
A total of 103 reports were submitted by 76 participants from industry, university, research, and government laboratories in Europe, North America, Asia, and Australia. Various methods were used for glycosylation analysis, including mass spectrometry, fluorescence detection, and capillary electrophoresis. Fifty-seven glycan compositions were reported at least six times; some labs differentiated between isomers. Consensus values were calculated for each of the 57 glycans. The number of glycan compositions identified by labs ranged from 4 to 48, with a median of 22. Most participants reported about the same number of glycans for NISTmAb and mod-NISTmAb. To ascertain the ability of labs to demonstrate comparability of two samples, the mod-NISTmAb/NISTmAb ratios were calculated. The ratios for the four most commonly reported glycans agreed within 8% to 30%. Notably, the extent of agreement between a participant’s reported values and the study’s consensus values is a strong function of the participant’s measurement repeatability.

Conclusions:
Determining variability of glycosylation profiles and assigning best values from a larger number of laboratories and range of measurement methods provides a baseline for comparison in the rapidly developing field of glycosylation analysis. We thank all the scientists who participated in this study.

Novel Aspect:
Variability and values for the glycosylation of a monoclonal antibody reference material from 100+ reports worldwide

References
Introduction:
We have implemented a platform utilizing acoustics to load samples into a time-of-flight mass spectrometer capable of analysing more than 100,000 samples per day. In this poster we demonstrate the use of our platform to screen a kinase and compare the data with a classical high-throughput screening technology. Since acoustic mist ionization mass spectrometry (AMI-MS) is a direct infusion system, kinases are a challenge due to the enzyme’s requirement for MgCl2.

Methods:
An acoustic transducer generates a nebulised spray from the wells of a 384-well plate located on a moving XY-stage. High voltage is applied to a charging cone above the well, inducing charge separation in the sample. The nebulised spray is drawn through a heated transfer interface into the MS and an ion beam is generated. To utilize the platform with a kinase enzyme, ammonium phosphate at high pH is added to precipitate out the Mg2+ ions.

Results:
AMI-MS was used to build a kinase enzyme assay to identify inhibitors from a subset of the AZ compound collection. We established a protocol using ammonium phosphate as a stop solution at the end of the assay to remove magnesium chloride and reduce suppression in the mass spectrometer. Having established the kinetic parameters for thistarget were screened a 60,000 compound subset using both ADP Glo™ kits and AMI-MS endpoints. There was a strong correlation between both assay formats, over 2000 compounds were identified as active in both formats. Surprisingly, there were only small clusters of compounds which appeared active in only one of the two assay technologies (38 unique to AMI-MS and 101 unique to ADP Glo™).

Conclusions
Our data demonstrates that AMI-MS is able to screen kinase targets where there is a requirement for magnesium chloride despite this being a direct infusion MS technology. AMI-MS compared very favourably with traditional kinase screening technologies but proved significantly cheaper as there was no requirement to purchase detection kits.

Novel Aspect:
This is the first example of AMI-MS being used to screen a kinase target. The data presented clearly demonstrate that AMI-MS is comparable with existing kinase technologies.

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Quantification of large molecular weight biotherapeutics in biological matrices relies mostly on enzyme-linked immunosorbent assays (ELISAs) mainly due to several advantages: good sensitivity (1-10 ppm), high-throughput, ease-of-use and low cost per sample. In the case of low-abundance protein impurities (HCPs) present in protein therapeutics (e.g. mAbs), these biological assays typically provide the total HCP concentration (usually expressed in ppm or ng HCP/mg mAb), but ELISAs cannot identify and measure individual HCP contaminants. Several MS-based assays have been recently developed to cover this knowledge gap [1-2]. Here we explored the capabilities of a single dimension chromatographic assay, coupled with mass spectrometry using two different data independent acquisition modes for detection of peptide precursors and their fragments.

Methods

The LC/MS assay described here relies on two data-independent acquisition mode implemented on a quadrupole/time-of-flight (QTOF) mass spectrometer. While in MSE mode all peptide ions produced by the electrospray source are transmitted by the quadrupole analyzer, in SONAR mode the quadrupole slides over the mass range of interest during the time required for recording a single MS-spectrum by the TOF-analyzer. Co-eluting precursor ions with different m/z are separated during the rapid quadrupole scan and their corresponding fragmentation spectra are acquired using an identical quadrupole separation. Mass spectra are recorded with high-resolution (~ 25,000) for precursors/fragments.

A monoclonal antibody (mAb), purified by Protein-A affinity-chromatography and then by SCX -chromatography (strong-cation-exchange) using four different elution protocols, was denatured/reduced/alkylated and digested with trypsin.

Preliminary data

LC/MS-assays have recently been adopted as orthogonal techniques to ELISAs for HCP analysis due to their flexibility and sensitivity. Here we describe an efficient analytical scale two step LC/MS assay that allows the identification and quantification of HCPs during mAb purification in a CHO (chinese hamster ovary) cell line. In the first step, called the Discovery Assay, the mAb digest (100-500 µg)is analyzed in SONAR mode on a C18 column (2.1 x 150 mm, operated at 200 µL/min flow rate) using a 90 min gradient. Two HCPs and 4 spiked protein standards were identified across 5 mAb preparations (one Protein A and 4 SCXfractions) following a CHO database search using Progenesis QIP 4.0 software. The same software was also used to build a spectral librarycontaining 13 CHO HCP peptides and 57 peptides from the spiked protein digestion standards. The spectral library contained the peptide RT, the precursor m/z and the MS/MS fragmentation spectrum of each peptide, thus facilitating subsequent HCP identification and quantification in other mAb preparations.

In the second step of the assay, the same 5 digests were analyzed in triplicate in a different acquisition mode (MSE) using higher throughput runs (30 min gradients, 45 min runs) and lower sample loads (10-20 µg of mAb digest). The
MSE acquisition is a data-independent acquisition mode which does not use precursor ion isolation. Instead, in MSE low-energy scans (for precursors) are alternated with high-energy scans (for fragments). The MSE data was searched against the spectral library (containing 70 peptides) to quantify and monitor all 6 previously identified proteins (2 HCPs and 4 spiked proteins). The sensitivity of both assays (discovery and monitoring) was 10 ppm. SONAR acquisition offers additional selectivity to MSE acquisition, by producing fragmentation spectra with minimized background interferences.

References:
Introduction: Covalent inhibitor of K-RAS G12C to fight lung cancer
The small GTPase K-RAS is the most prevalent oncogene in cancers. The Gly to Cys mutation at position 12 is frequent in lung cancer. The optimization of a drug, covalently bound to Cys12, would target the oncogene without affecting the wild type protein [1]. Mass spectrometry is well suited to the study of covalent complexes and we have implemented a series of tools to support a K-RAS G12C research program.

Methods: Mass spectrometry tools to study covalent complexes
Several LCMS/MS-based assays were developed either with recombinant protein or with cellular or tissue lysates: intact mass to rapidly screen or fully characterize the binding kinetics (kinact/Ki) of covalent complexes, peptide mapping to determine the binding site, and targeted quantitative proteomics to measure target engagement in culture cells and tissues.

Results: Optimization of K-RAS G12C covalent binders
To discover novel covalent inhibitors of K-RAS G12C, several thousands of covalent compounds were screened in an assay measuring the extent of covalent binding on a recombinant protein by intact mass LCMS. Hits were validated by peptide mapping experiments to ascertain that the Cysteine 12 residue was the site of interaction between the compounds and K-RASG12C. During a chemical optimization program, the newly synthesized compounds were evaluated in the intact mass LCMS assay, as a function of inhibitor concentration at a fixed incubation time (EC50) or at several times (kinact/Ki) [2]. Optimized compounds inhibited the growth of a K-RAS G12C dependent cell line. A targeted LCMS-based assay was implemented to directly and quantitatively determine the engagement of KRAS-G12C in cell lysates [3].

Conclusions:
Our LCMS toolbox has proven to be very robust and powerful to support the optimization of novel covalent inhibitors of K-RAS G12C. Although other biochemical or cellular assays were implemented for the research program, the LCMS assays provided the most direct and non-ambiguous results.

Novel Aspect:
An LCMS intact mass assay as a primary tool to support chemical optimization in a pharmaceutical discovery program.

References:
494 - CLINICAL VALIDATION OF A UPLC/MS/MS-BASED ASSAY TO CONCURRENTLY MONITOR FOUR DIRECT ORAL ANTICOAGULANTS IN DRIED BLOOD SPOTS

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Keywords: Clinical validation, direct oral anticoagulant, dried blood spot, liquid chromatography, tandem mass spectrometry

Introduction: Monitoring blood concentrations of direct oral anticoagulants (DOAC) is not required regularly [1]. However, concentrations of some DOAC correlate well with (un)desired outcomes [2,3]. Thus, analyzing individual DOAC exposure could be useful for risk assessment, e.g. when clearance pathways are impaired. Dried blood spotting (DBS) is a practicable sampling technique for regular monitoring, enabling blood sampling at home at any relevant time point [4].

Methods: DOAC concentrations were quantified using a previously published ultrahigh performance liquid chromatography/tandem mass spectrometry (UPLC/MS/MS) multi-compound assay [5], which was refined for DBS analysis. In a clinical study, approved by the responsible ethics committee, DBS from capillary whole blood and plasma were simultaneously sampled in 33 DOAC patients. Deming regression and Bland Altman analyses were used for evaluation.

Results: Our DBS assay fulfilled pertinent validation standards for a hematocrit range of 33-65 % and was linear within a calibration range of 2.5 (apixaban, rivaroxaban), 4.4 (dabigatran), and 9.3 ng/mL (edoxaban) to 750 ng/mL with only minor ion suppression (matrix effect ≤ 12.5 %). Inter-assay and intra-assay accuracies ranged between 88.3-110 % and corresponding precisions were ≤ 13.3 %. DOAC in DBS were stable up to 52 d at room temperature, if stored in the dark and with humidity protection (desiccant). Deming regressions yielded conversion factors for each DOAC (apixaban 1.46, dabigatran 1.51, edoxaban 0.95, and rivaroxaban 1.37), which were used to predict plasma concentrations from (capillary whole blood) DBS. Bland Altman analyses confirmed that plasma concentrations predicted from DBS agreed well with actually analyzed plasma concentrations (71 % for apixaban, 94 % for dabigatran, 100 % for edoxaban, and 88 % for rivaroxaban). Thus, meeting the EMA guideline’s demand that in > 67 % of all sample pairs’ concentrations must not deviate > 20 %.

Conclusions: UPLC/MS/MS for DOAC quantification from DBS constitutes a practicable and sensitive technique to monitor DOAC exposure. The results of the clinical validation study confirmed that blood concentrations from DBS can accurately predict plasma exposure. Thus, the present DBS assay is an attractive option to monitor ambulatory DOACs.

Novel Aspect: We present a DBS assay capable of analyzing all DOAC at once, with minor setting requirements regarding sample collection.

References:
Introduction:
Alzheimer's disease (AD) is a neurodegenerative disorder responsible for more than 70% of all dementia cases. This disease is characterized neurologically by progressive loss of memory, behavior, language, and visuo-spatial skills impairments. AD has become the focus of one of the most intensive investigation in the medicinal field because of worldwide universal phenomenon – population aging and considerable economic impact. Novel therapeutic strategies based on biologicals are currently under investigation recently. One of the innovative AD therapeutic in clinical trials is AADvac1 – immunotherapy designed to elicit an immune response against pathologically modified forms of tau protein, developed by Axon Neuroscience [1,2]. Herein, we present combination of simple acidic chemical cleavage (sample preparation step) with effective and very fast LC-MS/MS method for quantification of immunogen Axon Peptide 108 in AADvac1.

Methods:
Chemical cleavage:
A strategy based on chemical cleavage at aspartyl residues using dilute formic acid developed by Li et al. [3] was used in this study. Appropriate cleavage of the therapeutic peptide was obtained when the sample was incubated in 2% formic acid at 108°C for 2 h.

LC-MS/MS method:
The quantitative LC-MS/MS experiments were performed with a Waters ACQUITY UPLC system coupled to the Quattro Premier XE triple quadrupole mass spectrometer. ACQUITY UPLC BEH Shield RP18 column (2.1 mm x 100 mm, 1.7 µm particle size) was used for analysis. Column temperature was set for 30 °C. Mobile phase A consisted of 20mM ammonium formate/0.1% FA in MPW, and mobile phase B was acetonitrile. The flow rate was 0.5 mL.min⁻¹ and the injection volume was 5 µL. Mass spectra were acquired using positive electrospray ionization mode (ESI+) and selected reaction monitoring (SRM) mode. The following MS conditions were applied. The capillary voltage was 3 kV and the source temperature and desolvation temperature were 120 and 450 °C, respectively. The cone gas and desolvation gas flowed at 50 and 600 L/h, respectively. Argon was used as collision gas.

Results:
The effective non-enzymatic release step of Peptide 108 based on acid hydrolysis with the use of 2% formic acid was successfully tested and implemented. The main benefit of the chemical cleavage procedure performed with formic acid was high level of compatibility of the cleaved sample with the ESI-MS ionization. Moreover, formic acid was a part of used mobile phase. The developed and optimized LC-MS/MS method was then validated according to the ICH guideline Q2(R1) [4]. Calibration curve was linear within the range of 10 – 300 µg.mL⁻¹. Correlation coefficient was higher than 0.99. The intra-and inter-day precision were 0.92-5.03% and 3.05-10.36%, respectively. The recovery ranged in the interval of 99.73-101.10%. The developed and validated method was successfully applied to determine the amount of Axon Peptide 108 in several clinical batches of AADvac1. Three different batches of AADvac 1 drug product were analyzed and the determined amount of the therapeutic peptide ranged from 74.8 to 106.8 µg/mL.
Conclusions
We developed and tested an effective approach of chemical cleavage of peptide conjugated to the high molecular protein carrier which was connected with very effective, fast and simple LC-MS/MS analysis. Developed and validated LC-MS/MS method was successfully applied and the exact amount of Axon Peptide 108 presented in AADVac 1 was determined in final drug product. The obtained results were in good accordance with the declared amount. This relatively rapid, simple and robust method has a potential to be applied in to the field of biopharmaceuticals analysis.

Novel Aspect:
A simple and rapid approach based on combination of non-enzymatic cleavage and LC-MS/MS analysis was presented as a powerful tool for quantitation of therapeutic peptide against Alzheimer's disease in innovative biologic AADVac1. The use of chemical hydrolysis was associated with solubilization of the AADVac 1 adjuvant (alhydrogel) which was beneficial in term of simplicity. Moreover, it was clearly demonstrated that chemical hydrolysis is much more faster and cheaper in comparison to convenient approach based on enzymatic cleavage (e.g. asparaginase N).

References

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Development of a solid phase micro extraction tool for the determination of a small molecule–drug conjugate directed against carbonic anhydrase in cancer chemotherapy

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Keywords
Capillary gap sampler, ESI Mass spectrometry, Solid phase microextraction, Small molecule drug conjugate, Cancer chemotherapy.

Introduction
The targeted delivery of cytotoxic agents into tissues, especially for malignant cells is an attractive strategy to avoid dose limiting toxicity. Targeting moieties can be antibodies, aptamers and low molecular weight non-peptidic ligands. Previous studies showed the advantages of conjugating small molecules to the drug over attaching macromolecules. One of the common used target moiety for selective delivery of cytotoxic agent is acetazolamide. In this area, there is a pressing need to develop rapid techniques to quantify the amount of drug in the tissue in order to investigate the tumor targeting performance of the different ligands. So-called the “capillary gap sampler” is capable of automated and site-specific extraction of very small sample amounts, which is interesting for this application.

Methods
The capillary gap sampler is a miniaturized sampling device enabling automated low-volume sample handling, for direct interfacing to ESI mass spectrometry. It consists of a liquid bridge of several nanoliters formed between two capillaries, where one acts as the ESI-MS spray needle. Sample extraction is performed by a coated stainless-steel pin which is controlled by a robot arm. Selective drug extraction is possible through immobilizing carbonic anhydrase (CA) on the pin. This modification is performed by overnight reaction between protein with epoxy modified beads which are glued to the tip of the pin. Using fluorescence microscopy, bright field and blue channel images of beads carrying protein linked to 8-anilino, 1-naphthalene sulfonate and controls confirmed the attachment of the protein.

Results and discussion
The development started with optimization of the coating procedure, desorption solution, etc. In order to find the optimum desorption solution two tests were performed: in the first one, influence of pH on the binding of acetazolamide with CA was investigated. In the second test, different phases (ACN, MeOH, EtOH, Acetone 60% in water) were used for desorption. ACN was found to be the optimum desorption solution. Extraction of the drug from PBS was performed by dipping the CA modified extraction tool inside the solution. After a quick washing step with water, it enters into the liquid bridge, where the analyte desorbs and is sprayed into the ESI source. Peaks corresponding to the drug (drug+), (drug+Na++H+), (drug+2H+), (therapeutic warhead=cytotoxic agent coupled to
the targetingagent+H+) were observed in the spectrum. Finally, repeatability of the acetazolamide extraction using CA modified beads are evaluated by performing thirteen 5-minute extractions from 500nM acetazolamide in PBS solution. The relative standard deviation was below 10%, which confirms the repeatability of the method. Drug extraction studies from human plasma will also be shown.

Novel aspects
Development of a extraction method for evaluation of targeted drug delivery.
697 - IN VIVO ASSESSMENT OF THE CONTRIBUTION OF SURFACTANT REPLACEMENT THERAPY IN RABBITS BY STABLE ISOTOPES NATURAL ABUNDANCE APPROACH

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Keywords: GC-C-IRMS; stable isotope; 13C natural abundance; surfactant; Respiratory Distress Syndrome

Introduction:
Respiratory Distress Syndrome (RDS) is a breathing disorder affecting preterms due to lack of lung surfactant, a lipoprotein layer which prevents alveolar collapse [1]. Treatment with exogenous surfactant improves gas exchange and survival [2]. The aim of this study was to estimate the contribution of exogenous surfactant phospholipids to the rabbit alveolar pool in vivo by 13C natural abundance.

Methods:
In a feasibility study we measured the 13C/12C ratio of disaturated-phosphatidylcholine palmitate (DSPC-PA) in poractant alfa and in bronchoalveolar lavages of 20 rabbits. The contribution of exogenous surfactant was then estimated in a rabbit model of RDS [3] including 7 controls and 15 rabbits treated with 50, 100 or 200 mg/kg of poractant alfa. DSPC-PA 13C/12C ratio was measured by GC-C-IRMS.

Results:
The DSPC-PA 13C/12C ratio of poractant alfa was constant among 7 batches produced over a year with a mean value of -18.8±0.1 ‰ [Range: -18.9 ‰; -18.6 ‰]. The mean 13C/12C ratio of surfactant DSPC-PA recovered from the lung lavages of 20 rabbits was -28.8±1.2 ‰ [Range: -31.7 ‰; -25.7 ‰]. DSPC-PA in rabbits had a significant lower 13C abundance than poractant alfa, with a mean difference of 10.0‰, wide enough to distinguish the endogenous from the exogenous compound. The contribution of exogenous surfactant to the total alveolar surfactant was calculated in all treated rabbits and it ranged from a minimum of 42.9% in 50mg/kg group to a maximum of 90.2% in 200 mg/kg group, with a significant dose-effect.

Conclusions:
The DSPC-PA of exogenous and endogenous surfactant have a different natural abundance of 13C and this difference could be exploited to estimate vivo the alveolar contribution of exogenous surfactant in treated rabbits. Since the use of chemically synthetized tracers is not required, it could be useful in human research and in surfactant replacement studies in preterm newborns.

Novel Aspect:
This is a novel method to measure in vivo the contribution of exogenous surfactant to the alveolar pool by using stable isotopes at natural abundance.

References:
Introduction:
In the arena of biotherapeutic analysis, the analysis of host cell proteins (HCP) at the ppm level is critical. ELISA is currently the gold standard for QC applications, but the advantages of mass spectrometry are abundant. We show how PASEF (parallel accumulation and serial fragmentation), as implemented on the timsTOF PRO QTOF, can be applied to HCP analysis for highly sensitive detection with enhanced data quality.

Methods:
The NISTmAb Reference Material 8671 and the Universal Proteomics Standard (UPS1, Sigma) were reduced, and digested. Peptides were separated on an Intensity Solo 2 1.8µm C18 column using an Elute UHPLC coupled to a timsTOF Pro ion mobility QTOF mass spectrometer (all Bruker Daltonics). A 150 minute gradient was used. For nanospray, a nanoElute UHPLC was fitted with an IonOpticks C18 column using a 210 minute gradient.

Results:
The UPS1 standard was used to make a 5-step 1:3 dilution series in a constant background of NIST mAb over a concentration range from 0.3 to 934 ppm. PASEF enabled the detection of UPS1 proteins down to low single digit ppm concentrations in the presence of 25 µg NIST mAb. Linear response for the UPS1 protein beta-2-microglobulin in the concentration range from 132 ppm to 1.6 ppm was observed. The dilution series indicated that PASEF enabled detection of HCPs in the range of 1 to 100 ppm of the therapeutic protein. Nano UHPLC was also evaluated for its suitability for HCP identification. This setup has already been established as the new benchmark for bottom-up proteomics applications [1, 2]. More than 200 HCPs were identified in 1.5 µg NIST mAb, including expected and previously unreported HCP proteins. The depth of HCP coverage and sequence coverage was further extended to >280 HCP identifications by employing an alternate digestion method in which only the HCPs are digested allowing the mAb to be removed prior to analysis [3].

Conclusions
PASEF improved the sensitivity of peptide mapping enabling the detection of HCPs at sub 100 ppm levels. PASEF coupled to nanoLC facilitates detection of previously unreported trace level HCPs. The quality of MS/MS sequence spectra provided by the timsTOF Pro with PASEF allows high confidence in protein ID even when only 1-2 peptides are sequenced.

Novel Aspect:
Sensitive HCP assay for routine use enabled by time and space focusing of precursor ions with a PASEF scan.

References

Lubeck et al., Bruker Application Note 131
Introduction:
Deamidation is one of the most common modifications which can be induced in monoclonal antibodies during production and prolonged storage. It is critical to evaluate and monitor these biologically relevant product quality attributes (PQAs) as deamidation can cause structural changes, reducing biological activity and efficacy of the biotherapeutic. Here, we monitored the degree and site of deamidation induced in pH stressed antibody digest.

Methods:
The trastuzumab samples were exposed to pH stress by increasing the pH of digestion buffer from nearly acidic (6.8) to alkaline (8.8). All measurements were carried out in replicates on using high flow liquid chromatography and a high resolution mass spectrometer using a data dependent (DDA) acquisition strategy. The complete data processing was performed with BioPharmaView™ software using the MAM workflow and reference ranges were set for pass/fail criteria.

Results:
The monoclonal antibody digest was exposed to higher pH levels in order to induce deamidation. All asparagine sites were monitored and defined for quantification using MAM workflow in BioPharmaView™ software. The lowest levels of deamidation were observed with the neutral or nearly acidic pH (6.8) as compared to the alkaline pH (8.8) values. The MAM workflow in the BioPharmaView™ software enabled the accurate and automated quantitation of all deamidation sites within the defined criteria of pass/fail using neutral pH values as reference points. The accuracy of the method was assessed by spiking the forced deamidated samples into the control sample at different levels. The sample with the lowest deamidation levels (pH 6.8) was used as control due to lack of samples with nodeamidation. Excellent linearity was observed with the increasing levels of spiking thus verifying the accuracy of the method.

Conclusions:
Using BioPharmaView™ software with the attribute calculator for the MAM workflow, levels of deamidation were calculated for all possible deamidation sites within trastuzumab. The data was automatically processed and showed the susceptibility of some residues to pH whereas others are far less sensitive. The results can be used to refine monitoring of attributes to those that are more susceptible to environmental changes.

Novel Aspect:
MAM workflow in BioPharmaView™ software for the quantitation of pH induced Deamidation.

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296 - UNREVEALING PHASE I METABOLISM OF COMBRETASTATIN A4 BY A MULTI-TOOL MS-BASED APPROACH

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Keywords: RLM, SPME, combretastatin A4, electrochemistry

Introduction:
Drug metabolism remains the main point of interest for clinicians, as comprehensive information regarding metabolic pathways and the mechanisms of action of new drugs are essential for recognition of their possible toxicity and biological activity in patients. The multi-tool analytical approach based on electrochemistry, in silico predictions and in vitro studies with the use of rat liver microsomes were employed for recognition of combretastatin A4 metabolism.

Methods:
In silico prediction was carried out using MetaPrint2D-React for recognition of transformations at primary sites of metabolism. For electrochemical reaction the ROXY™ system was used. It conducts redox reactions which are the most common during I phase metabolism. The rat liver microsomes was analyzed via two different extraction approaches protein precipitation and solid phase microextraction. OrbitrapHRMS was used for instrumental analysis.

Results:
The use of a high resolution mass spectrometer enabled a non-targeted investigation of metabolic transformations of the parent drug and identification of previously reported [1] as well as unreported metabolites. In total fourteen putative metabolites of combretastatin A4 were found. The main paths of CA4 metabolism are O-demethylation and aromatic hydroxylation and those metabolites were found by all aforementioned metabolism generating Methods: There was no differences found between PP and SPME analysis of RLM in terms of identified metabolites. The use of SPME allowed performing time course analysis without need of sample multiplication. The EC reaction resulted in generation 3 metabolites reported also by RLM and one exclusive metabolite not found by other techniques.

Conclusions
The high mass accuracy and fragmentation patterns supported by in silico data showcase the applicability of this approach as a ultimate tool for further identification of putative metabolites detected in microsomes extracts or via EC reactions, especially in cases where authentic standards are not available to confirm the tentative results attained via untargeted analyses.

Novel Aspect:
In total of eight metabolites of combretastatin A4 not reported elsewhere were identified via non in vivo based approaches.

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References:
APPLICATION OF TRIPLE QUADRUPOLE MS FOR DRUG-TO-ANTIBODY RATIO AND MOLECULAR WEIGHT DETERMINATION OF ANTIBODY-DRUG CONJUGATES.

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Keywords: Antibody drug conjugate (ADC), RPLC, drug-to-antibody ratio (DAR), triple quadrupole MS, Middle-down

Introduction:
Antibody-drug conjugates (ADCs) are an increasingly popular class of therapeutic drugs with an inherently high heterogeneity from a large arsenal of drugs, linkers and antibodies. RPLC-MS using QTOF or Orbitrap analyzers have been used successfully to determine the average drug-to-antibody ratio (DAR) for several constructs. Here we have investigated the potential for the very common triple quadrupole (QqQ) analyzers as an alternative for this application.

Methods:
Deglycosylated Trastuzumab and in-house synthesized ADCs generated from Trastuzumab by disulfide reduction with TCEP, followed by conjugation of vcMMAE to the resulting thiols were used for the comparison. Waters Premiere QTOF and Waters XevoTQ-S Micro in combination with Waters Aquity UPLC system and Waters Xbridge C4 column was used for the RPLC separation for the comparison. Mobile phases consisted of 0.5% FA with H2O or ACN.

Results:
Both ADCs and monoclonal antibody (mAb) were analyzed with RPLC-MS on a QTOF and QqQ instruments. Having reduced the disulfide bonds to separate the light- and heavy chains the charge envelopes moves to lower m/z so the loss of ions by a limited m/z range was small. Consequentially the loss of any ions above 2000 m/z in the QqQ was found to have no evident effect on the assigned molecular weights after deconvolution. Comparing the two instruments, preliminary data indicates that the differences in spectral quality related to differences due to age and type of ionization source had bigger effect than the loss of part of the charge envelope on the DAR determination and LOD.

Conclusions:
Preliminary data implicates that for average DAR determination of ADCs the QqQ instruments can be a cheaper and more readily available option for smaller labs in early stages of ADC development. The limited m/z range of the QqQ has this far proven to have no major effect on DAR value determination.

Novel Aspect:
We have shown that triple quadrupoles can be used for accurate DAR determination of disulfide reduced ADCs and mAbs.
Keywords: electrochemical chamber, auranofin, redox reaction, pharmacokinetics

Introduction:
Gold complexes belong to a group of rapidly emerging novel therapeutic and diagnostic compounds with improved specificity for tumor tissue and strong antiproliferative potency. They are usually responsible for the cell growth-inhibiting effects related to the mitochondrial activation of apoptosis. Auranofin used in the treatment of rheumatoid arthritis was selected for the experiments because of documented activity against ovarian cancer, HIV, E. Histolytica and M. Tuberculosis.

Methods:
Redox activity of selected gold(I) complex was investigated by means of a ROXY ERC system (Antec, The Netherlands) consisting of a potentiostat, equipped with an electrochemical micro-reactor cell (μ-PrepCell, Antec, The Netherlands) and an infusion pump (KD Scientific, model KDS100, USA, Figure 1c). The Roxy system was online hyphenated to an Agilent 6460 LC/MS ESI triple quadrupole mass spectrometer (Santa Clara, USA) as a detector.

Results:
Reduction and intermediate electrochemical conditions corresponding to conditions of cytosol and blood were found optimal to obtain most probable reactive forms for auranofin: (tEP)2-Au and tetra(tri and di)AtgS-Au, which can interact with bioligands in human organism. Obtained products are in agreement with forms of auranofin bound to GSH and human albumin. Products of deacetylation were minor and observed mainly in the presence of sodium cations. Moreover, sodium ions were found to enhance creation of disulfide bridges during anodic process. ERC–ESI MS results additionally allow to state that deacetylation and loss of Atg or tEP groups by auranofin are irreversible as is disulfide formation. Nitrile group attaching to gold and its influence on transformation of auranofin can mimic the behavior of ligands typical for physiological conditions. Ligand exchange can be proposed as the most probable mechanism for metabolic changes of auranofin.

Conclusions
Electrochemical reaction chamber (ERC) coupled to ESI MS was found to provide significant information considering the transformations of auranofin. Moreover, molecule-specific mass spectrometry delivers complementary data to square-wave voltammetry, which aids in understanding the nature of electrochemical conversions of complex or unstable compounds.

Novel Aspect:
For the first time ERC-ESI MS was used to study the transformations of cytotoxic metal transition complexes such as auranofin.

This work was financially supported by the Polish National Center of Science (NCN) within the project DEC-2013/09/B/ST4/00961

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Introduction:
Alzheimer’s disease (AD), the most common form of dementia (%70). Current drug treatments aim to prevent cognitive decline. No new drug have been approved by FDA since 2003.Studies have revealed that alkaloids with anticholinesterase activity are a promising resource in drug discovery.[1] The aim of the project is to discover natural compounds for the treatment of AD and investigate their effects on neurodegeneration in a comprehensive manner.

Methods:
Natural compounds (2 different doses) were administered for 7 days, to 12 m old (pathological phase) 5xFAD transgenic mice (n=8). Littermate mice were used as control group. FDA approved AD drug Galantamine is also used as positive control. Morris water maze (MWM) was performed to investigate changes in learning and memory impairment. Action mechanism of drugs were investigated in 3 different regions of the brain which are cerebellum, cortex and hippocampus via LC-MS/MS based protein expression analysis.

Results and Conclusion:
2 of 6 compounds showed significant improvement in learning and memory according to MWM test. The compound which improves the condition most, was used for further proteomic investigation. Protein expression changes between control and drug-administered groups were determined by LC-MS/MS analysis. In each samples, approximately 1250 proteins were identified and differentially expressed proteins between groups were subjected to pathway analysis to enlighten the molecular mechanisms affected by the drug.

Novel Aspect:
This is the first study that nLC-MS/MS based proteomics approaches were used in the research of molecular effect mechanism of novel compounds on 5xFAD mouse model.

References
Simultaneous determination of aripiprazole, dehydro-aripiprazole, olanzapine, risperidone, paliperidone, quetiapine and clozapine in human plasma by LC-MS/MS

Keywords: antipsychotics, LC-MS/MS, SPE, pharmacokinetics, quantification, method validation

Introduction: A simple and sensitive LC–MS/MS method was developed and validated for the simultaneous quantification of aripiprazole (ARI) and its active metabolite dehydro-aripiprazole (DARI), olanzapine (OLA), risperidone (RIS) and its active metabolite paliperidone (PAL), quetiapine (QUE) and clozapine (CLO) in human plasma. These drugs have been widely used in the treatment of schizophrenia and schizoaffective disorders [1]–[3].

Methods: Stable isotopically labeled internal standards - [2H8]-ARI, [13C,2H3]-OLA, [2H4]-RIS, [2H4]-PAL, [13C4]-QUE and [13C,2H3]-CLO - were used for all analytes. Three-step phospholipids-eliminating microelution-solid-phase extraction was used for analyte extraction. The chromatographic separation was performed during 8 min under gradient conditions through a mobile phase consisting of 0.2% formic acid and acetonitrile with a flow rate of 0.6 ml/min.

Results: The analytical method has been validated according to the recommendations of European Medicines Agency (EMA) and Food and Drug Administration (FDA) through tests of precision, accuracy, stability, sensitivity and specificity. The coefficient of variation was not more than 20% for the lower limit of quantification and was below 15% for the rest of quality controls. The method is able to detect the drug levels in the ranges of 0.18-120 ng/ml for ARI, 0.25-80 ng/ml for DARI, 0.5-1000 ng/ml for CLO, 1-100 ng/ml for OLA, 0.2-30 ng/ml for PAL and 0.5-160 ng/ml for QUE during therapeutic drug monitoring. Comparing with protein precipitation (PPT), our SPE sample preparation method resulted to be effective in removing phospholipids from plasma.

Conclusions We have developed a sensitive LC-MS/MS method to determine the levels of 5 antipsychotics and 2 active metabolites with the usage of stable isotopically labeled internal standards in human plasma. The method is currently being utilized in pharmacokinetic studies. Its application could help to optimize the effectiveness of treatment and manage side effects or toxicity leading to individualized therapeutic decisions for each patient.

Novel Aspect: The present method applies effective phospholipids’ removal during SPE and therefore results in exceptional recoveries and low matrix effect.

References
861 - LC-MS IDENTIFICATION AND QUANTIFICATION OF BLEOMYCIN IN SERUM AND TUMOR TISSUE

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Keywords: Bleomycin, metal complex, serum, tumor, LC-MS analysis

Introduction
Bleomycin (BLM) is a cytotoxic antibiotic that appears as a mixture of more than ten structurally strongly related glycopeptides, most abundant being BLM-A2 and BLM-B2 fractions. In vivo BLM chelates with several metals, in particular with copper. Aiming to investigate its biodistribution and pharmacokinetics as a part of a wider electrochemotherapy study, we developed a specific and sensitive mass spectrometry-based analytical method for determination of BLM in serum and tumor tissues.

Methods
Frozen tumors were grinded to fine powder, the suspension was sonicated, the supernatant filtered and loaded onto a preconditioned solid phase extraction (SPE) cartridge. Reversed-phase SPE of diluted serum and tumor suspensions was performed as the enrichment step. BLM behavior was investigated with UHPLC coupled to a QTOF MS[1]. To improve quantification characteristics the instrumental method was later translated to UHPLC coupled to QTRAP MS.

Results
The optimized sample preparation method used Oasis HLB™ cartridges at acidic pH of the sample matrix, water as the wash solvent, and 40% methanol followed by acetonitrile for BLM elution. For UHPLC separation we selected BEH Amide stationary phase, which clearly and distinctively separated the Cu complexes of BLM-A2 and BLM-B2 fractions.

The investigation of BLM chelating properties implies that Cu complexes form in vitro and in vivo, and offer a viable form for the quantitative determination of BLM in biological samples. The amount of Cu available in the serum is sufficient to quantitatively chelate metal-free BLM even at its highest concentrations. Metal-free BLM-A2 shows a 2+ charged fragment ion at m/z 707.76, but it is a “transient” species that forms the Cu complex. The latter shows an abundant ion at m/z 707.21, and m/z 707.71 as the second isotope ion in the typical Cu isotopic pattern. In the complex mixture of several BLM fractions and their metal complexes, and considering also background ions from the biological tissue matrices, this second isotope ion may be misinterpreted and incorrectly assigned to the metal-free BLM-A2, which could be the reason why the attempts to determine BLM with MS have been so scarce.

Conclusions
Except in the controlled metal-free environment BLM is not found in a free form, but is highly prone to form chelates. Thus, MS analysis results in the apparent disappearance of BLM characteristic fragment ions. In addition, the chelates may be hidden at first sight, since they tend to form double charged ions. Choosing a HRMS proved crucial for recognizing BLM MS behavior, since it enabled us to follow the isotopic pattern of the double charged ions in the clusters.

Novel Aspect
The LC-MS analytical method for determination of bleomycin in serum and tumor tissues is developed for the first time.

References
INTRODUCTION: Meloxicam belongs to oxicam class of nonsteroidal anti-inflammatory drugs (NSAIDs) and is widely used to treat pain or swelling caused by osteoarthritis or rheumatoid arthritis. After oral administration of meloxicam, it is absorbed with bioavailability of 89% and has long half-life (22–24 hour). Meloxicam is extensively metabolized to four inactive metabolites by cytochrome P450 (CYP) 2C9 with a minor contribution of CYP3A4. The primary metabolic pathway of meloxicam is oxidation and 5'-carboxy meloxicam has been known for the major metabolite of meloxicam. The aim of the study was to develop and validate a more rapid and sensitive analytical method for simultaneous determination of meloxicam and 5'-carboxy meloxicam based on LC-MS/MS system.

Methods: After addition of 100 μL of 1 M hydrochloride acid, 200 μL aliquot of human plasma was extracted with an internal standard (piroxicam) by simple liquid-liquid extraction using methyl-tert butyl ether. The chromatographic separation of each analyte was achieved on a reversed phase Luna C18 column (50 mm × 2.0 mm i.d., 5 μm particles) in a run time of 3 min, using a mixture of methanol and 10mM ammonium formate buffer (pH3.5) (80:20, v/v) at a flow rate of 200 μL min⁻¹. The tandem mass spectrometry was operated in multiple reaction monitoring acquisition mode using positive ion mode.

Results: This method was linear over the concentration range of 2–3000 ng/mL for meloxicam and 0.5–50 ng/mL for 5'-carboxy meloxicam, respectively. The lower limit of quantifications of meloxicam and 5'-carboxy meloxicam were 2 ng mL⁻¹ and 0.5 ng mL⁻¹, respectively.

Conclusions: The present analytical method showed better sensitivity and reduced running time than a previous study and successfully applied to a pharmacokinetic study after administration of 15 mg oral dose of meloxicam to healthy male subjects.

Novel Aspect: A rapid, simple, sensitive analytical HPLC-MS/MS method for the determination of meloxicam and 5'-carboxy meloxicam in human plasma was developed and successfully validated. Our study showed 4-fold more sensitivity for both analytes and reduced run time, compared to the only study for the simultaneous determination of meloxicam and 5'-carboxy meloxicam.
SIMULTANEOUS DETERMINATION OF TRAMADOL, O-DESMETHYLTRAMADOL, AND N-DESMETHYLTRAMADOL IN HUMAN PLASMA BY LIQUID CHROMATOGRAPHY-TANDEM MASS SPECTROMETRY AND ITS APPLICATION TO A PHARMACOKINETIC STUDY

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Keywords: tramadol; O-demethyltramadol; N-desmethyltramadol; HPLC-MS/MS; Human plasma; Pharmacokinetics

Introduction: Tramadol is a centrally acting analgesic drug and indicated for the treatment of moderate to moderately severe pain. To determine the tramadol and its two main metabolites, O-desmethyltramadol (ODT) and N-desmethyltramadol (NDT), in human plasma, a sensitivity-enhanced LC/MS/MS method was developed in this study.

Methods: A simple, rapid and sensitive high performance liquid chromatography analytical method coupled with tandem mass spectrometry (HPLC-MS/MS) for the simultaneous determination of the tramadol, ODT and NDT was developed and validated. Metoprolol was used as an internal standard (IS) and the analyte was extracted with methyl tert-butyl ether (MTBE). The chromatographic separation of tramadol, ODT, and NDT was achieved on a reversed-phase Luna C18 column (50 mm × 2.0 mm i.d., 5 μm particles), using a mixture of 10 mM ammonium formate buffer (pH 3.5)-methanol (30:70, v/v) at a flow rate of 200 μL/min. The total run-time was 2.5 min and the retention times of tramadol, ODT, NDT and IS were 0.88 min, 0.82 min, 0.90 min, and 0.85 min respectively. The analytes and IS were ionized using a positive ion mode and analyzed using multiple reaction monitoring (MRM) of the transitions at m/z 264.3→58.1 for tramadol, 250.3→58.2 for ODT, 250.3→44.1 for NDT, and 268.2→116.1 for IS.

Results: The weighted (1/x2) calibration curves were linear over plasma concentration range 0.20-400 ng/mL for tramadol (r = 0.999) and 0.25-200 ng/mL for ODT and NDT (r = 0.998 and r = 0.997, respectively). The lower limit of quantifications (LLOQ) of tramadol, ODT, and NDT using 100 μL of human plasma were 0.02 ng/mL, 0.25 ng/mL, and 0.25 ng/mL, respectively. This HPLC-MS/MS method showed improved sensitivity for quantification of tramadol, ODT, and NDT using lower volume of human plasma and reduced running time, compared with previously described analytical Methods.

Conclusions: The method was successfully used to a pharmacokinetic study following the administration of a single 100 mg oral dose of tramadol to healthy male subjects.

Novel Aspect: To best our knowledge, there was only one case report using achiral determination of tramadol, ODT and NDT in human plasma employing HPLC-MS/MS and has lower sensitivity than this method.
Introduction:
In vivo lung perfusion is a novel technique designed to localize chemotherapy to the lung tissue suffering metastasis. Effectively, increased doses of chemo agents can be used while reducing systemic exposure to the individual thus decreasing associated side effects. As there are currently no techniques available to determine drug tissue concentration during IVLP without taking biopsies, SPME emerged as a feasible strategy for in vivo quantification of doxorubicin.

Methods:
In vivo lung perfusion (IVLP) was used to administer doxorubicin for treatment of metastasized lung tumor in a human being. BioSPME fibers were inserted in vivo into three sections of the lung to monitor drug concentration, distribution and effect in lung tissue during IVLP. Liquid chromatography tandem mass spectrometry was used for drug quantification, while high resolution mass spectrometry was employed for untargeted analysis to determine markers of tissue stress or damage.

Results: (Limit 900 characters without spaces)
An administered dose of 5 µg/mL of doxorubicin in perfusate solution resulted in a drug tissue concentration 40 times higher than the administered dose, reaching a concentration of 200 µg/mL within the first hour of IVLP. This increase was marked by a ~50% decrease in the perfusate drug concentration. Subsequent analysis of the perfusate solution as well as the lung tissue at hourly intervals showed a decreasing trend in the concentration in both biological matrices. Untargeted metabolic profiling processed via multivariate analysis using Metaboanalyst 4.0 showed an interesting feature at m/z 166.0864 tentatively identified as L-phenylalanine, an endogenous metabolite that could serve as a possible marker of tissue stress or damage since it is known to be a metabolotoxin at high concentrations.

Conclusions
Targeted analysis for doxorubicin quantitation and metabolic profiling to assess chemotherapeutic effect in lung tissue could be achieved using SPME. A decreasing trend of doxorubicin concentration in perfusate is linked to its continuous accumulation in tissue during IVLP. Furthermore, it is hypothesized that the decreasing doxorubicin concentrations in lung tissue may be due to intracellular uptake of the drug, making it less available to the SPME fibers that extract via free concentration.

Novel Aspect:
For the first time, concurrent semi-quantification of doxorubicin and chemical tissue biopsies in vivo in human lung during surgery can be achieved via SPME.
References


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Assessment of antibody-derived therapeutics at the intact and middle-up level by CESI-MS

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Keywords: CE-MS, CESI, antibody-based pharmaceuticals, nanobodies, proteoforms

Introduction: (Limit of 400 characters)
Antibody-based pharmaceuticals often contain complex structural heterogeneity which requires enhanced analytical methods for reliable characterization of variants and degradation products. In this poster we will describe how CE-MS (CESI-MS which integrates CE and electrospray ionization (ESI) into a single device) in combination with high-resolution MS detection has been used for profiling antibody therapeutics.

Methods: (Limit of 400 characters)
By using a neutral capillary coating (to provide near-zero electroosmotic flow) and an acidic background electrolyte intact model proteins were detected with overall migration-time RSDs below 2.2% (using 3 different capillaries). Samples, including mono- and bivalent nanobodies, and three monoclonal antibodies (mAbs) were tested.

Results: (Limit 900 characters)
Intact nanobodies were resolved from their degradation products, which could be assigned to deamidated, cleaved, and truncated forms at the C-terminal tag with excellent resolution of isomeric deamidated products obtained. The mAbs were analysed intact and after digestion by the IdeSendoproteinase (middle-up approach). CE-MS of intact mAbs resolved clipped species (e.g. light chain and light chain-heavy chain fragments) from the native protein as well as glycoforms containing sialic acids from their non-sialylated counterparts. For IdeS-digested mAbs, F(ab)2 and Fc/2 where efficiently resolved. While migration of Fc/2 fragments were fairly similar for the three mAbs, the migration of the F(ab)2 strongly depended on the mAb. All Fc/2 charged variants, which included glycoforms containing sialic acids and other PTMs such as loss of C-terminal lysine or deamidation of Asn, were nicely separated in less than 20 min. This allowed a detailed and reliable assessment of the Fc/2 heterogeneity (18-33 proteoforms) for the mAbs studied.
DETECTION AND CHARACTERIZATION OF STABLE IMINIUM ION REACTIVE METABOLITE USING HIGH RESOLUTION MASS SPECTROMETRY.

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Keywords: Drug Metabolism, Reactive Metabolites, High Resolution Mass Spectrometry

Introduction: Iminium ion reactive metabolites

Xenobiotics including drugs may be metabolized to form reactive metabolites that covalently bind to protein or DNA. Such bioactivation is suspected to play a causative role in various adverse drug reactions. Thus, early detection of reactive metabolites has become an important part of the drug discovery process. Several tertiary amine-bearing drugs undergo P450- or MAO-catalyzed oxidation to unstable iminium ion species which readily hydrolyze to aldehydes, both of which being potentially reactive. However, due to their inherent chemical instability, the use of trapping agents (e.g. cyanide, methoxyamine) is required to put them into evidence. In this poster, we report unusual iminium ion metabolites which appear sufficiently stable to be directly detected and characterized using liquid chromatography (LC) coupled with high resolution mass spectrometry (HRMS).

Methods: In vitro incubations and metabolites identification

10 µM incubations of compound P, bearing a piperazine moiety, were performed with human liver microsomes in potassium buffer (0.1 M, pH 7.4) for 60 minutes with and without 1 mM solution of potassium cyanide. The reaction was initiated by addition of NADPH after 5 minutes of preincubation and stopped with an equal volume of acetonitrile.

10 µM incubations of compound P were also performed using fresh rat and cryopreserved human hepatocytes in suspension at cell density of 1 million cell/mL. An aliquot of each sample was taken at various time points and reaction was stopped with an equal volume of acetonitrile. After centrifugation at 13000 RPM for 10 minutes, all the samples were transferred to an HPLC vial and submitted to the Q-Exactive HF (ThermoFisher AG, Reinach) LC-MS system. After a preliminary full scan experiment in positive ion mode, representative samples, using a blank matrix without compound as a control, were chosen and then processed using Compound Discoverer 2.0 (ThermoFisher AG, Reinach) to identify the main metabolites. After the selection of molecular ions of interest, production reaction monitoring (PRM) experiments were performed to elaborate the fragmentation pattern of each metabolite. To refine structural elucidation, a deuterium exchange experiment was also performed, in which the number of exchangeable proton present in the molecule were established.

Results: Metabolic Profiling

Metabolic profiles were similar between human microsomes and hepatocytes. No conjugated phase II metabolite was observed whereas parent drug (P) was the most prominent entity detected in either matrices. Several prominent phase I metabolites were also seen. This included the N-dealkylated product M1, which was further oxidized to M2 and M3, the N-oxide M4 and a doubly dehydrogenated product M5. In addition, the dehydrogenated M7 and amide derivatives of M1 (M8 and M9) and of P (M10) were also detected in hepatocytes. In the rat cells, the nitrones derivative of M1 was the main entity detected. Parent drug (P) M1, M2 and M3, M4, M5, M8, M9, M10 were also observed. Based on fragmentation patterns, all biotransformations were assigned to the piperazine ring.
Both M5 and M7 did not exhibit any shift in the deuterium exchange experiment, suggesting that these metabolites were already charged and did not require an ionization proton for MS detection. Metabolite M7 was successfully trapped with the iminium trapping agent cyanide in human liver microsomes, and the concomitant lowering of M5 abundancy was observed.

Conclusions:
The involvement of an intermediary iminium ion metabolite in the metabolic pathway of P was initially suspected from characteristic downstream metabolites of such species, the dealkylated M1 and the amide derivative M10 only seen in hepatocytes, which would be consistent with a commonaldehyde oxidase-mediated detoxification process. This was further substantiated by the successful trapping of the postulated iminium ion in the presence of cyanide. However, the detection and characterization of M7, which appears to be the iminium ion itself, was quite unexpected. The potential conversion to a dimer or an enamine derivative were excluded based on MS and deuterium exchange data, respectively. The exact position of the unsaturated bond on the piperazine ring could not be allocated with more precision. The presence of the pyrazinium metabolite M10 suggested that M7 was also undergoing secondary metabolism in the incubation matrices.

Novel Aspect: Based on these results M7 was assigned as an unusually stable iminium ion species. This metabolite appeared not only stable in solution and during LC-MS analysis, but it could still be detected after several days of sample storage in autosampler. The isolation and further characterization of this iminium ion metabolite by alternative analytical techniques (e.g. NMR) would be required for definitive structural elucidation.

References:
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20 - PHYTOCHEMICAL COMPOSITION OF EXTRACTS FROM WOOD AND LEAVES OF LEBANESE CEDAR (CEDRUS LIBANI) USING GC/MS

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Keywords: Lebanese cedar, extraction methods, Characterization, GC/MS

Introduction: (Limit of 400 characters)
Lebanon Cedar is a native Mediterranean species with a pleasant odor and valuable wood. Despite the efforts devoted to the study of this plant, it remains little characterized from the analytical, technological and biological point of view. The aim of this study is to determine the phytochemical profile of leaves and wood of cedrus libani by using GC/MS and extract all components by maceration, soxhlet and ultrasound-assisted extraction (UAE) techniques.

Methods: (Limit of 400 characters)
Lebanese cedar leaves and wood samples were collected from a natural reserve in Nabatieh, southern Lebanon. These samples were extracted using three different methods: ultrasound assisted extraction, maceration and Soxhlet extraction. Four different extraction solvents were used: hexane, ethyl acetate, acetonitrile and methanol. The extracts thus obtained were analyzed by gas chromatography coupled with mass spectrometry (GC/MS).

Results: (Limit 900 characters)
The results show that among the chemical classes extracted, five are interesting: terpenes, polyphenols, phytosterols, vitamins and cortisol. Hexane appears to be an ideal solvent for the extraction of terpenes. Methanol is an excellent solvent for the recovery of polyphenols. About the phytosterols and vitamins, the four solvents are able to extract them. As for cortisol, only methanol makes it possible to obtain it. On the other hand, ultrasound assisted extraction seems the best method of extracting the five chemical classes of interest. However, Soxhlet extraction due to the high level of hydrocarbons extract does not have the ability to use for the extraction of a very large number of compounds as compared to the other two Methods: For maceration, its weak point is that it cannot extract the cortisol. In addition, several compounds of medical and industrial importance were extracted from lebanese cedar, such as vitamin E, vitamin A1, 24,25-dihydroxyvitamin D3, and phytol.

Conclusions (Limit of 400 characters)
Analysis of these results shows that the ultrasound assisted extraction using methanol solvent of leaves and wood was the best method in order to obtain the five interesting families: terpenes, polyphenols, phytosterols, vitamins and cortisol. The extraction method was optimized and the identification of compound was completed by the GC/MS using the library of NIST and should be completed by LC/HRMS and LC/RMN by putting in evidence its analytical fingerprint.

Novel Aspect: (Limit of 150 characters)
The results illustrate the importance of optimizing the extraction of the principal families present in the leaves and wood of libani cedrus prior to their measurement using GC/MS.
References:

A COMBINATION OF CHEMICAL PROTEOMICS AND DARST-BASED APPROACHES TO DISCLOSE NATURAL PRODUCTS BIOLOGICAL TARGETS.

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Keywords: target identification, affinity based chemical proteomics, DARTS (drug affinity responsive target stability), natural molecules.

Introduction: Disclosing Natural Products Interactome
The identification of the biological and potential therapeutic targets of small molecules, along with their off-targets, is a significant concern. Here, a combination of the affinity-based chemical proteomics (AP-MS) and a simple, universally applicable approach based on the drug affinity responsive target stability (DARTS) has been applied to disclose the interactome of Arzanol, a natural prenylated phloroglucinylpyrone, used in folk medicine to treat infections and inflammatory diseases [1].

Methods: Combining AP-MS and DARST approaches
AP-MS [2] is based on the preparation of an Arzanol bearing solid support, the isolation of its potential targets from a crude cell extract, SDS-PAGE of Arzanol bound targets and their identification by MS. The DARTS approach [3] takes advantage of a reduction in the protease susceptibility of Arzanol targets, easily revealed by SDS-PAGE-LC-MS analysis or by immunoblotting, without Arzanol modification or immobilization.

Results: Brain Glycogen Phosphorylase as a novel Arzanol target.
AP-MS revealed brain glycogen phosphorylase (bGP) as the major target of Arzanol together with the muscle GP isoform and ATP-dependent 6-phosphofructokinases. DARTS experiments followed by both nano-LC-MS and immunoblotting confirmed bGP as a direct interactor of Arzanol. bGP catalyzes the rate-limiting step of glycogen mobilization in brain; its allosteric modulation by AMP, ADP, ATP and phosphorylation, finely tunes its activity in response to different signals. Thanks to AP-MS competition assays, the interaction site of Arzanol on bGP has been disclosed.

Conclusions: Expanding Arzanol potential bioactivity
The natural heterodimeric polyphenol Arzonol is known for its anti-inflammatory and antioxidant properties [4]. Up to now, a full screening of its biological most relevant interactors was still missing and, thus, a combination of chemical proteomics and DARTS experiments has been carried out on this intriguing natural compound, disclosing its interaction with bGP. Brain glycogen metabolism plays a critical role in major brain functions such as learning or memory consolidation: alteration of glycogen metabolism and glycogen accumulation in the brain contributes to neurodegeneration

Novel Aspect: DARST as the ideal approach to support AP-MS data.
In the past, AP-MS has been widely employed to identify targets of chemically reactive drugs. DARTS strategy is an ideal alternative and/or complement to AP-MS working on native unmodified molecules.

References

INVESTIGATION OF AMYLOID-BETA 1-42 REACTIVITY AND STABLE ADDUCT(S) FORMATION BY MASS SPECTROMETRY-BASED APPROACH

Introduction:
Amyloid β-peptide 1-42 (Aβ42) aggregation is a central event in the pathogenesis of Alzheimer’s disease (AD). Carbon monoxide-releasing molecules (CORMs) are able to carry and release controlled amount of CO and are known to exert anti-inflammatory/apoptotic activities [1]. The study focus on the evaluation of the inhibitory properties of the carbon monoxide-releasing molecule 3 (CORM-3) towards Aβ42 aggregation and the investigation of its mechanism of action.

Methods:
Aβ42 samples were prepared as previously reported [2]. CORM3/Aβ42 adduct(s) formation and inhibition of Aβ42 oligomerization were monitored by ESI-Quadrupole-Time of Flight (Q-ToF) flow injection analysis using reserpine as internal standard. Thioflavin T (ThT) assay [3] was used to monitor fibril formation, while circular dichroism (CD) spectroscopy was applied to follow Aβ42 conformational changes [4].

Results:
MS analyses showed that CORM3 can form stable adduct(s) with Aβ42 monomers in a dose- and time-dependent manner. Confirming the evidence reported in a previous study [5], adduct formation involves histidine residues on Aβ42 skeleton and the Ru(II)(CO)2 portion of CORM3. Inhibition of Aβ42 aggregation strongly depends on CORM3/Aβ42 ratio and was confirmed by both ThT assay and CD analysis. The latter offered further insights into the mechanism of action of CORM3, highlighting that CORM3 could prevent the conformational shift toward an amyloidogenic β-sheet rich conformer.

Conclusions:
CORM3 emerged as a promising inhibitory agent toward Aβ42 aggregation. The application of a MS-based method allowed confirming the formation of CORM3/Aβ42 adduct(s) and highlighted CORM3 inhibitory activity. Combination of MS and spectroscopic data, such as fluorescence and CD, shed light on the mechanism of action of this compound. Furthermore, MS and ThT studies quantitatively contributed in identifying optimal CORM3/Aβ42 ratio for best inhibitory effect.

Novel Aspect:
The work highlighted CORM3 is endowed with a good inhibition capacity toward Aβ42 aggregation, in addition to known anti-inflammatory properties.

References
Introduction
Focal adhesion kinase (FAK) is a non-receptor tyrosine that regulates migration, proliferation and survival of cells. A large number of studies have strongly implicated FAK in the development of colon cancer. The novel potential functions of FAK in the regulation of epithelial-mesenchymal transition (EMT) has been studied extensively in invasive and metastatic cancers. The study focuses on new binding partners of FAK as therapeutic targets for colon cancer.

Methods
Human colon cancer HCT-116 was cultured, harvested and lysed. The protein concentration was measured by BCA kit. FAK expression was confirmed by Western blot. FAK and binding partners were captured by immunoprecipitation method and separated by SDS-PAGE. Gels were excised and digested with trypsin. Tryptic peptides were injected to nano-LC/MSMS system and data were searched against the Mascot search engine. The binding candidates were analyzed with Western blot.

Results
FAK was expressed highly in colon cancer cell HCT-116 compared to the other cancers and normal colon cell. Immunoprecipitation of FAK protein was optimized in the manner of bead selection, cell lysate amount, antibody amount, washing conditions, and elution volume. Gel-LC/MSMS data and mascot search revealed about 350 protein identity. FAK was found with the highest score indicating the successful pull-down and digestion. Among the matched proteins, reportedly known binding partners of FAK include growth factor receptor-bound protein 14 GRB14, intergrin-linked protein kinase ILK, Ras-related protein RAB-22A, Rho-related GTP-binding protein RhoU. New candidates for FAK binding partners proposed in our result include SACS, sacsin; SYNE3, nesprin; ZYX, zyxin; PLK1, serine/threonine protein. The new binding partner candidates were confirmed using Western blot.

Conclusions
The further work include the effect of growth factors, FAK inhibitors on colon cancer cells to elucidate the upstream regulators or downstream regulators involving the FAK signaling pathways. New binding partners of FAK once validated and characterized will be potential biomarkers for colon cancer diagnosis and useful for the development of therapeutic targets.

Novel Aspect
The literatures have not shown evidences for interaction between new binding candidates of FAK. Necessary confirmation would be novel for therapeutic target development.

References
460 - COMPREHENSIVE N-GLYCAN STRUCTURE CATALOGUE OF THERAPEUTIC ANTIBODIES

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Keywords: monoclonal antibody, N-glycan, reference, mass spectrometry

Introduction:
Glycosylation of monoclonal antibody (mAb) therapeutics plays vital roles in drug’s biological activity and safety. Monitoring of glycosylation affected by various environment conditions is highly required to access QA/QC of mAbs. Here, we developed a LC/MS based N-glycan library for rapid and high-throughput identification in various mAbs.

Methods:
The N-glycan catalogue was constructed using human immunoglobulin G and five different mAb therapeutics produced by various cell-based expression systems. Reduced native N-glycans were separated and analyzed by porous graphitized carbon (PGC)-chip nano-LC/MS. Accurate masses were used to compositionally profile N-glycans and their structures were further elucidated by CID MS/MS. Major glycans (G0F, G1F, and G2F) on mAbs were quantified by UHPLC-HILIC-FLD.

Results:
Representative commercial mAbs (adalimumab, bevacizumab, infliximab, rituximab, and trastzumab) produced by CHO cell and Sp2/O cell were selected to construct detailed N-glycan catalogue for rapid characterization. In parallel, human IgG was used as the referenceto confirm human-like glycan structures. The majority of the N-glycans from five commercial mAbs were characterized by matching the RT and accurate masses with the structure of glycans found in a human IgG reference. Corresponding compositions were further confirmed using structural connectivity of the individual monosaccharide residues by tandem MS. In particular, non-human glycans found in non-human mammalian cell system were newly identified by accurate masses, RTs, and tandem MS. Antibody N-glycan catalogue containing over 70 distinct glycan compositions provides structures, relative quantity, and chromatographic retention time (RT) based on PGC column.

Conclusions:
We created the N-glycan catalogue for rapid identification of glycans in mAbs. The combination of accurate masses and LC retention times enables real-time identification for glycan structures. Product ions of each N-glycan by tandem MS were also recorded in the catalogue. N-glycan catalogue of mAbs can be used as a valuable reference in order to evaluate mAb variants and/or biosimilars for both developmental and regulatory purposes.

Novel Aspect:
Comprehensive N-glycan catalogue makes it possible to identify their structures in a high-throughput manner by matching LC retention times and accurate masses.
Introduction:
Sequence variants (SVs) are unintentional amino acid substitutions and contribute to the overall heterogeneity of biotherapeutics. The presence of these replacements can be at differing levels, dependent on the cause of the variant, making identification difficult. Here, we monitor the low level SVs using a highly sensitive, high resolution mass spectrometer, the SCIEX TripleTOF® 6600, coupled to built-for-purpose BioPharmaView™.

Methods:
NIST mAb reference standard (#RM8671) was digested using standard conditions. First, digested NIST was run using a 3 hour gradient on an ExionLC™ AD system coupled to a TripleTOF® 6600 and data was acquired in DDA mode. Data was searched using ProteinPilot™ including a search for SVs. Subsequently NIST was acquired using SWATH® acquisition. The data was processed in BioPharmaView™ software to identify and relatively quantify the sequence variants.

Results:
NIST mAb was digested and run using a 3 hour reversed-phase gradient in data-dependent acquisition mode. The data was searched with ProteinPilot™ with an emphasis on IgG antibody and SV identification in order to look for variants. Once the SVs were identified, these sequences were input into BioPharmaView™ as impurities to monitor with SWATH® acquisition on a shorter gradient. Using the peptide mapping workflow including MAM analysis in BioPharmaView™, SVs can be identified and also quantified based on the non-variant peptide despite the shorter gradient and their low abundance. The data-independent SWATH® acquisition ensures that MS/MS data is available on the whole mass range throughout the gradient.

SVA requires a mass spectrometer with a high linear dynamic range, high sensitivity and speed to ensure that the variants are seen at MS level and also fragmented resulting in a good quality MSMS data.

Conclusions
SVs contribute to the overall heterogeneity of biologics and are a measure of product, and production stability along with potential detrimental effects on physicochemical properties, tertiary structure, and efficacy of the product. Misfolded proteoform variants may be toxic impurities. The ability to detect these variants at low level assists manufacturers in determining the potential consequences of the variant and differences from the expected product.

Novel Aspect:
Sequence variant analysis using MAM workflow.
**142 - EPITOPE PEPTIDES IDENTIFIED BY ONLINE BIOSENSOR-MS NEUTRALIZE PATHOPHYSIOLOGICAL ANTIBODIES AND OPEN NEW CLINICAL THERAPY APPROACHES FOR LYSOSOMAL DISEASES**

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Epitope peptides identified by online biosensor-MS neutralize pathophysiological antibodies and open new clinical therapy approaches for lysosomal diseases

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Keywords
Biosensor-MS combination; Antibody epitope identification; Lysosomal diseases; Enzyme replacement therapy; Neutralizing epitope peptides.

Introduction
Epitope analysis is a key step in the development of therapeutic antibodies. Lysosomal Storage Diseases (LSDs) are successfully treated by Enzyme Replacement Therapy (ERT); however substantial, life threatening effects arise from antibodies that block the infused enzyme. A new online proteolytic excision SPRMS combination (PROTEX-MS) enables simultaneous structure identification and affinity quantification of antibody epitopes. Here we report a new therapeutic concept by identification of antibody epitopes upon ERT. Synthetic epitopes against pathophysiological antibodies open new clinical therapy approaches by (i), hyposensitization of patients, and (ii) molecular apheresis to deplete antibodies.

Methods
Epitopes from antisera of Fabry disease (FD; α-Galactosidase deficiency) patients were identified using the PROTEX-SPR-MS combination. Tryptic peptide mixtures were loaded onto the SPR-MS; after washing out nonbinding peptides, epitope peptides were eluted with 0.01% TFA into the affinity interface, ESI-MS was performed with a Waters QuattroUltima MS. The epitope peptide, α-Gal(309-332) was identified from 2 FD patients and revealed high affinity (KD, 39 nM) comparable to the full length enzyme (KD, 16 nM). Linear, stabilized epitope peptides were prepared by SPPS.

Results
Key tools of the online PROTEX SPR-MS Epitope Analyzer are (i), a new SPR suitable for direct application of ESI-MS without any buffer change (T-SPR); (ii), a new high pressure proteolytic system with substantially enhanced...
efficiency of protease digestion. The PROTEX SPR-MS analyzer was applied to the epitope elucidation and affinity characterization of antibodies upon ERT in FD patients, and provided the epitope, αGal(309-332), in which the lysine residues K-315 and 326 were shielded upon antibody binding. The epitope αGal(309-332) was synthesized by solid phase peptide synthesis (SPPS) and purified by HPLC on a semipreparative C4-column, yielding homogeneous peptide with >95 % purity. SPR determination of the epitope peptide and the full length enzyme provided high affinities (KD, 39 and 16 nM, respectively). The αGal(309-332) epitope contained two internal lysine residues that were not cleaved upon proteolytic epitope excision, consistent with the shielding upon antibody binding.

Conclusions
The clinical application potential of the PROTEX SPRMS Epitope Analyzer was shown in epitope elucidation from therapeutic and pathophysiological antibodies of lysosomal enzymes, with affinity constants (KD) amenable from milli- to nanomolar ranges. Epitope peptides in FD patients are currently developed for antibody neutralization in clinical ERT by systemic application and apheresis at physiological conditions. Thus, the identification, chemical synthesis, and biochemical evaluation of antibody epitopes open provide new clinical treatment approaches for reversing the immunogenicity and reconstituting the therapeutic efficacy of ERT in lysosomal diseases.

Novel Aspect
Antibody epitope identification in ERT of lysosomal diseases opens new clinical therapy to neutralize antibodies and reconstitute therapeutic efficiency.

Introduction:
Pharmaceutical companies always try to reduce the time spent on Drug Discovery to push forward compounds of interest into Clinical Development. To achieve the pre-clinical phase, Analysts need to provide the right and critical physicochemical data to the chemists.

Thanks to hyphenated techniques, we have developed in house-procedures and methods to test the stability of compounds, only looking for information which are relevant for the developability of a compound.

Methods:
Depending on the compounds to be tested, we develop methods in SFC/MS for the chiral stability if compounds are pure enantiomers, after a screening to define the best separation. Chemical stability is monitored in UHPLC/MS either in LR or HRMS. If a compound is found unstable, High-Resolution equipment allows the identification of degradation compounds.

Our protocols are set up after discussion between chemists and analysts depending on the information they need.

Results:
The chemical stabilities are monitored in UHPLC/UV/MS, on a UPLC/UV/MS (Quattro-micro) or a UHPLC/UV/MS (Q-Exactive) instruments, UV results are reported in tables and peak areas are compared to areas obtained at the beginning of the study, this is useful to discriminate precipitation and degradation. If degradation occurs, MS data are examined to give information about the degradation compounds to the chemists, if needed, HRMS data (full MS and after fragmentation) can provide structural information.

For the chiral stability, the same solutions are run in SFC/MS. SFC/UV having a low sensitivity, solutions are analyzed in MS in SIM (Selected Monitoring ions) mode. Most of the time, this technique allows to detect less than 1% of distomer but to improve the sensitivity on poorly ionizable compounds or to discard salts coming from the highly charged buffers a step of Solid Phase Extraction (SPE) on cartridges can be added.

Conclusions
To push forward or discard compounds showing good or bad pharmaceutical potencies, the combination of different hyphenated techniques available in our laboratory helps the analytical chemists to fully describe the stability of a compound, depending on the product itself and the information needed by the chemists.

Novel Aspect:
The combination of MS coupled to different chromatographic techniques are useful to obtain critical physicochemical data of pharmaceutical compounds.
A Highly Selective and Sensitive LC-MS/HRMS Assay for Coproporphyrin-I and –III, Emerging Endogenous Biomarkers of OATP, in First-in-Human Clinical Trials

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Keywords: High Resolution Mass Spectrometry (HRMS), Transporter Biomarkers, Organic Anion Transporting Polypeptides (OATP), Coproporphyrins, Drug-Drug-Interactions (DDI)

Introduction (Limit of 400 characters): The evaluation of transporter biomarkers in early clinical trials could help identify NCE candidates with true drug-drug interaction (DDI) liabilities and thus reduce the number of costly clinical DDI studies being conducted. Coproporphyrin-I and Coproporphyrin–III are two such endogenous isomeric compounds which have emerged as potentially viable biomarkers of organic anion transporting polypeptide (OATP) mediated DDIs.

Methods (Limit of 400 characters): A UHPLC-MS/HRMS assay was developed using, previously unreported, CP-I/CP-III doubly charged ions as precursor ions to improve the assay sensitivity over the singly charged precursor ions. The TOF-MS/MS selectivity of the assay was improved by monitoring and post-acquisition summing the responses from the major doubly charged product ions. The LC-MS/HRMS assay, developed and qualified, supported the quantitative determination of CP-I/CP-III in human plasma.

Results (900 characters): In this study, we used both singly- ([M+H]+) and doubly-charged ([M+2H]2+) precursor ions of CP-I/CP-III respectively at m/z 655.3 and 328.1 to evaluate high-resolution MS (TOF-MS) for untargeted and MS/HRMS for targeted quantification data on a high-speed Triple TOF 6600 LC-MS/MS system. For the first time, we were able to demonstrate that the doubly-charged ([M+2H]2+) precursor ions of CP-I/CP-III are present and detectable with 5-10 times more intensity. Calibration and quality control samples were prepared to cover a range of 50-1000 pg/mL in 3-times charcoal stripped plasma, and parallelism to native plasma established. The LC-MS/HRMS assay, developed and qualified, supported the quantitative determination of CP-I/CP-III in human plasma with an assay range of 0.05-10 ng/mL. In control pooled human plasma, levels of CP-I and CP-III ranged from 0.45 and 1.1 ng/mL and from 50 and 500 pg/mL, respectively. In control pooled human urine, levels of CPs were at least 10-fold higher than those detected in plasma.

Conclusions (Limit of 400 characters): The NCE in this study had been shown to inhibit OATP in vitro and the probe drug DDI study was incorporated into the clinical study. At the highest dose of the NCE, CP-I showed a 1.37-fold increase in plasma AUC0-48hr versus placebo, which was comparable to the plasma AUC0-48hr ratio change observed with atorvastatin (1.62). These results support that UHPLC-MS/HRMS assay is sensitive and selective to measure endogenous biomarkers of DDIs.

Novel Aspect (Limit of 150 characters): Clinical application of UHPLC-MS/HRMS assay for endogenous OATP biomarkers, CP-I and CP-III, and correlation of AUC0-48hr ratio changes with those from atorvastatin.
Introduction:
Monoclonal antibodies (mABs) and antibody drug conjugates (ADCs) are a fast growing class of therapeutics. ADCs and mABs are complex heterogeneous populations of molecules. The NIST IgG mAB was used to validate our Agilent LC-nanoESI-CHIP-MS system performance. We additionally monitored the structural stability and degradation products of several intact and reduced forms of the Infliximab, Panitumumab, Rituximab, Kadcyla (ADC) and others.

Methods:
For de-salting Amicon ultra 100 kDa spin filters, reduction with 4.4 molar eq. TCEP. For LC-MS Agilent CHIP cube on Agilent 6520, sample captured (0.2 µL inj on 40 nL Zorbax 300SB-C8, 5 µm) using 4µL/min H2O + 0.1 % formic acid (FA), then eluted off trap column onto the analytical column (43 mm x 75 µM with Zorbax 300SB-C8, 5 µm) using ACN:H2O 90:10 with 0.1% FA. Processing with Agilent MassHunterMaxEnt deconvolution with mass using NIST Mass and Fragment Calculator, v1.32 [1].

Results:
Evaluation of the system using the NIST IgG showed expected glycoforms for the intact protein [2]. Overall the mass accuracy for both intact and reduced mABs and ADC were within acceptable ranges (-12.1 and 20 ppm, respectively). For the Kadcyla ADC heterogeneous light chain D0, D1 and D2 proteins were shown with distinctive 957 Da emtansine payloads [3, 4]. In cases where TCEP was used to reduced mABs, partial or uncompleted reduction of disulfides was observed. This could have been due to potential steric hindrance of TCEP and inaccessibility of sequestered hydrophobic disulfides [5]. The inter-month mass stability and accurate mass reproducibility of an ADC suggested experiments should be run alongside controls for comparison. For ADCs under alkaline conditions and elevated temperatures the drug payload distribution was shown to be affected.

Conclusions
LC-nanoESI-MS is a useful analysis method, amongst other orthogonal techniques, to determine heterogeneous distributions of intact (non-reduced) and reduced forms of both mABs and ADCs.

Novel Aspect:
LC-nanoESI-CHIP-MS system was suitable for intact and reduced analysis of mABs and ADCs and able to monitor drug payload stability on ADCs under accelerated stress conditions.

References
Development of a Hybrid LC-MS/MS Method for the Quantification of Antibody-Conjugated Payload in In-vivo and In-vitro ADC Samples

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Keywords: Hybrid Method, LC-MS/MS, ADC, Antibody Conjugated Payload, Bioanalysis, NBE

Introduction
Antibody Drug Conjugates (ADC) are a novel class of targeted therapeutics that consist of a potent cytotoxic drug (payload) covalently bound to a monoclonal antibody (mAb) through a cleavable or non-cleavable linker [1,2]. ADCs can selectively deliver potent drugs through an antigen-antibody recognition mechanism. This step is followed by ADC-receptor complex internalization, enzymatic or chemical cleavage of the linker or protease degradation of Ab to intracellularly release the drug. The goal of ADCs is to improve therapeutic window by avoiding limitations related to classical antibody (Ab) based therapeutics or chemotherapy that uses potent cytotoxic small molecules.

Due to their multi-component structure and inherently heterogenous nature, both antibody related analytes (total antibody, conjugated antibody) as well as small molecule related analytes (free payload, antibody-conjugated payload and total payload) are needed to understand ADC disposition in vivo. The bioanalytical assays to support ADC pharmacokinetics studies thus often require a combination of antibody (ligand binding assay, LBA) and small molecule analysis (LC-MS/MS) platforms.

Antibody-conjugated payload analyte describes the payload that is still conjugated to the antibody. Recently, there is a growing attention in Ab-conjugated payload determination to better define the ADC PK profiles [3,4,5].

Methods
A specific LC-MS/MS method was established and qualified for Ab-conjugated payload analyte for an ADC bearing a payload covalently bound through a cleavable linker. The method consists of an immunocapture of the ADC on a spin plate containing Protein A that selectively binds IgG antibodies. Subsequently a hydrolysis step was applied to the immobilized ADC on the plate to generate free payload. Eluates form the plate were subjected to a protein precipitation technique (PPT) step and supernatants are finally analyzed by LC-MS/MS to quantify the free toxin.

Results
The analytical method was qualified for linearity, accuracy, precision, selectivity, carryover, stability (freeze/thaw, benchtop, autosampler), and for dilution range.
To verify the reliability of the obtained results, a set of samples previously tested using conjugated payload method, were assayed by an orthogonal conjugated antibody assay, successfully qualified. This protocol, exploiting Meso Scale Discovery (MSD) technology, was developed and qualified to quantify ADC molecules in animal species (mouse, rat and cynomolgus plasma). A goat human IgG-heavy and light chain, monkey-adsorbed biotin labeled antibody was used as a capture antibody and a Sulfo-tag labeled monoclonal antibody against the toxin (was used as detection. The biotin labeled ADC target receptor was used as capture reagent instead of the goat anti human IgG to quantify the ADC in human plasma.

Conclusions
A good agreement between LBA conjugated Ab and LC-MS/MS conjugated payload results was found confirming the reliability of the two assays. The conjugated payload method was applied to support in-vitro (plasma stability studies) and in-vivo (PK) studies.
Novel Aspect
A novel hybrid LC-MS/MS method was developed and applied for the determination of Antibody Conjugated Payload in ADC bioanalysis. The procedure consists of an immunocapture step, digestion and detection of free toxin using an LC-MS/MS method.

References

For information please contact: scientific@ismc2018.it
Introduction:
Tuberculosis (TB) is the first cause of death from infectious diseases worldwide. Only a single anti-TB vaccine is currently available for clinical use, but its efficacy is not achieved with certainty [1]. The aim of this work is to provide a basis for the rational design of a neo-glycoconjugate vaccine against TB.

Methods:
All proteins, glycoconjugates and antibodies used in this study were characterized by MALDI-TOF MS. The antibody epitope of recombinant Ag85B antigen was determined by proteolytic extraction-MS. Antibodies from sera of TB-patients, vaccinated subjects and a healthy control were employed, together with a commercial monoclonal antibody. Affinity binding constants were defined by SPR biosensor analysis.

Results:
The MS epitope determination and SPR analysis allowed the identification and characterization of the interactions between Ag85B antigenic protein from Mycobacterium tuberculosis (MTB) and antibodies from different sources, both qualitatively and quantitatively. It was possible to use human clinical samples to determine the Ag85B epitope. The identification of the same binding areas of the protein interacting with a monoclonal antibody and with antibodies from human sera allowed to validate the simplified monoclonal model, confirming its applicability in SPR experiments. Therefore, an alternative screening method for the immunological evaluation of antigenic proteins from MTB has been developed. With this in vitro approach, the effects of the introduction of conservative mutations in Ag85B protein, as well as Ag85B conjugation with saccharidic moieties, were studied.

Conclusions:
The combination of two analytical approaches yielded the identification of different assembled epitope regions on recombinant MTB antigens, their affinity binding constants in the interactions with specific antibodies and revealed the importance of protection from excessive glycosylation. These findings provide a molecular basis for an improved rational design of a new potential glycoconjugate vaccine against TB.

Novel Aspect:
A qualitative and quantitative investigation of antigen-antibody interactions was performed to characterize a potential vaccine against TB.

References
Introduction
The real-time monitoring of the concentration of doxorubicin (DOX) in lung tissue aims to enhance the effectiveness of applied chemotherapy while reducing severe side effects [1]. To precisely assess the level and biodistribution of DOX, in vivo solid-phase microextraction (SPME) has been introduced as minimally invasive technology that integrates sampling, extraction and sample clean-up, and allows for repeated sampling of different tissue compartments [2-4].

Methods
In optimized SPME conditions, sterile 15 mm (45 µm thickness) mixed-mode fibers underwent preconditioning in acetonitrile/water mixture (80:20, v/v) for 60 min. Next, the fibers were directly introduced into lung tissue for 20 min extraction of DOX under static conditions, followed by a desorption step in acetonitrile/water (80:20, v/v) with 0.1% formic acid for 60 min and finally the obtained extract was subjected to LC-MS/MS analysis.

Results
In SPME method development, several parameters that could influence the extraction efficiency of DOX (e.g., selection of optimal preconditioning and desorption solvents, fiber sterilization) were evaluated. SPME extraction in lung tissue reached equilibrium in 20 min. The entire procedure was performed without internal standard correction, enabling the methodology to be implemented in in vivo SPME sampling. Under optimized conditions, SPME exhibited excellent linearity (R² ≥0.99) within the range of 2.5-50 µg/g, a LOQ of 2.5 µg/g, good precision (RSD<14.7%) and accuracy (±103.2). Relatively short sampling time ensures that alterations in the level of DOX can be monitored during chemotherapy, and that a near-real-time profile of drug biodistribution in the lung tissue can be provided. Due to the minimal invasiveness of SPME probes to the living system, sampling can be performed several times in different tissue compartments during the treatment of cancer metastases in lungs with the use of in vivo lung perfusion (IVLP) [5].

Conclusions
An analytical system based on SPME sampling coupled to LC-MS/MS method was developed to accurately and precisely quantitate the level of the anticancer drug, DOX, by direct sampling of lungs. The optimized SPME method facilitates near to real-time monitoring of the concentration and biodistribution of DOX in the lung tissue of a living organism (in vivo SPME) during IVLP in the operating room.

Novel Aspect
In vivo SPME method for repeated sampling of DOX under equilibrium conditions without internal standard correction was developed to monitor drug level in different lung areas.
References

MONITORING OF THERAPEUTIC DOSES OF ANTIPSYCHOTIC DRUGS IN SALIVA AND SERUM SAMPLES WITH THE USE OF SOLID-PHASE MICROEXTRACTION TECHNIQUE.

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Keywords: solid-phase microextraction, atypical antipsychotic drugs, therapeutic drug monitoring, LC-MS/MS

Introduction
The analysis of biological fluidsto directly control the psychoactive substances is especially important in patients vulnerable to drug overdosing, e.g. pediatric and geriatric patients [1]. To provide fast and reliable information about the drug concentration, solid-phase microextraction (SPME) is introduced as a high-throughput sample preparation technique that facilitates accurate quantitation of the level of drugs in biological matrices [2,3].

Methods
Under optimized SPME conditions, the extraction of atypical antipsychotic drugs (risperidone, olanzapine, clozapine, quetiapine and aripiprazole) and internal standard (promethazine) from saliva and serum samples was performed with the use of C18 blades for 60 min at 1000 rpm. Then SPME blades were rinsed with nanopure water for 10 sec, and next desorption was performed for 10 min at 1000 rpm. Finally obtained extracts were analyzed with the use of LC-MS/MS system.

Results
SPME conditions for the analysis of antipsychotic drugs in biofluids were optimized in order to select the most effective extraction phase, and finally C18 blades were chosen, as they provided good recoveries and the lowest carryover effect. Desorption of the analytes was performed in optimal desorption mixture (ACN/H2O/FA, 80:20:0.1, v/v/v) and reached equilibrium within only 10 min. The obtained kinetic profile of antipsychotic drugs in saliva samples was compared to the kinetic profiles of these drugs obtained for serum samples. Although the level of antipsychotics in saliva samples was much lower (ng/mL) in comparison to the concentration of drugs measured in serum samples (µg/mL), the kinetic profile of analyzed compounds was similar. Therefore, SPME may be implemented for direct monitoring of the level of antipsychotic drugs in non-invasively collected saliva samples and can be used as alternative to traditional methods utilizing serum samples for the measurements of these drugs.

Conclusions
High-throughput method based on SPME technique coupled to LC-MS/MS was developed for quantitative analysis of five antipsychotics in human saliva and serum samples. The optimized SPME method facilitates non-invasive monitoring of analyzed drugs present at low level in saliva samples, without the need for blood collection. SPME allows for a control of drug intake and dose modification, and may be a part of personalized therapy during long-term treatment.

Novel Aspect
SPME technique for direct monitoring of therapeutic level of atypical antipsychotic drugs in saliva and serum samples was developed.

References
MULTI-ATTRIBUTE MONITORING (MAM) TO IDENTIFY DIFFERENCES IN TRASTUZUMAB FROM 2 MANUFACTURERS.

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Keywords: Mass Spectrometry, Biologics, Trastuzumab, MAM, Biosimilars

Introduction:
Biosimilars are a growing industry, which require comparison of the safety and efficacy of the new biotherapeutic to the original. Certain CQA’s, such as high mannose content glycans, can have a detrimental effect on the function of the product. Using trastuzumab from two different manufacturers, a comparison of the biosimilars was performed, acquiring data using SWATH® and processing using BioPharmaView™ MAM workflow to identify the levels of change in the sample.

Methods:
Peptide digests of trastuzumab from two manufacturers, were run using an ExionLC™ AD with a Phenomenex®Kinetex® column C18, 100A, 1.7 µm, 2.1 x 150 mm at 60°C. A: 0.1 % formic acid in water, B: 0.1% formic acid in acetonitrile using a linear gradient of 5 to 40 over 120 min at a flow rate of 0.2 mL/min using SWATH acquisition.

Trastuzumab (intact and reduced) were acquired using a 20 min gradient.

All data was processed using BioPharmaView MAM and intact workflow.

Results:
Trastuzumab peptide mapping was acquired on the X500B using SWATH acquisition. The data was processed in BioPharmaView with the glycosylations setup as attributes for monitoring and the subsequent glycosylation percentages were calculated and compared against each other for the two manufacturers versions of trastuzumab. Differences in the glycosylations were identified at the peptide level. The same samples were acquired as intact protein and the levels of glycosylations and the variances were compared to the data acquired in peptide mapping. The result was a confirmation that some levels of glycans differed between the two samples showing how the workflow can be used to monitor for changes in biologic in production or can be used to compare biosimilar to the original biotherapeutic.

Conclusions
With the increasing number of biosimilars under development; robust, reliable workflows are required to fully characterize any Critical Quality Attributes which may differ from the originator. Certain process-specific post-translational may have a negative effect on the efficacy and clearance of the therapeutic therefore a Multiple Attribute Method allows monitoring of these in a single workflow.

Novel Aspect:
Comparison of 2 biosimilar trastuzumab using multi-attribute monitoring.

For information please contact: scientific@imsc2018.it
Introduction: ADME genes (ADME: absorption, distribution, metabolism, and excretion) encode for the involved phase I-II enzymes and transporters, and they respond adaptively to xenobiotic exposure. To predict in vivo drug response in ADME proteins we applied a Parallel Reaction Monitoring (PRM)-based targeted proteomics approach to quantify the proteins in 3D human liver spheroids challenged with different dose regimes of several hepatotoxicants up to two weeks of treatment.

Methods: After isolation from drug-treated 3D liver spheroids, proteins were digested and analyzed by Liquid Chromatography - Mass Spectrometry (LC-MS) on a Q Exactive™ HF instrument (Thermofisher Scientific) in PRM mode. Raw MS data were directly imported into Skyline for peptide/protein quantification. Since ADME proteins are structurally represented in PBPK models the measured changes in protein abundance may be directly integrated in the corresponding models.

Results: To design targeted PRM assays for the ADME proteins, we focused mainly on the ADME genes that are involved in the pharmacokinetics of the tested liver toxicants. MS coordinates such as precursor and retention time information were selected from spectral libraries built from MS/MS spectra acquired by data-dependent analysis experiments of protein digests isolated from pooled drug-treated 3D liver spheroids. In total, dozens of PRM assays were developed and optimized for the detection of the ADME proteins. Overall, we could identify and quantify with high confidence the different ADME proteins with either two or three proteotypic peptides across all the drug-treated 3D liver spheroids. We demonstrated high reproducibility in protein abundance between biological replicates across all time points and dose regimes. We showed that the abundance profile of ADME proteins were drug and dosage regime specific. The observed changes were integrated in the various PBPK models to describe cellular adaptation in response to xenobiotic exposure.

Conclusions: This allowed in particular to simulate the to-be-expected effect of multiple drug administration on drug pharmacokinetic over time. As such the presented approach presents an important tool to predict in vivo drug response in ADME proteins after multiple administration in a joint approach of model-based assay design and targeted proteomics.

Novel Aspect: We report quantitative and qualitative changes of ADME proteins in drug-treated 3D human liver spheroids by label-free PRM assays.
471 - LC-MS/MS ANALYSIS AND RAT PHARMACOKINETICS OF THE NOVEL CYCLIN-DEPENDENT KINASE INHIBITORS BP-14 AND BP-20

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Keywords: cyclin-dependent kinase inhibitor; BP-14; BP-20; pharmacokinetics; tissue distribution

Introduction:
BP-14 and BP-20 are novel cyclin-dependent kinase inhibitors, structurally related to roscovitine, with potent antiproliferative activity [1]. A simple LC-MS/MS method for determining them in biological material was developed and validated following EMA guidelines. The method was applied in a pharmacokinetic study in rats following i.v. and i.d. administration including plasma pharmacokinetics, tissue distribution and excretion (renal and biliary).

Methods:
Chromatographic separation was performed on reversed phase BEH C18 column (100 x 2.1 mm, 1.7 µm) by gradient elution with mobile phases composed of 15 mM ammonium formate pH 4.0 and methanol at flow rate 0.25 mL/min at 40 °C. The analytes were detected based on their multiple reaction monitoring transitions in positive electrospray ionisation mode m/z 473.07 > 157.93 for BP-14, m/z 499.62 > 184.2 for BP-20 and m/z 355.5 > 90.86 for roscovitine (internal standard).

Results:
The method provided good linearity within concentrations 1 - 10000 nmol/L for BP-14 and 10 - 25000 nmol/L for BP-20 in plasma and 100 - 10000 nmol/L for BP-14 and 10 - 25000 nmol/L for BP-20 in bile and urine (R2>0.9989). The results of validation fit within the EMA acceptance limits in terms of within-run and between-run accuracy and precision. Both compounds were stable in a set of stability tests in matrices (plasma, bile and urine). Plasma concentrations of both compounds after i.v. administration followed a typical bi-exponential decline, consisting of a rapid distribution and slow elimination phase. The bioavailability after i.d. administration was determined 0.63% for BP-14 and 1.58% for BP-20. The distribution into tissues (adipose tissue, kidney, lungs, spleen, liver, muscle, brain) was the highest for the adipose tissue and the lowest for brain. The biliary excretion of the parent BP-14 and BP-20 accounted for 4.81% and 10.6% of the doses, respectively, and renal excretion for less than 0.5% in both cases.

Conclusions:
Similar pharmacokinetic profiles of both compounds were obtained after i.v. administration, with a rapid initial decrease in plasma concentrations, intensive distribution to adipose tissue and approximately 5-10% excretion of the parent drugs through urine and bile. Their bioavailability after i.d. administration was low.

Novel Aspect:
The results represent first information on pharmacokinetics of BP-14 and BP-20 in rats and will help to design a new generation of drugs with strong anticancer activity.

References:
Identification of 5-Fluorocytosine and Its Metabolites in Complex Samples by Liquid Chromatography Coupled with Mass Spectrometry.

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Keywords: mass spectrometry, gene therapy, chemotherapeutics, metabolites, complex samples

Introduction

5-fluorocytosine (5FC) is an anti-mycoticum which can be used as precursor for anti-cancer drug 5-fluorouracil (5FU) in cancer treatment by the procedure "suicide gene therapy" [1]. In recent years, the use of high performance liquid chromatography (HPLC) coupled with mass spectrometry (MS) is dominated for purpose of identification and determination of 5FC and its metabolites in complex samples [2], [3].

Methods

All results were obtained by high resolution LC-MS-IT-TOF mass spectrometer (Shimadzu, Kyoto, Japan) with electrospray ionization. Chromatographic separation was performed on Ascentis C18 (100x2.1 mm; 3 μm) column (Sigma-Aldrich). The samples for analysis was prepared and delivered by the Laboratory of Molecular Oncology.

Results

The developed HPLC-ESI-IT-TOF-MS method was applied to the analysis of samples of mesenchymal stem cells, tumor cells and their control samples to which 5FC was not added. In spectra obtained by HPLC-ESI-IT-TOF-MS analysis of samples (media of tumor or mesenchymal stem cells) were present molecular ions which corresponding to the 5FC in positive ionization mode and to the 5FU and 5-fluoro-2-deoxyuridine monophosphate (5FdUMP) in negative ionization mode. The presence of 5FC's metabolites was conditional upon to presence genes which encoding excretion of enzymes cytosine deaminase (CD) or uracil phosphoribosyltransferase (UPRT). In spectra obtained from analysis of sample without addition of gene CD or CD/UPRT were present only molecular ions corresponding to 5FC and none molecular ions which corresponding to its metabolites in negative ionization mode.

Conclusions

The paper dealt with the possibilities of high performance liquid chromatography coupled with mass spectrometry for identification of 5-fluorocytosine and its major metabolites in biological samples. Developed HPLC-ESI-IT-TOF-MS method was successfully applied for analysis of mesenchymal stem cells, tumor cells and their control samples.

Acknowledgements

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Novel Aspect

Obtained results can bring new look on the treatment efficiency of metabolic conversion 5FC during suicide gene therapy.

References

Efficient dereplication with high resolution high accuracy mass spectrometry for the discovery of novel bioactive natural products

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Abstract

Introduction: Antimicrobial resistance poses a serious threat worldwide and new antibiotics are urgently required. Meanwhile, the pace of discovery of novel antibiotics has slowed down significantly recently due to the frequent rediscovery of known compounds. Microbial genome mining as a new approach can potentially rejuvenate the stalled antibiotics discovery pipeline. High resolution mass spectrometry provides one of the most efficient ways to carry out dereplication.

Methods: Secondary metabolites produced by producing microorganisms are extracted from spent fermentation medium and analyzed with high resolution LC-MS/MS. Molecular formulae for all major peaks identified in chromatograms are generated based on isotopic pattern and accurate measurement, these will be used for database searching, both known and potentially novel compounds can be further identified through MS/MS and UV absorbance.

Results. Rapid advances in bioinformatics enables the complete or partial structure of novel natural product to be predicted based on gene sequence, combining with genetic manipulation of bacterial strains, the final biosynthetic products can be identified through a LC-MS/MS based comparative metabolomics approach. The discovery and biosynthetic investigation of coelimycin P1, an unusual yellow-pigmented metabolic product of the cpk cryptic polyketide biosynthetic gene cluster from Streptomyces coelicolor M145 will be discussed.

Novel Aspects: Efficient de-replication with high resolution high accuracy Q-TOF mass spectrometry is critical for future bioactive natural product discovery.

Key words: High resolution, mass spectrometry, dereplication, drug discovery

References.
Introduction:
Recently has been proved that mesenchymal stem cells (MSCs) are able to package and deliver paclitaxel through their extracellular vesicles, suggesting the possibility of using MSCs to develop drugs with a higher cell-target specificity. The main objective of this study was to exploit the ability of mass spectrometry in order to perform structural studies on paclitaxel molecule because this drug follows a specific fragmentation pathway, which cleaves the molecules in two major fragment ions. These fragments are considered markers in biological fluids.

Methods:
The bile was collected every hour from ½ h to 3 h. An aliquot of 300 mL was picked up from every samples, diluted with acetonitrile 1:1(v:v) and then centrifuged. The supernatant was evaporated to dryness. The residue, dissolved in 100 mL with 0.02 M ammonium acetate buffer and acetonitrile 65:35 was directly injected into LC-MS/MS system. The entire effluent from the column of the control and pooled bile samples were analyzed in mass spectrometry. Product ion LC-MS/MS scans were obtained.

Results:
It is possible to take advantage of paclitaxel mass spectrometric behavior to establish the main features of drug metabolism, given that the hydroxylation process takes place on the two major fragment ions. Paclitaxel has extensively been studied both in the laboratory and in the clinic. However, its complete metabolic pathway in rats and humans have not yet been fully understood.

We concluded that the simply observation of intense molecular ions, even if well separated from the chemical noise, is not enough to determine the presence of paclitaxel analogs as well as their chemical modifications. Tandem mass spectrometry allowed to detect the presence of 9 metabolites, distinguishing them from the other endogenous contaminants.

These metabolites were recognized as three di-hydroxytaxol, four mono-hydroxytaxol, one deacetyltaxol and one containing the taxane ring.

With this methodology, we were able to identify four new metabolites of paclitaxel belonging to the di-hydroxy and mono-hydroxy series.

Conclusions
The assessment of the most important paclitaxel metabolites represents the first structured approach to pharmacokinetic studies (PKs) of this drug used in modern cancer therapy that is based on the development of new formulations. Pharmaceutical formulations such as liposome, micelle, emulsion and nanoparticles are potentially useful for drug delivery systems. The pivotal characteristics of new drug delivery systems, such as engineered nano-materials, have to include biodegradability, biocompatibility and non-toxicity for human cells [1-2].

Novel Aspect:
Paclitaxel has been recently loaded in different types of engineered nano-materials because they are demonstrated to penetrate specific tissues and reach directly the tumor site.

650 - COLLISION-INDUCED DISSOCIATION STUDY OF ANTIMICROBIAL PEPTIDE DENDRIMERS

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Keywords: Antimicrobial Peptide dendrimers, Collision-induced dissociation, MS/MS sequencing

Introduction
Peptide dendrimers are non-natural branched peptides with multibranched topologies that exhibit interesting antimicrobial properties [1]. In particular, dendritic architectures composed of lysine (K) and leucine (L) were demonstrated to be active against a broad panel of multidrug-resistant pathogens [2]. The renowned efficiency of CID for MS/MS sequencing of linear peptides was evaluated for structural characterization of a KL peptide dendrimer.

Methods
High resolution MS and MS/MS experiments were performed after positive ion mode electrospray ionization (ESI), using a Synapt G2 HDMS instrument. Lysine-Leucine (KL) peptide dendrimers were dissolved in acidified methanol to promote formation of protonated species.

Results
MS/MS was previously demonstrated to be very powerful at characterizing dendritic structures [3], shown to exhibit a typical dissociation behavior consisting of iterative losses of repeating building blocks from outside branches and then internal arms down to the inside core [4]. MS/MS data recorded on the KL-peptide dendrimer suggested a different scenario, with the dissociating precursor ion to be considered as a peptidic backbone with pendant ramified segments rather than a dendritic structure. On the one hand, CID data recorded at low collision energy allowed the main backbone to be sequenced, and hence the branching points to be localized. On the other hand, raising activation energy permitted to characterize the structure of the pending peptidic segments. Moreover, charge states observed for product ions allowed some insights in the actual location of protons in the quadruply charged studied species.

Conclusions
This study showed that KL-peptide dendrimers behave as a branched peptide rather than a dendritic structure upon CID. Dissociation rules to be established from MS/MS data obtained for this first generation molecule are currently evaluated for higher generation KL peptide dendrimers.

Novel Aspect
First report of MS/MS structural characterization KL-peptide dendrimers.

References
Introduction:
Antibody-Drug Conjugates (ADCs) combine the target specificity of monoclonal antibodies to the high cytotoxicity of a small molecule toxin selectively killing tumor cells while minimizing toxicity to normal tissues. ADCs are generated by covalently binding the selected toxin through a linker to specific antibody residues, usually Cysteine or Lysine, which increases the complexity of the products generating heterogeneous mixtures of isomers and isoforms.

Methods:
The characterization of the ADC product is fundamental because drug loading and distribution can affect the safety and efficacy of the ADC. Mass spectrometry can be applied for the identification of the ADC modified residues and, in combination with chromatographic techniques as UV-LC or HIC, for the determination of the Drug-Antibody Ratio (DAR) and the distribution of the toxin in the different populations generated by the cysteine conjugation process.

Results:
At NMS a new proprietary molecule suitable for conjugation has been developed and used to derivatize a commercial antibody to generate a cysteine conjugated ADC with an average DAR of ~3.5. The addition of the drug at the interchain cysteine residues results in a heterogeneous population of ADCs that differ in the number of drugs per antibody and in the presence of positional isomers.
The peptide mass fingerprinting strategy has been applied for the identification of the modified residues after the complete enzymatic digestion of the ADC and to characterize the ADC at the peptide level looking at primary sequence and post-translational modifications.
The DAR distribution analysis and the identification of the positional isomers present in the sample have been achieved by the combination of HIC and RPLC-MS. The intact DAR species separated by HIC on the bases of their hydrophobicity have been collected and analysed in denaturing conditions by RPLC-MS in order to separate and identify the ADC fragments composing each isomeric form.

Conclusions
The application of analytical technologies, especially mass spectrometry, have been applied for the analysis of a cysteine conjugated ADC produced at NMS. These technologies represent very useful tools for the characterization of these heterogeneous products during the drug development process to guarantee the batch to batch reproducibility.

Novel Aspect:
Mass spectrometry, in combination with allied separation techniques, has been applied for the characterization of an NMS proprietary drug conjugated antibody.
THE EFFECTS OF LOW TEMPERATURE STORAGE ON THE STRUCTURE OF HERCEPTIN®: TO FREEZE, OR NOT TO FREEZE: THAT IS THE QUESTION

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Keywords: IgG1, Herceptin®, cryo-stability, IM-MS, intrinsic-fold

Introduction:

For research and drug development mAbs are usually examined as fresh solutions stored between 2 – 4°C. By contrast the manufactured product is freeze-dried and stored at much lower temperatures. Here we use IM-MS and HDX-MS to probe structural differences between lots of the IgG1 mAb, Herceptin® as well as the effect of cold storage on the intrinsic conformation of this protein and its glycoforms.

Methods:

DT-IM-MS measurements were used to compare refolded mAbs against the freeze-dried API. Collision induced unfolding (CIU) measurements were performed using an RF-confining linear drift field Synapt G2 along with circular dichroism (CD) to provide stability profiles. HDX-MS analyses enabled the dynamic properties to be compared. We compare data obtained from fresh, freeze-dried and reconstituted solutions.

Results:

The fresh Herceptin® sample has a significantly broader collision cross section distribution (CCSD 45 – 100 nm2) compared with the frozen originator lots (60 – 80 nm2) despite being identical in terms of protein sequence. This is the case across 3 lots of Herceptin® as well as a lower purity (‘research grade’ trastuzumab) sample, which are extensively characterised with IM-MS as well as HDX-MS. Data from the renatured protein is compared with these two extreme cases in order to determine if freeze-storage fundamentally alters the protein fold. In each case the disulphide bridges are mapped. Global conformational stability is further probed using collisional activation prior to ion mobility analysis, and the profiles compared for lot-to-lot variability and storage effects. CD is used to examine the differences in secondary structure. As controls we have analysed the NIST mAb standard[1] and an intact mass check standard from Waters, using the same combination of mass spectrometry and biophysical techniques.

Conclusions:

We see significant differences in the conformational profile of Herceptin® depending on its treatment and between lots. This indicates that harsh storage conditions such as deep-freezing, do indeed restrict flexibility in the global conformation of Herceptin®. We present data that shows if complete denaturation of both the fresh and frozen mAb can be used to restore flexibility and consider the implications of these findings for drug processing following discovery.

Novel Aspect:

(150 characters max.)

Insights into the effects of cold storage upon the stability profiles, intrinsic-fold and global conformations of multiple Herceptin® lots.

References
Introduction:
The identification of therapeutic inhibitors for protein tyrosine phosphatase 1B (PTP1B) is an attractive approach to treat type II diabetes, but it has failed so far due to insufficient selectivity [1]. Covalent inhibitors that target residues only present in PTP1B might overcome these limitations. We studied the reactivity of cysteines in PTP1B and designed and characterized potential ligands.

Methods:
Mass spectrometry: 2-(4'-(iodoacetamido)anilino)naphthalene-6-sulfonic acid (IAANS) was used for modification of accessible cysteine residues of PTP1B. The number and position of the reactive residues were determined by LC-MS1 and LC-MS2. Reaction of PTP1B with the designed ligands was studied in the same way. Other methods: molecular modelling, in vitro phosphatase assay, X-ray crystallography.

Results:
First, the accessibility of different cysteine residues was determined by incubating PTP1B with IAANS under native conditions and analyzing the resulting products by LC-MS. We found Cys32 as potential target for specific inhibition of PTP1B. Next, a library of molecules was designed for covalent inhibition of PTP1B and was screened with an in vitro enzymatic assay. Several inhibitors were selected for mass spectrometry experiments to evaluate their reactivity towards Cys32. Despite a having a high potency, no alkylation of Cys32 with these ligands could be detected under the same conditions as used for IAANS. X-ray crystallography analyses revealed that, although the inhibitors occupy the intended pocket, their orientation hinders the reaction with Cys32.

Conclusions:
We identified Cys 32 as potential target for PTP1B inhibition and designed inhibitors to alkylate it. Although these substances are very potent in enzymatic in vitro assays and bind the expected pocket, neither X-ray crystallography nor mass spectrometry could demonstrate covalent modification of PTP1B. Therefore we conclude that the observed activity is due to non-covalent interaction.

Novel Aspect:
Use of IAANS to test the accessibility of cysteine residues
Handling a native protein (6 reduced Cys) without reducing agent

References:
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For information please contact: scientific@imsc2018.it
Therapeutic drug monitoring and drug discovery: Is it all known or are we innovative and game changing?

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Keywords: Quantitation, omics, instrumentation, drug discovery

Introduction:
Nowadays, mass spectrometry, mostly hyphenated with either GC or LC, is THE prime technology applied in therapeutic drug monitoring (TDM) and drug discovery (DD). The accurate quantitation of drugs and related metabolites are essential for personalized treatment scenario’s in the clinic, while both quantitative and qualitative data of these drugs and associated metabolites or PD effects are required in drug discovery.

Methods:
In order to address all questions in both TDM and DD a wide range of instruments, spanning from single quads till high resolution MS-systems, like e.g. the orbitrap and even FTICR systems, are applied in conjunction with state-of-the-art separation sciences to provide reliable data supporting decision making in either continuation or stopping further development or treatment.

Results: Here we address several of the challenges we are facing in the two application areas and present some of the solutions. Next to small molecules, more and more larger molecules, both as proteins but also Post-Translational Modifications (PTM) need to be monitored, quantified and related to either beneficial or adverse effects from initial PK until lead optimization and further development. Also in TDM, next to the traditional small molecules, proteins, antibodies, drug-antibody complexes etc. are getting more attention in the clinic. All these new area’s are relying on sensitivity, specificity and both spectral and chromatographic resolution. Hence, a diversity of applications will be addressed. Besides, the growing interest in applying not only targeted but also omics-like strategies to profile off-target effects, pharmacodynamics and drug related compounds is gaining popularity in early screening already. In TDM on the other hand, these technologies are far from routine at the moment.

Conclusions
Although, new technology, either being hardware, software is developed, there still is a need for higher sensitivity, higher acquisition speed, higher dynamic range and higher resolution in both TDM and DD. Here some examples are shown addressing some of the challenges.
Next, we show some of the novel applications rising at the horizon to further improve reliable and quantitative as well as qualitative information, required to make better and faster decisions on medication or progression of novel drugs in further development.
Ultrafast detection of drugs and metabolites in urine by Flow Injection Analysis (FIA) coupled to Magnetic Resonance Mass Spectrometry (MRMS)

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Ultrafast detection of drugs and metabolites in urine by Flow Injection Analysis (FIA) coupled to Magnetic Resonance Mass Spectrometry (MRMS)

Keywords: Metabolomics, Drug detection, Flow injection analysis, Screening

Introduction:
Detection of metabolites and drugs in body fluids such as plasma or urine by LCMS is a routine method in metabolomics and doping analysis. Routine UPLC-MS measurements are performed typically in 15 min. Therefore, the number of analyzed samples is highly limited. In this work, a fast method for detection of drugs and their metabolites in urine using FIA-MRMS is presented. Roughly 250 samples can be measured in 24h using this technique.

Methods:
Six pooled urine samples were purified by SPE using Merck LiChrolutEN SPE cartridges. Samples were extracted with methanol from SPE cartridges and diluted 1 to 100 for FIA. Each sample was analyzed in 5 minutes by FIA-MRMS using a solariX 2xR (Bruker Daltonik, Bremen) in ESI using positive and negative ion mode. Analysis of data was performed with MetaboScape 3.0 (Bruker Daltonik, Bremen).

Results:
The data of the ESI(+) and ESI(-) were combined for feature analysis. More than 2100 features have been found for the pooled urine samples. More than 90% of the detected features could be assigned with a molecular formula. 300 drug candidates have been detected in the urine samples using a HMDB urine database with a mass error tolerance of only 0.5 ppm. The detected drugs have been compared with the medication of the patients. Several drugs have been found only in one or a few pooled urine samples. By comparing the relative abundances of features of all samples, possible metabolites of drugs could be identified.

Conclusions:
Drugs and their metabolites can be detected by FIA-MRMS in a few minutes. This workflow is much faster than the conventional workflow using UPLC-MS. This method could even be used for quantification when internal drug standards are added. Due to the complexity of the samples ultra-high mass resolution as well as very accurate mass detection is a prerequisite for this workflow.

Novel Aspect:
FIA-MRMS can be used for fast detection of drugs and metabolites in urine.
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Keywords: liquid chromatography-tandem mass spectrometry, tyrosine kinase inhibitors, µ-elution solid phase extraction, endogenous phospholipids, therapeutic drug monitoring

Introduction:
Therapeutic drug monitoring (TDM) can improve efficacy and safety (1). Thus, we developed a liquid chromatography-tandem mass spectrometry (LC–MS/MS) method for monitoring 11 tyrosine kinase inhibitors (TKIs) used in chronic myeloid leukemia (imatinib, dasatinib, nilotinib, bosutinib, ponatinib), polycythemia vera (ruxolitinib), chronic lymphocytic leukemia (ibrutinib) and rheumatoid arthritis (filgotinib, tofacitinib, baricitinib, peficitinib).

Methods:
Only 200 µL of human plasma was needed for the extraction of analytes and their stable isotope-labeled internal standards. µ-elution solid phase extraction was optimized and compared to simple protein precipitation. Gradient elution on Poroshell 120 EC-C18 column, flow rate of 0.6 mL/min and 60ºC was applied for compounds separation. The mobile phase consisted of 0.1% formic acid in water, pH=2 (solution A) and 0.1% formic acid in acetonitrile (solution B).

Results:
Acquisition lasted for 8 minutes followed by a re-equilibration time of 4 minutes. Mass spectrometer was operating in the positive ionization mode. Dynamic multiple reactions monitoring scan was applied to improve method sensitivity. Endogenous phospholipids can strongly affect MS analysis. Therefore, the monitoring of m/z 184 > 184 (glycerophosphocholines) and m/z 104 > 104 (lysophosphatidylcholines) as common in-source collision-induced dissociation ion fragments, were included in the method. Caffeine was added to the assay as well. According to the recommendations of regulatory agencies [2,3,4], tests of precision, accuracy, recovery, matrix effect, process efficiency, stability, sensitivity (great LLOQs), and selectivity were performed and enabled us to validate the method. Sample preparation applied in the present assay showed high efficiency in phospholipids removal. More than 99% of main plasma lysophosphatidylcholines and more than 89% of glycerophosphocholines were eliminated compared to protein precipitation.

Conclusions:
This method enables simultaneous plasma monitoring of 11 TKIs, caffeine and two main plasma phospholipids. High effectiveness in endogenous phospholipids elimination ensures the reliability of the assay. Present LC–MS/MS method is currently used in our clinical practice, being applied to TDM of dasatinib, imatinib, nilotinib and ponatinib. TDM of TKIs helps to individualize dose adjustment and manage adverse effects in patients.

Novel Aspect:
The method monitors endogenous phospholipids (glycerophosphocholines and lysophosphatidylcholine) as well as caffeine in addition to 11 TKIs in a simple run.
References
A.08 LIFE SCIENCES - METABOLOMICS

1326 - LIQUID EXTRACTION SURFACE ANALYSIS AND DIRECT ESI/NANOESI MASS SPECTROMETRY FOR HIGH THROUGHPUT URINARY METABOLOMICS APPLIED TO MALARIA

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Keywords: Liquid Extraction Surface Analysis, flow injection mass spectrometry, direct nanoESI mass spectrometry, urinary metabolomics, biomarker discovery

Introduction:
Direct electrospray/nano-electrospray ionisation mass spectrometry (ESI/nanoESI-MS) have great potential as high-throughput approaches for untargeted urine metabolomics [1-5]. Here, we compare standard liquid chromatography-mass spectrometry (LC-MS) with ambient ionisation MS methods of liquid extraction surface analysis MS (LESA-MS), flow injection ESI-MS (FIE-MS) and nanoESI-chip-based infusion for clinical urinary metabolomics.

Methods:
LESA-MS, chip-based infusion and FIE-MS using a high resolution-MS were developed and validated against a standard LC-MS method [6]. A mixture of 35 urinary metabolites were used to optimise and establish a high throughput sample preparation and data analysis protocols and the methods were then applied for biomarker discovery in malaria (n=88).

Results:
The analysis of malaria/control (n=41/47) urine samples was validated using pooled QC samples interspaced within the run. Malaria generated 7526 and 576 ions from LC-MS and FIE-MS, respectively. OPLS-DA showed clear separation and clustering of malaria from the controls with cross-validation of R2Y=0.810/0.993, Q2=0.538/0.583, sensitivity (80%) and specificity (77%) for LC-MS/FIE-MS, indicating comparable results. Altered levels of 30 and 17 metabolites were identified by LC-MS and FIE-MS, respectively, including pipecolic acid, taurine, 1,3-diacetylpropane, N-acetylspermidine and N-acetylputrescine and may have the potential of being used as biomarkers of malaria.

Conclusions:
The developed direct ESI/nanoESI-MS demonstrated high throughput capability combined with the ability to differentiate between urine samples in disease and health state. LESA-MS requires no sample preparation and only involves placing urine samples onto a slide surface, gives the method a further credit of being very simple and easy to use. Therefore, these methods are recommended to be considered as a fast diagnostic/screening tool in clinical practice.

Novel Aspect:
High-throughput surface analysis and direct ESI/nanoESI-MS methods for clinical biomarker discovery.

Advanced data processing for direct ESI/nanoESI-MS.

References:
1337 - METABOLIC CHARACTERIZATION OF THP-1 MACROPHAGES POLARIZATION USING LC-MS-BASED METABOLITE PROFILING

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Keywords: Macrophages, THP-1, metabolomics, LC-MS, polarization.

Introduction:
Macrophages are heterogeneous and plastic immune cells that change their phenotype and function in response to environmental cues. M1 and M2 macrophages represent pro and anti-inflammatory phenotypes which show distinctively different functions [1, 2]. LC-MS metabolomics study was applied to characterize the metabolic profiles of M1 and M2 macrophages. This can be translated clinically to interrogate and manipulate macrophages phenotype.

Methods:
THP-1 cells macrophages were polarized with either GC-MSF, LPS and IFN-γ or M-SCF and IL-4 to generate M1 or M2 polarised cells respectively. Unpolarised cells (M0) were set as a control. Then, LC-MS-based metabolite profiling was performed on an Accela system coupled to an Exactive (Thermo Fisher Scientific) operating with electrospray ionization (ESI), and ZIC-pHILIC (4.6 x 150mm and 5μm particle size, Merck Sequant) was used for chromatographic separation.

Results:
The results showed that M1 and M2 macrophages have distinctive metabolic profiles. Clear separation was shown in OPLS-DA models between M0, M1 and M2 macrophages. Moreover, various metabolic pathways were significantly disturbed upon polarization including sphingolipids and pyrimidine metabolic pathways in M1 THP-1 macrophages. Whilst arginine and proline, alanine, aspartate and glutamate metabolisms, were the most significantly perturbed metabolic pathways in M2 THP-1 macrophages. Relevant key metabolites were successfully selected as potential biomarkers for each of functional phenotype, such as [SP (22:0)] N-(docosanoyl)-sphing-4-enine, [SP (24:0)] N-(tetracosanoyl)-sphing-4-enine, [SP (16:0)] N-(hexadecanoyl)-sphing-4-enine, cytidine, cytosine, and 5-hydroxy trytophan for M1 macrophages and N-acetyl glutamate, N-acetyl aspartate, and 4-imidazolone-5-propanoate for M2 macrophages.

Conclusions
LC-MS metabolic profiling was used to characterize M1 and M2 macrophages. This approach efficiently demonstrated the distinct metabolic profile for M1 or M2 macrophages and revealed potential biomarkers for each phenotype. These biomarkers can be translated to explain the mechanisms of macrophages polarization and consequently, manipulate the cells phenotype. For example M2 is required to resolve autoimmune diseases, where M1 is active against tumor cells.

Novel Aspect:
Discovery of biomarkers by LC-MS metabolomics can lead to understand polarization mechanism and then, development of targeted therapies for treating various diseases.
References

For information please contact: scientific@imsc2018.it
1379 - INVESTIGATING THE DIFFERENCES IN THE MOLECULAR EFFECTS OF FREE AND NANOPARTICLE LOADED METHOTREXATE ON PROLIFERATIVE AND NONPROLIFERATIVE CANCER CELLS USING ADVANCE CELL BASED METABOLOMICS

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Keywords:
Metabolomics, LC-MS, PLGA, Nanoparticles, Methotrexate

Introduction:
Polymer nanomedicine has drawn the attention of researchers in recent years, especially drug loaded NPs. However, the impact of drug loaded NPs at the molecular and cellular levels could be different from the free drugs. These discrepancies might be attributed to different levels of uptake or cellular trafficking routes, yet it is not fully understood. Metabolomics is a relevant tool for understanding such mechanisms involved in the response drug delivery systems.

Methods
Methotrexate (MTX) loaded and blank PLGA nanoparticles were fabricated using the conventional nanoprecipitation technique. The fabricated NPs in addition to free MTX were dosed to THP-1 macrophages and A549 lung epithelial cancer cells for 24 hours, after that the cellular metabolism was quenched and the intra cellular metabolites were extracted. Global LC-MS based metabolic profiling was used to study the metabolic changes resulted upon different treatments.

Results
THP-1 cells were sensitive to the NPs more than to MTX and most responses can be linked to the phagocytosis process, the NPs resulted in down regulation in TCA cycle and in glycolysis, at the same time redox homeostasis impairment was recorded. A549 cells were sensitive to MTX more than to nanoparticles and the responses metabolic responses indicated apoptosis. MTX has resulted in nucleotide metabolism impairment, oxidative stress and shift in the energy metabolism towards fatty acid oxidation (FAO), this shift in energy metabolism leads to accumulation of acetyl CoA as end product of FAO, and because TCA cycle is malfunctioning, acetyl CoA is transformed to Acetoacetate which leads to ketoacidosis and apoptosis. The NPs did not have the same impact on A549 cells which might be due to the fact that the epithelial cells are not phagocytic. Being not proliferative might explain the resistance of activated THP-1 cells to MTX, especially because MTX is an antifolate agent that exert its action during the S phase in proliferation process.

Conclusions
Global metabolic profiling approach was used to study the effects of MTX and MTX loaded PLGA NPs on THP-1 and A549 cells successfully. THP-1 cells were sensitive to the NPs more than to MTX and most responses can be linked to the phagocytosis process. A549 cells were sensitive to MTX more than to nanoparticles and the responses lead to apoptosis.

Novel Aspect:
This work highlights the utility of metabolomics approach in understanding the difference in the molecular activity of a therapeutic agent when dosed as in free form or in NPs.

References


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Measuring trehalose 6-phosphate (Tre6P) in different plant materials – the technical challenge of working at the femtomole scale

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Key words: Trehalose 6-phosphate (Tre6P), High-Performance Anion-Exchange Chromatography (HPAEC), Tandem mass spectrometry (MS/MS), Stable-isotope labelled internal standards (SIL-IS).

Introduction

Trehalose 6-phosphate (Tre6P) is an essential metabolite in plants. It acts as a signal of sucrose availability, thereby linking growth and development to carbon status. The amounts of Tre6P in plant tissues are extremely low (between 10 and 1000 pmol·g-1FW in Arabidopsis thaliana), and plants contain several isomeric disaccharide monophosphates. HPLC coupled to tandem mass spectrometry (LC-MS/MS) is the most suitable technique for measuring Tre6P, offering both high sensitivity and high specificity.

Methods

Frozen plant material is extracted with chloroform/methanol (3:7, v/v) as in [1]. Each sample is spiked with a mix of [2H2]Tre6P and other stable-isotope labelled internal standards (SIL-IS). Compounds are initially separated on an IonPac AS11-HC-4 with an alkaline eluent containing 5% methanol (ICS 5000+; Thermo Scientific Dionex). Detection and quantification is then achieved by tandem mass spectrometry (QTrap 6500; Sciex) [1,2].

Results and Conclusions

We achieve high specificity by simultaneous measurement of multiple product ions in the third quadrupole. The use of SIL-IS allows correction for ion suppression and other matrix effects in plant extracts which typically contain up to 25,000 different compounds [3]. In addition to Tre6P, we concurrently measure other phosphorylated intermediates, nucleotide sugars and organic acids, providing a comprehensive overview of central carbon metabolism.

Novel aspects

This technique allows accurate determination of low abundance signal metabolites and metabolic intermediates in plants that cannot be measured using other Methods:

References

Identification of novel long chain N-acylhomoserine lactones of chain length C20 from the marine phototrophic bacterium Rhodovulum sulfidophilum

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Ajinomoto Co., Inc., Institute for Innovation, Kawasaki (1) - Toyohashi University of Technology, Department of Environmental and Life Sciences, Toyohashi (2) - Waseda University, Graduate School of Science and Engineering, Tokyo (3)

Keywords: N-acylhomoserine lactone (AHL), quorum sensing (QS), C20-HSL, precursor ion scan, high resolution mass spectrometry

Introduction:
Gram negative bacterial quorum sensing (QS) is mainly regulated by an extracellularly produced N-acylhomoserine lactone (AHL). AHL consists of a lactone ring and an acyl chain, which generally varies from C4 to C18 in length. The marine photosynthetic bacterium Rhodovulum sulfidophilum produces extracellular nucleic acids involved in its flocculation [1], and may be regulated through AHL signaling.

Methods:
AHL extracts from cultured by R. sulfidophilum were obtained by TLC and analysed with liquid chromatography tandem mass spectrometry (LC-MS/MS) systems. In order to profile and detect AHLs, precursor ion scanning was performed using quadrupole-ion trap mass spectrometer. For both identification of the compounds and differential profiling analysis, high resolution accurate mass data was obtained by quadrupole-Orbitrap hybrid mass spectrometer.

Results:
AHL fraction containing long acyl-chain homoserine lactone (HSL) exceeding C10 was obtained from the reverse-phase thin layer chromatography-fractionated cultured supernatant of the marine photosynthetic bacterium R. sulfidophilum. We performed precursor ion scanning analysis and detected two kinds of long chain AHLs with chain length C20. By fragmentation search analysis to detect compounds with a homoserine lactone ring moiety for data dependent acquisition, a minor AHL, presumed to be 3-OH-C18-homoserine lactone, was also found. Among the detected C20-HSLs, N-(3-Hydroxyicosanoyl)-L-homoserine lactone (3-OH-C20-HSL) was structurally identified and 3-OH-C20:1-HSL was strongly suggested (paper in submission).

Conclusions:
We found that R. sulfidophilum produced two novel C20 long chain AHLs.

Novel Aspect:
This is the first report on structural analysis of novel AHLs with the longest yet-described acyl chain length of C20.

Reference:
INTRODUCTION

The endemic Colombian fruits are an abundant source of secondary metabolites especially of phenolic compounds [1], [2]. Borojoa patinoi and Bactris gasipaes, two fruits from the region, are possible sources of this type of metabolites. Besides, there isn’t any research focused on the identification of phenolics in these two fruits. The aim of this research is the total quantification and identification of phenolic compounds in these two fruits.

METHODS:

The samples were acquired at Buenaventura’s City market located at the Pacific coast of Colombia. The extraction was performed using methanol acidified with formic acid [3], [4]. The separation was carried out using a Kinetex C18 column (150 mm x 3 mm i.d., 3 µm) [3]. The mobile phase used was 0.1% formic acid in water and methanol. The Ion Trap was operated in data-dependent, full scan and MSn mode to obtain fragment ion m/z with a collision energy of 35% and an isolation width of 2 m/z.

RESULTS:

The extraction of polyphenols was previously optimized for HPLC-ESI-MS/MS analysis. The extraction was carried out according to the obtained profiles from the metanolic extracts allowing the identification of new compounds by means of data bases [5] [6]. In addition, by means of the Folin-Ciocalteu method the total content of phenolic compounds was quantified in these extracts. Additionally, the antioxidant activity was measured by the ABTS and DPPH Methods: The anti-inflammatory activity by FOX method [7] and the anti-cholinesterasic activity by de Ellman method [8].

CONCLUSIONS

At the structures elucidation ten new compounds were found for Bactris gasipaes among which 8 polyphenols stand out. In Borojoa patinoine nine new compounds were found eight of which are polyphenols. IC50/EC50 values were also found using quercetin as a positive control for antioxidant activities and anti-inflammatory assay. EC50 values were determined for the anticholinesterasic activity using Eserine as positive control.

NOVEL ASPECT:

This is the first study focused on the search for polyphenols for these two Colombian fruits.
References

Fatty acid β-oxidation fuels tricarboxylic acid cycle to sustain cell proliferation.

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Keywords: Mitochondria, mtDNA, fluxomics, β-oxidation.

Introduction: Mitochondria play a central role in energy metabolism and possess their own genome (mtDNA). Mitochondria control cell cycle and differentiation where they orchestrate and finely tune all metabolic transitions. In line with this, mtDNA is vital for cell life since it encodes for 13 OXPHOS subunits, mt-tRNAs and mt-rRNAs. The goal of this project is to identify new metabolic patterns by which cells survive to an important metabolic deficit, such as lack of mtDNA.

Methods: For this study we took advantage of p0 cells (mtDNA partially depleted cells). By LC-MS/MS, we performed steady state metabolomics and metabolic tracing. Specifically, we labelled the cells with U-13C6-glucose, U-13C5-glutamine and U-13C16-palmitate.

Results: Our results indicate that C2C12, Neuro2a, Hepa 1-6 and C3H/10T1/2 undifferentiated p0 cells have different metabolic phenotypes compared to wild type control cells with normal mtDNA levels. Specifically, steady state metabolomics and metabolic tracing analysis indicate that mtDNA downregulation differentially impairs metabolic profile of p0 cells in a cell specific fashion. Specifically, metabolomic and fluxomic analyses indicate that β-oxidation fuels the TCA cycle to replenish the reducing cofactor NADPH through the activity of the malic enzyme 1 (ME1).

Conclusions: Our data indicate that mtDNA depletion to a similar extent, differentially affects the metabolic profile, depending on the cell type.

Novel Aspect: Our findings provide new insights into undisclosed metabolic pathways activated to face important metabolic deficits. Specifically, we directly link for the first time the β-oxidation of fatty acid to NADPH, aspartate and asparagine levels and cell proliferation.
DIAGNOSTIC PERFORMANCE OF METHYLMALONIC ACID TESTING BY LCMS/MS COMPARED TO HOLOTRANSCOBALAMIN BY IMMUNOASSAY FOR VITAMIN B12 DEFICIENCY

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Keywords: Methylmalonic acid, LC-MS/MS, Holotranscobalamin, Immunoassay, Vitamin-B12 deficiency

Introduction:
Measurement of total-B12 has been suggested to lack sensitivity to detect vitamin B12 deficiency. Methylmalonic acid (MMA) and Holotranscobalamin (Holo-TC) are recommended to be more sensitive markers for B12 deficiency. Our preliminary aim was to develop an LC-MS/MS method to measure MMA. We attempted to evaluate the diagnostic accuracy of HoloTC for the detection of B12 deficiency based on the surrogate reference criterion of a raised MMA (>500nmol/L).

Methods:
We employed a simple protein precipitation technique using acid, and a fast-chromatographic method using Ascentis RP-amide column and gradient mobile phase (3% - 90% v/v methanol with 0.1% formic acid) on Sciex 6500 QTRAP in negative mode, MRM of 117 - 73. Serum samples were tested for MMA (LC-MS/MS) and Holo-TC (immunoassay, Abbott-Architect / Roch-Cobas). Using ROC curve analysis and MMA as surrogate, the diagnostic accuracy of Holo-TC was evaluated.

Results:
We successfully developed an LC-MS/MS method for MMA measurement with an analytical range of 0.14 - 86.4 µmol/L. Serum samples, n=50 including 10 normal (>250pmol/L), 10 abnormal (<140pmol/L), 30 equivocal (140-250pmol/L) total B12 were tested for MMA and Holo-TC. Overall, we observed an increase in MMA (>500nmol/L) in 10% of patients, excluding patients with renal failure or known alternative reason for elevated MMA (1). Of this group, Holo-TC was low (<25 pmol/L) in 20% and equivocal (25-50pmol/L) in 80%. When MMA cut-off is 500nmol/L, the positive predictive value of Holo-TC<25pmol/L was 20%, and the negative predictive value of Holo-TC>50pmol/L was 100%. Compared to Abbott, results for Holo-TC by Roche was discordant in 4% of patients. All patients with low total B12 (<140pmol/L) had equivocal Holo-TC and normal MMA (<500nmol/L).

Conclusions:
Both MMA and Holo-TC have limited abilities to predict B12 deficiency. The use of these markers in combination, together with clinical details, may provide the most reliable representation of B12 status.

Novel Aspect:
To our knowledge, this is the first study that comparing several methods of measurement for detection of vitamin B12 deficiency.

References:
Introduction:
Gas chromatography coupled to mass spectrometry (GC-MS) is widely used in clinical data analysis due to several major advantages: speed, resolution and available databases. Several data preprocessing tools for GC-MS are available in R [1] and C [2]. However, free open source pipelines are less common in this field. Clinical data requires often times certain specifications and code editing thus emphasizing the need of open sourcing.

Methods:
Pycmetrix is a framework built in python for preprocessing of GC-MS data and chemometrics data analysis. The package includes the possibility of deconvolution, quantification and interaction with a database via mysql.

Results:
The framework was applied to GC-MS data processing of several patients diagnosed with MCADD. The quantification feature was validated using the calibration curves and showed very good results. A feature selection method was further developed for a fast and automated diagnosis. The data from 20 healthy patients and 20 MCADD were used to create a model in a lower feature space. Using the tool described herein, we were capable to accurately quantify and classify the data.

Conclusion:
The pipeline proved to be efficient in working with multiple data sets in one run. The hardware requirements for the data analysis were reasonable, making the preprocessing and data analysis possible even on a low cost PC or laptop.

Novel Aspect:
Pycmetrix includes the features not available in other python packages such as: effect size based feature selection, supervised projection pursuit, Bayesian hypothesis testing and other useful options.

References
Keywords: chemometrics, GC-MS data analysis, framework, python, Bayesian

Introduction:
Gas chromatography coupled to mass spectrometry (GC-MS) is widely used in clinical data analysis due to several major advantages: speed, resolution and available databases. Several data preprocessing tools for GC-MS are available in R [1] and C [2]. However, free open source pipelines are less common in this field. Clinical data requires often times certain specifications and code editing thus emphasizing the need of open sourcing.

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Novel Aspect:
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References
617 - THE UTILIZATION OF TANDEM MASS SPECTROMETRY TECHNIQUES IN DIAGNOSIS OF PATIENTS WITH HOMOCYSTINURIAS.

Josef Bártl (1) - Jakub Krijt (1) - Petr Chrastina (1) - Jakub Hodík (1) - Markéta Paulová (1) - Renata Pinkasová (1) - Jitja Sokolová (1) - Viktor Kožich (1) - Karolína Peškova (1)

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Keywords: tandem mass spectrometry, homocystinuria

Introduction:
Homocystinurias (HCYU) are rare genetic disorders characterized by accumulation of homocysteine (Hcy) in the body due to defects in a) the remethylation (RMD) pathway or b) transsulfuration pathway (cystathionine β-synthase deficiency -CBSD). Metabolism of Hcy depends also on supply, transport and intracellular metabolism of B vitamins. We developed tandem mass spectrometry methods enabling differential diagnosis of various types of HCYU.

Methods:
FIA-MS/MS newborn screening (NBS) method for amino acids (AA) in dried blood spot (DBS) and LC-MS/MS determinations of: 1) Hcy, methionine (Met) and methylmalonate (MMA) in DBS. 2) Cystathionine and other AA in plasma after derivatization with alkyl chloroformates. 3) S-adenosyl methionine (SAM) and and S-adenosyl homocysteine (SAH) in plasma. 4) CBS activity in plasma using stable isotope substrate and determination of the deuterated product cystathionineD2.

Results:
The established reference ranges were as follows: Hcy (DBS): < 12 μmol/L; Met (DBS): 7-33 μmol/L; MMA (DBS): < 5 μmol/L; Hcy (plasma): 3.5-15 μmol/L; cystathionine in plasma (P): 80-1000 μmol/L; Met (P): 12-40 μmol/L; SAM (P): 50-160 nmol/L; SAH (P): 26-80 nmol/L and CBS activity: 100-1000 nmol/hour/L plasma. By introducing new LC-MS/MS methods we diagnosed patients with CBSD presented by accumulation of Hcy and Met and decreased metabolites below the CBS block (i.e. cystathionine and cysteine), RMD including methylene tetrahydrofolate reductase deficiency and intracellular methylcobalamin synthesis defects characterized by decreased production of Met and SAM, accumulation of Hcy and an increased flux of metabolites through the transsulfuration pathway and B vitamins deficiencies presenting with elevated Hcy and MMA. Within NBS program between July 2016 and December 2017, we analyzed samples from 128531 newborns and detected 3 patients with maternal vitamin B12 deficiency and 1 patient with CBS deficiency.

Conclusions:
We developed LC-MS/MS methods approach for diagnosing patients with HCYU enabling us to discriminate between primary enzyme deficiencies and vitamin B deficiencies and distinguish between defects in the RMD and transsulfuration pathway of Hcy. Methods employing DBS are also used in NBS program and have the clinical utility for monitoring of therapy in patients with hyperhomocysteinemias due to ease of collecting and shipping DBS samples by the patients.

Novel Aspect:
The LC-MS/MS methods help to enhance efficacy in diagnosis procedure for hyperhomocysteinemias and simplify monitoring of patients under treatment.

Acknowledgement:
This work was supported by the grant Nr. 16-30384A from the Czech Health Research Council and RVO VFN64165 from the Ministry of Health of the Czech Republic.
Introduction:
The analysis of the metabolites can present an analytical challenge, particularly those involved in the central carbon metabolism and neurotransmission. Often, structural isomers can’t be differentiated by MS or MS/MS alone, making a separation crucial. Unfortunately, the LC separations prove difficult for some metabolites; some hydrophilic analytes are poorly retained by RP-LC, while column-to-column reproducibility can sometimes be more challenging with HILIC separations.

Methods:
In contrast, capillary electrophoresis (CE) is well suited towards metabolomics analyses. CESI-MS integrates CE and electrospray ionization (ESI) into a single device and this combines the benefits of a high-resolution separation with the increased MS sensitivities that result from the ultra-low flow rates. In this case, we demonstrate CESI-MS for the analysis of these challenging metabolites.

Results:
A single protocol was established for the separation and detection of both cationic and anionic metabolites. Electrophoretic separations were performed using 30 kV to generate a field strength of 333 V/cm and normal and reversed CE polarities were employed for cationic and anionic metabolites respectively. MS compatible background electrolytes (BGEs) facilitated highly efficient separations, achieved in less than 30 mins. Enhanced sensitivity was achieved using transient isotachophoresis with CESI directly coupled to either a SCIEX TripleTOF® 6600 or QTRAP® 6500+ system. Cationic metabolites were characterized using normal polarity with positive ESI while the anionic metabolites were analyzed from the same sample by simply switching the polarity of the CE and MS.

Conclusions:
The CESI-MS technology proved powerful for the targeted analysis of the central carbon metabolism, particularly the small organic acids of the tricarboxylic acid (TCA) cycle and the isobaric phosphorylated sugars of the pentose and glycolysis pathways. In addition, CESI-MS was used to detect metabolites involved in neurotransmission such as those in the tryptophan pathway. Despite an injection volume of less than 50 nL, low nanoMolar concentrations were easily detected.
1236 - PHENOLICS, ALKALOIDS, LIPIDS AND PROTEINS OF OCHROMA PYRAMIDALE, TAIPIRIRA GUIANENSIS AND INGAEDULIS, HOST PLANTS OF THE CATERPILLAR LONOMIA DESCIMONI.

Felipe Benavides (1) - Chiara Carazzone (1)
Universidad de los Andes, Chemistry Department, Bogotá D.C., Colombia (1)

ords: Lonomia, Host-plant, Secondary Metabolites, Primary Metabolites.

Introduction
The Lonomia caterpillars are venomous animals found in Latin America and Colombia owns a great variety species of this genus.[1][2] Very few works has been realized about the Lonomia caterpillars in Colombia and their host plants. The aim of this investigation is to study of the polyphenols, alkaloids, lipids and proteins content of Ochroma pyramidale, Tapirira guianensis and Inga edulis, host plants of the caterpillar Lonomia descimoni.

Methods
The specimens were collected in the Colombian Amazon jungle. The leaves were macerated with liquid nitrogen and the extraction was performed with different solvents depending on the metabolites of interest (polyphenols[3], alkaloids[4], lipids[5] and proteins[6]). The extracts were filtrated and injected onto HPLC-ESI-MS/MS system. The spectra and chromatograms were analyzed for the identification of the metabolites present in the extracts.

Results
The extraction conditions for the lipids[3] and the proteins[4] were optimized previously to the analysis by HPLC-ESI-MS/MS. The alkaloids and polyphenols extraction conditions were performed according to [5] and [6], respectively. The profiles obtained of the different extracts of the leaves of the three plants understudy allowed the comparison of the metabolites present. The tandem mass spectra of the metabolites were assigned and, when possible, identified. Additionally, we could establish that despite the presence of some metabolites with the same m/z relation in the three plants, these corresponded to different compounds, since the MS/MS fragments of these were different.

Conclusions
The chromatograms and the MS / MS spectra allowed us to observe differences between the polyphenol, alkaloid, lipid and protein profiles of the three plants under study. Only a few compounds are present in all plant species, therefore it would be interesting to investigate the effects of these metabolites on the diet of the caterpillar.

Novel Aspect
In spite of the Colombian biodiversity, most species remain uninvestigated. This is the first study on the host plants of Lonomia descimoni, a venomous caterpillar.

References
Introduction:
Since successful introduction of immunosuppressants, organ transplantation is a routine procedure, which saves lives of patients with end-stage organ failure worldwide. The demand for grafts is much higher than the available pool of organs, therefore various strategies are explored to address this issue. The one presented here is metabolic profiling of organ with low-invasive method based on solid phase microextraction and high resolution mass spectrometry.

Methods:
The study of kidney grafts were performed on pig model. In one group organs were subjected to warm ischemia and in the second group kidneys were non-ischemic. Solid phase microextraction probes of ca. 0.2 mm diameter and 7 mm of coating length were selected for untargeted extraction of metabolites. Sampling was performed before organ harvesting, during preservation with normothermic perfusion and after reperfusion. Analysis were done on UPLC-Q-Exactive Focus.

Results:
The proposed analytical strategy enabled metabolomics of living organ with very low-invasiveness and tissue biopsy-free sampling. The results of LC-MS analysis revealed significant differences in metabolome of kidney obtained from donors after cardiac death (DCD, ischemic organs) and heart beating donors (no ischemia). Comparison of the data from organs subjected to warm ischemia showed increased level of lysyl residue oxidation products. The effect was most pronounced after reperfusion indicating increased risk of reperfusion injury in DCD. On the other hand, monitoring of changes during normothermic perfusion showed that level of some metabolites inducing oxidative stress speaking during ischemia drops to initial values suggesting that the used preservation strategy can successfully reverse some adverse reactions initiated by ischemia. This finding is supported by putative identification of the nephroprotective compounds reported earlier in the literature as the ones possessing anti-ischemia-reperfusion injury activity.

Conclusions
The results obtained indicate that novel analytical strategy proposed for assessment of graft quality carries a great potential for future improvement of pre-transplant diagnostics. It offers a possibility of selection of panels of biomarkers, which after required validation, could be used for fast on-site screening with SPME-MS platform in the future.

Novel Aspect:
The applicability of SPME-HRMS-based strategy for graft assessment was proven using a porcine model of kidney transplant.
Laura Brunelli (1) - Elisa Caiola (2) - Francesca Falcetta (2) - Silvia Giordano (1) - Mirko Marabese (2) - Massimo Brogini (2) - Roberta Pastorelli (1)

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Keywords: NSCLC, metabolism, co-occurring genetic lesions, mass spectrometry

Introduction:
NSCLC is a heterogeneous disease, with multiple oncogenic mutations (e.g. KRAS, LKB1). NSCLC patients have a variety of clinical courses, which are partly due to differences in co-occurring molecular events [1]. Since both KRAS mutation and LKB1 loss have an impact on cellular metabolism, it is fundamental to discern the metabolic effects induced by single genetic events from those induced by their co-occurrence, for novel therapeutic interventions.

Methods:
We knocked-out the LKB1 gene in well-characterized NSCLC cell clones harbouring KRAS WT or mutant G12C proteins. We obtained an isogenic system in which KRAS mutation and LKB1 loss were individually or concomitantly present. The effects of these genetic makeups on cell metabolism were investigated in these NSCLC cells by means of an integrated survey of proteomics, metabolomics, 13C-Glucose/Glutamine flux analysis coupled with functional in-vitro strategies.

Results:
Using untargeted (FIA-LTQ Orbitrap) and targeted (p180KIT, HPLC-Triple Quad 5500) metabolomics approaches we demonstrated how isogenic NSCLC cells harbouring both KRASG12C/LKB1 loss had an impact on the same metabolic pathways (glycolysis, TCA, urea cycle) used by their single lesion counterparts, but were able to exploit these metabolic routes through a heightened metabolite production. The enhanced metabolite production triggered by the co-occurrence was not reflected in a different enzymatic asset in terms of both numbers and magnitude of enzymatic changes. Furthermore, metabolic flux analysis showed how the metabolic dynamics of glycolysis and TCA cycle differed between clones harbouring the dual lesion from those with the single lesions. The enhanced metabolic activity of cells with both genetic lesions rendered the viability of these cells susceptible to energetic stress caused by both glycolysis inhibition and by nutrients limitation (glucose/glutamine) than those harboring single oncogenic lesions.

Conclusions:
We demonstrate how the metabolic rewiring observed in presence of KRAS/LKB1 co-occurring lesions, whilst failing to improve cellular fitness, was accompanied by reduced cell growth rates compared to those of wild type or single lesion cells. Furthermore, the cells with both lesions demonstrated greater susceptibility towards survival impedance caused by 2-DG treatment or glucose/glutamine limitation.

Novel Aspect:
This observation raises the prospect that energy stress may affect NSCLC cellsharbouring co-occurring lesions, which may render them more susceptible to cytotoxic drugs.

References:
Bhattacharya S. Socinski M.A., and BurnsetT. ClinTransl Med. 4:35; 2015
CONVERSION OF COENZYME A THIOESTERS BY NATIVE CHEMICAL LIGATION – A NEW APPROACH FOR CHARACTERIZING AN IMPORTANT CLASS OF BIOMOLECULES

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Keywords: Coenzyme A thioesters, native chemical ligation, fragmentation patterns, chiral separation

Introduction
Acyl-coenzyme A esters (acyl-CoAs) are very important biomolecules which play a central role in different metabolic processes, including β-oxidation of fatty acids, amino acid degradation and the citric acid cycle. Although numerous techniques for the analysis of acyl-CoAs have been developed, a straightforward approach for the unambiguous characterization of isomeric acyl-CoAs is still lacking.

Methods
Transformation of CoA-thioesters has been achieved using the native chemical ligation (NCL) reaction [1]. Different acyl-CoAs were incubated with L-cysteine and sodium 2-mercaptoethanesulfonate (MESNa) in 0.05M phosphate buffer (pH 7.4) at 70 °C. Products were analyzed without further modification and after the cleavage of the disulphide bond by a reductive agent with LC- ESI-TOF in positive mode.

Results
In this research the replacement of coenzyme A from CoA-thioesters with L-cysteine by NCL is demonstrated. Initially, transthioesterification between acyl-CoAs and L-cysteine occurs to form products that further undergo an irreversible amide formation. Under the reaction conditions applied the thiol-containing amide is oxidized to a disulphide with MESNa, which results in higher water solubility. All products detected showed mass spectrometric fragmentation patterns corresponding to cleavage of the amide and disulphide bonds, which allow their straightforward identification in mixtures. After reduction of the disulphides all products showed a fragmentation pattern characteristic of cysteine derivatives. Additionally, we performed the NCL reaction on the racemate of 2-methylhexanoyl-CoA and successfully separated diastereomeric products on an achiral column.

Conclusions
The methodology described enables detailed structural characterisation of the acyl-residues in acyl-CoAs by generating transformation products through the NCL reaction which deliver more informative mass spectra. Furthermore, it provides a possibility for separation and assignment of configuration of alpha-substituted chiral compounds on an achiral column using L-cysteine as a chiral auxiliary.

Novel Aspect
Selective conversion of CoA thioesters to the cysteine containing products is a novel aspect for characterization of an important class of biomolecules.

References
Enhanced Metabolite Identification Using An Orbitrap Tribrid Mass Spectrometer

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Introduction

High resolution mass spectrometry (HRMS) is an essential tool for metabolite identification. HRMS with sophisticated data acquisition features can provide the critical information for metabolite structure elucidation and alleviate the difficulties of matrix complexity.

Here we present a study for metabolite ID using Orbitrap Tribrid MS with data acquisition features designed to significantly improve small molecule structure analysis, which include automatic background ions subtraction, inclusion/exclusion lists generation, and real-time decision-making to optimize MSn data acquisition quality and speed.

The high quality information-rich MSn data generated using this improved HRMS was processed using structure analysis and data mining software “Mass Frontier” and “Compound Discoverer”.

Methods

Amprenavir, Bosentan, Lopinavir, and Ritonavir were selected as model compounds. The compounds, at concentration of (5 µM), were incubated with human liver microsomes (HLM) in the presence of GSH and UDPGA. Incubations without drug or without NADPH were chosen purposely to test the background subtraction feature.

The LCMS analyses were performed on Vanquish Flex Binary UPLC system with DAD detector coupled to an Orbitrap Tribrid Mass Spectrometer. Mobile phases were composed of: A: H2O/0.1% formic acid, and B: ACN/0.1% formic acid with gradient on Hypersil column (2.1X100 mm 1.9 µm).

High resolution full scan and MSn data were collected in a data-dependent fashion with polarity switching. The data acquisition method was incorporated with features optimized for small molecule analysis.

Preliminary Data

These HIV drugs were selected because of their extensive metabolism. Using the Orbitrap Tribrid Mass Spectrometer's special features for small molecule data acquisition: the automatic background subtraction, inclusion/exclusion list generation, and real-time decision-making optimization of MSn spectra acquisition, the MS provides a wealth of information for metabolite identification. The automatic background subtraction feature is especially useful for identification of metabolites present in a complicated biologic matrix.

The new and enhanced features that use the high quality MS/MS fragments to trigger the MSn acquisition significantly improved MSn data quality, because multi-stage MS/MS (MSn) information is essential for confident metabolite structure elucidation. For example, HRAM full scan data show that Ritonavir metabolites include four mono-oxidation products, two N-dealkylation products, and four di-oxidation products. In order to determine the structure and the sites of biotransformation of these metabolites, high quality MS/MS and higher order MS/MS spectra were obtained using the novel data collection features of Orbitrap Tribrid MS.

Major and minor metabolites, both phase I and II, of these model compounds formed in the HLM microsomal incubations were quickly detected by processing the HRAM and higher level MS/MS (MSn) spectra of metabolites using Mass Frontier and Compound Discoverer.
This workflow offers significant improvements in speed and confidence of routine drug metabolite identification and other small molecule structure characterization applications.

Novel Aspect
Small molecule specific acquisition features of OrbitrapTribrid MS improve MSn data quality and increase confidence for metabolite identification.

Options:
A graduate student is presenting author on this abstract? No
A post-doc is presenting author on this abstract? No
An undergraduate student is presenting author on this abstract? No

Poster:
Drug Metabolism: Quantitative Analysis

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608 - TARGETED APPROACH FOR DETERMINATION OF METABOLITES OF FLAVONOIDS AND ISOFLAVONOIDS IN BIOLOGICAL SAMPLES

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Keywords: quercetin, UHPLC-MS/MS, MEPS, μ-SPE-PT, isoflavonoids

Introduction:
Rutin is metabolized into two groups of metabolites: flavonoid type with higher molecular weight (quercetin, quercetin-3-O-glucuronide, tamarixetin and isorhamnetin) and polar weak phenolic acids (4-methylkatechol, phloroglucinol, 3,4-dihydroxyphenylacetic acid, homovanilic acid, 3-hydroxyphenylacetic acid, 3-(3-hydroxyphenyl)propionic acid). Tectoridin and tectorigenin are isoflavonoids.

Methods:
The UHPLC method with mass spectrometry (MS/MS) detection was optimized for the separation of all 13 analytes. For the pre-treatment of rat plasma samples, microscale extraction approaches including μ-SPE-PT (micro-solid phase extraction in pipette tips) and MEPS (microextraction by packed sorbent), but also non-selective PP (protein precipitation) were used.

Results:
The metabolites with significantly different physicochemical properties make the simultaneous determination quite challenging. Considering the critical pair of isomers (tamarixetine and isorhamnetin), BEH Shield RP C18 (2.1 x 100 mm; 1.7 μm) column and gradient elution with methanol and 0.1% aqueous solution of formic acid offered the best selectivity. The Xevo TQ-XS mass spectrometry was employed and the conditions for all compounds were also thoroughly optimized. Due the instability of some phenolic acids (homovanilic acid, 3,4-dihydroxyphenylacetic acid) in ion source, the Xevo TQ-S mass spectrometer detector was replaced with Xevo TQ-XS. This mass spectrometer contains different type of ion optics more suitable for targeted analytes. All compounds were analyzed in the mode SRM (selective reaction monitoring), while SIM was often needed at other platforms.

Conclusions
The UHPLC-MS/MS method was optimized and validated in the terms of sensitivity, linearity and matrix effects. Using Xevo TQ-XS mass spectrometer, all analytes could be quantified with linearity range between 0.1 – 1000 ng/mL.

Novel Aspect:
Due the instability of some phenolic acid analytes in ion optics, the Xevo TQ-S detector was replaced with Xevo TQ-XS.

This work was supported by the STARSS project (Reg. No. CZ.02.1.01/0.0/0.0/15_003/0000465) co-funded by ERDF, by SVV 260412 and by GACR 301/17/05409S.
Exploring Serum Targeted Metabolomics Mass Spectrometry Profile Analysis to Implement Risk Criteria for First-Degree Relatives (FDR) of Gastric Cancer (GC) Patients.

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Keywords: Metabolomics, Gastric cancer; first degree relatives; Biomarkers; Early diagnosis; introduce

Introduction: A positive family history is a strong and consistently reported risk factor for gastric cancer (GC) [1]. The main purpose of this study was to investigate serum metabolomic profiles to find additional biomarkers that could be integrated with serum pepsinogens and gastin test to improve the diagnosis of GC and the selection of first-degree relatives (FDR) at higher risk of GC development.

Methods: The targeted serum metabolomic profile included 188 metabolites, covering amino acids, biogenic amines, acylcarnitines, phosphatidylcholines, sphingomyelins and hexoses. Metabolite profiles were performed by liquid chromatography and flow injection tandem mass spectrometry analysis. Differential metabolomic signatures among the FDR (n=54) and GC (n=71) groups were investigated by univariate and multivariate analysis.

Results: Forty metabolites mainly belong mainly the phospholipids and acylcarnitines classes were significantly altered between FDR and GC patients. Nine metabolites resulting from the training set were further confirmed in the validation set. Compared with FDR, GC patients were characterized by lower levels of hydroxylated sphingomyelins (SM(OH)22:1, SM(OH)22:2, SM(OH) 24:1) and phosphatidylcholines (PC ae 40:1, PC ae 42:2, PC ae 42:3) and by higher levels of acylcarnitines derivatives (C2, C16, C18:1). The sensitivity and sensibility of these metabolomic biomarkers to distinguish potential cancer conditions was 73.47% and 83.78% respectively with AUC of ROC curve of 0.811 that improves to 0.90 when integrated with age and the serum level of serum pepsinogen II.

Conclusions: The results of the study for the first-time described serum metabolomic profiles that discriminate GC patients from FDR sharing the same environment and a similar genetic background. As compared with FDR, the GC patients showed specific serum metabolomic signatures characterized by an increase in specific acylcarnitines and a decrease in a distinctive subclass of sphingolipids.

Novel Aspect: The study underlines the role of the use of the individual's serum tandem mass spectrometry metabolomics profile to complement the triage of FDR at higher risk of GC development

References
793 - PHYTOCHEMICAL SCREENING OF FAGIOLI BIANCHI DI ROTONDA BEANS DOP (PHASEOLUS VULGARIS L.) AND EVALUATION OF THEIR BIOLOGICAL ACTIVITY FOR FUNCTIONAL FOOD PROPERTIES

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Keywords: Fagioli Bianchi di Rotonda, FT-ICR-MS, Phytochemical Screening, Hypoglycemic Activity, Metabolomics

Introduction: The aim of this study was to evaluate the metabolome of Fagioli Bianchi di Rotonda beans (Phaseolus Vulgaris L.), cultivated in Basilicata (Southern Italy), labelled with DOP mark quality, in order to promote their nutraceutical properties. In this study, a metabolomeuntarget analysis by HRMS was performed.

Methods: High-resolution mass spectra were acquired on a Bruker (BrukerDaltonik GmbH, Bremen, Germany) solariX XR Fourier transform ion cyclotronresonance mass spectrometer (FT-ICR-MS) equipped with a 7 Tesla superconducting magnet and anESI source in the negative ionization mode. The nutritional compositions including starch, fat, protein and phytochemicals were analyzed.

Results: A shotgun approach, based on direct infusion negative-ion ESI ultrahigh resolution mass spectrometry (FT-ICR-MS), was employed for the rapid analysis of metabolites occurring in all extracts of Fagioli Bianchi di Rotonda beans. Such non-targeted analysis generates a tremendous amount of data and requires visualization strategies to convert lists of accurate m/z values into metabolomic context. Visualization strategy using van Krevelend diagram was adopted. This diagram displays the hydrogen/carbon (H/C) vs. oxygen/carbon (O/C) ratios of these elemental formulas and provide a qualitative description of the molecular complexity of Fagioli Bianchi di Rotonda data. This plot enabled the localization of chemical species according to class metabolites, as carbohydrates, glycosylated compounds, peptides, polyphenols and saponins. The antioxidant activity and α-glucosidase inhibition activity of extracts were evaluated. All the samples possessed strong ABTS free-radical-scavenging capacity and α-glucosidase inhibition activity.

Conclusions: In this study, an identification of different classes of secondary metabolites in Fagioli Bianchi di Rotonda, ecotypes Bianco and Tondino, was carried out. Some of these compounds are known to show bioactivities interesting for human health. Results of biological activity assays highlighted the importance of the use of such food product for nutraceutical applications.

Novel Aspect: This study shed some light on metabolic profiles of Fagioli Bianchi di Rotonda and their nutraceutical properties. Results can be used for comparative studies.

References

1133 - TWO-DIMENSIONAL GAS CHROMATOGRAPHY AS A POWERFUL TOOL FOR DISCRIMINATING VOCs IN MONOVARIETAL AND COMMERCIAL EXTRA VIRGIN OLIVE OIL

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Keywords: GCxGC-TOF-MS, EVOO, VOCs, oils quality, LOX pathways, terpenes.

Introduction:
The unique and special flavour of Extra Virgin Olive Oil (EVOO) can be attributed to VOCs such as the C6 and C5 aldehydes, which are responsible for green aromatic notes, but also terpenes and sesquiterpenes, which tend to generate greater aromatic complexity in quality oils. The identification of the compounds responsible for flavour or off-flavour attributes is considered one of the keys for EVOOs quality control and a good indicator of of olive oil quality changes.

Methods:
Sixteen monovarietal EVOOs produced from different cultivars (purchased directly from producers) were compared with 25 blended commercial EVOOs (purchased in national grocery stores).

EVOO VOCs were analysed with HS-SPME-GCxGC-TOF-MS[1]. A total of 1254 putative compounds were detected, with 123 being significantly different in monovarietal and commercial oils. Compound identity was confirmed with Kovats retention index and by comparing spectra compounds to NIST 2.0, Wiley 8 FFNSC 2 (Chromaleont, Messina, Italy).

Results:
Principal component analysis (PCA) showed clear separation between the two classes of samples. Specifically, commercial samples were more influenced by compounds such as alcohols, acids (acetic acid and formic acid) and other acetates frequently responsible for certain unpleasant notes. The monovarietal samples were more influenced by compounds deriving from LOX pathways (aldehydes C6 and C5), as well as terpenes and sesquiterpenes, such as valencene, copaene and ocimene for example, which generally generate pleasant wood, green and lemon notes. Furthermore, differences in the composition of monovarietal samples were evaluated. Two varieties (Casaliva and Tonda Iblea) were clearly distinguished from the rest of the monovarietal samples, underlining that the cultivar and pedoclimatic conditions can influence VOC composition.

The preliminary results show that HS-SPME-GCxGC-TOF-MS is a fast and very powerful tool for revealing differences between the aroma of different classes of EVOO products.

Conclusions:
The present investigation demonstrates that volatile compounds can be used as reliable indicators of Extra Virgin Olive Oil quality. In detail, between commercial and monovarietal oils, significant qualitative differences were found in a large number of volatile compounds, highlighting the possibility to use terpenes, sesquiterpenes and aldehydes how markers for quality olive oil aroma.

Novel Aspect:
Two-dimensional gas chromatography is a novel method to discriminate EVOOs quality and aroma. It applicability can reveal the differences of a large number of volatile compounds, analyzing the macro and micro differences in this matrix.

References:
Acknowledgements: The research was supported by the Autonomous Province of Trento (Italy) and we thank Agririva – Agraria Riva del Garda for kindly providing the samples.
Introduction: Pancreatic ductal adenocarcinoma (PDAC), one of the most aggressive solid tumours of the pancreas, is characterized by a remarkable resistance that also depends on the presence of the so-called cancer stem cells (CSCs) [1,2]. It is evident that, to make an impact on pancreatic cancer, it is necessary to eradicate CSCs with targeted therapies and, for this reason, a complete molecular characterization of CSC biology is fundamental.

Methods: The MIAPaCa-2, PaCa3 and Panc-1 CSCs and relatives parental cells were cultivated and subjected to metabolomics and proteomic analyses. Different protocols have been evaluated to improve the identification of metabolites from adherent and suspended cell lines. Different extraction approaches and different derivatization approaches have been evaluated and compared.

Results: The results obtained indicate that CSCs are characterized by downregulation of succinate, fumarate, and malate and by upregulation of glutamate, in comparison to the parental cell lines. A reduction of Krebs cycle intermediates and an increase of a Krebs cycle-related metabolite may suggest a switch to a less oxidative metabolism. The results of the proteomic analyses are still in the collection phase. Preliminary data suggest that a total of 121, 188 and 186 proteins are modulated in Panc-1, PaCa-3 and MIA PaCa-2 CSCs as compared to the parental cells with p-value \( \leq 0.05 \) and fold change \( \geq 1.5 \), respectively.

Conclusions Altogether our findings may clarify some critical aspects of the metabolic network signature of PDAC and may serve as a relevant pool of metabolites and proteins from which biomarkers and therapeutic targets can be identified. Further analysis of biological replicates and validation of results are required to confirm our preliminary data.

References

Introduction:
In this study, we want to set up a targeted assay to measure the concentration of six steroids from salivary samples of pregnant women, beginning from the first trimester of pregnancy to a few weeks postpartum. Saliva has potential clinical applications in maternal monitoring and represents a less invasive alternative to serum or plasma for the measurement of steroid hormones. However, little information is available regarding the range of salivary steroid concentrations during pregnancy [1, 2]. Steroid hormones in saliva derive primarily from free steroid present in the general circulation and their concentrations can be regarded as the bioavailable fraction. Our aim is to establish an accurate MS-based assay for quantification of six steroids in saliva, reaching great sensitivity and precision.

Methods:
Steroid hormones are extracted from the biological matrix employing a methanol extraction protocol. After reconstitution in an aqueous buffer containing an internal standard, they are analyzed using a targeted approach by SRM technology on a nanoLC-MS system (Thermo TSQ-Quantiva coupled to a Waters M-class UPLC). Our targets include cortisol, cortisone, estradiol-17β, estriol, progesterone, dehydroepiandrosterone (DHEA), and testosterone. A minimum of three transitions are selected per each target compound, with optimized collision energy, to ensure high selectivity and great sensitivity. The LC is set up in RP mode, with a C18 column (200µm x 5cm), a flow rate of 2 µL/min and a gradient of 10 minutes. Calibration curves are included in the analysis for accurate quantification. The data will be investigated using a combination of Xcalibur Quan Browser and TraceFinder.

Results:
We have fully developed and optimized an SRM assay to include our six steroid targets in the analysis. For the purpose, commercially available reference compounds were directly infused into the MS to select the best precursor ion and three to four fragment ions, optimizing collision energy (among other parameters) per each fragment. Also, the LC gradient was optimized to have each peak, corresponding to each compound, chromatographically well resolved. Calibration curves containing all our compounds simultaneously, plus an internal standard to normalize for instrument variations, were built ranging from 0.4 to 400 nM. The curves showed good linearity and, for most of the compounds, the LLOQ was below 1 nM. A test phase was conducted on saliva samples (100µL) collected from pregnant women upon awakening at different stage of pregnancy. These test samples were employed to refine the extraction procedure and evaluate sample recovery.

Conclusions
Targeted metabolomics employing SRM assays allows us to investigate a broad panel of steroid hormones in biological fluids, such as saliva sample. The study will shed light on the course of the steroid concentrations in saliva across pregnancy and into the early postpartum period.

Novel Aspect: (Limit of 150 characters without spaces)
Our MS assay will represent a fast and reliable tool to measure steroid concentrations in the saliva of pregnant women.

References

Introduction: Rhizosphere: the belowground black-box
The plant root is embedded in a multiple organism network: the rhizosphere. For reactions towards different abiotic stresses [1, 2] or to interact with the microbial soil communities and neighboring plants [3-7], roots release metabolites called exudates. Until now, there is little known about these chemicals in natural habitats. Therefore, we applied non-targeted metabolomics combined with ecology to analyze exudate patterns in natural grassland communities.

Methods: Exudate analysis under field condition
Ten common European grassland species were grown under field conditions, exudates were directly collected from roots and subjected to non-targeted metabolite profiling by UPLC coupled to ESI-Q-TOF mass spectrometry and the combination of different software. Selected compounds were chemically classified on the level of metabolite families by the use of MetFamily. The method for exudate isolation was proven to be appropriate by control experiments.

Results: Exudates: species specific and environmentally influenced
Using our approach, more than 2800 compounds from samples of field grown plants were annotated. Different statistical tools revealed the release of semi-polar metabolites in a growth form dependent manner with species-specific compound patterns. This is more distinct in forbs than in grasses, which showed a less differentiated metabolite composition. In order to identify the main factors responsible for the exudate patterns, the species itself, plant functional traits, neighboring plants and environmental conditions were considered. In case of the five forbs, target species identity was determined as the main driver for exudate composition, while environmental conditions are decisive in case of the five grasses. Nevertheless, there are no specific metabolite classes released by single species. Rather, most of the detected specific metabolites could be putatively classified as members of the typically defense related metabolite families of flavonoids, coumarins, isoprenoids and phenylpropanoids, but also sugar related metabolites.

Conclusions: MS exudate analysis under field condition is feasible
Our analysis demonstrates the possibility to obtain and analyze root exudates in field experiments. The species specificity of root exudate patterns appears either to be driven by interspecific differences or by the environmental conditions, depending on the investigated growth forms. The putative classification on the level of metabolite families delivered a broad spectrum of metabolites of the secondary metabolism.

Novel Aspect: Novel method collection for analyzing exudates
We demonstrate the feasibility of analyzing exudates under field conditions by mass spectrometry and the benefits of EcoMetabolomics study.
Introduction
Metabolomics focuses on the chemical processes central to cellular metabolism. Mass spectrometry and specifically data dependent workflows tend to be the choice for the measurement of these metabolites. Data independent techniques such as SWATH® Acquisition are different in that they allow for unbiased data collection and MSMS of every single mass precursor can be collected allowing for information rich datasets. However, unambiguous metabolite identification can be increasingly challenging due to the lack of databases, chemical noise and isobaric compounds. The SWATH analysis of the Isotope Ratio Outlier Analysis (IROA) labeled Internal Standard (IS) provides the first mechanism for simultaneous and unambiguous compound identification and quantitation for unbiased metabolomics analysis.

Methods
A biochemically complex Internal Standard (IS) which contains 100’s of biochemicals, each with an IROA isotopic pattern, was added to clinical samples to accurately identify and quantitate complex mixtures without the need for baseline separation, and overcome variances introduced sample-to-sample or by ion suppression. SWATH fragmentation of the IROA peaks completely differentiates fragments, and artifacts. The identification of all IROA compounds and their fragments by ultra-high-resolution mass measurement make it possible to determine the empirical formula for all fragments. Data were collected a TripleTOF® 6600 System in SWATH® Acquisition using a variable window strategy which defined windows of varying mass ranges to be applied in areas of the chromatogram where there are many ions co-eluting.

Preliminary data
Applying a variable window SWATH Acquisition strategy to an IROA Internal Standard (IS) spiked sample made it possible to unambiguously identify and accurately quantify hundreds of biochemicals in a single unbiased metabolomics analysis using a TripleTOF® high resolution mass spectrometer. The IS contains 500+ well characterized compounds, which migrated in an HPLC separation with their natural abundance isotopomers, to provide for both identification and standard quantities for accurate measurement even in a non-baseline separated, “unbiased” metabolomics separation. Using traditional DIA, all compounds with the same retention time are fragmented without selection. SWATH DIA subjects all ions within a discrete m/z window to fragmentation allowing specific precursor ions to be selected, making it easier to analyze fragmentation spectra. However, a corresponding spectral library of metabolites is required for accurate identification. Here, we present SWATH-IROA DIA whereby uniquely-labeled IROA metabolites were captured within discrete SWATH windows, and subjected to fragmentation. IROA fragments and adducts were shown to have the identical labeling patterns of their precursor ions, with defined formulae. All artifactual (non-IROA) peaks from the SWATH window were eliminated and data was quantitated based on MSMS peaks. The combination of IROA and SWATH allow a path in which a basic metabolomic-style system may be used for the accurate clinical quantitation of several hundred compounds in a single sample without the need for a base-line separation. Specific software was developed to automatically find, quantitate and identify all natural-abundance peaks that corresponded to their known IROA isotopomers. The identification of compounds of unknown identity is simplified because all fragments are identified by their complete formula making the mode of fragmentation of the parent compound clear. Fragments are identified as they share the daughter fragments of their parent compounds.
Untargeted Metabolomics in Plant Biochemistry

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Keywords: Untargeted metabolomics, LC-MS, plant science, substance annotation, functional genomics

Introduction: Search for differentially accumulated metabolites in plant science

A frequent task not only in plant sciences is to find biomarkers for certain conditions (“treatment vs. control”), find chemical differences and similarities between cultivars, subspecies or species and to aid functional genomics by determining the effects of single mutations on the metabolome. This is done by untargeted metabolomics, a technique that works without prior knowledge of the metabolites’ identity.

Methods: Experimental setup for UPLC-HR-Q-ToF MS

For this task, we established a workflow starting from an appropriate experimental setup including numerous quality checks for liquid chromatography coupled to High Resolution Q-ToF MS and MS/MS. This is followed by data analysis with XCMS[1] and MetaboScape (Bruker) including appropriate statistical Methods: A combination of tools is applied for substance annotation and identification.

Results: A combination of tools is a start to a nearly comprehensive annotation

Our approach extracts substances of significance with a very high confidence. Limited by the extraction protocol and LC method, the focus is on semipolar compounds. After determining relevant features, the challenge is the identification of these unknown metabolites: In-house libraries are the basis for batch processing of whole feature tables in MetaboScape. This batch search is extended by publicly available databases (Massbank [2], GNPS [3], ReSpect). Further annotation is done using the in-silico fragmentation tool MetFrag[4]. It is clear that most of the estimated 200,000 metabolites in the plant kingdom are not yet included in databases and therefore, cannot be annotated easily. But, in many cases it is already sufficient to know the compound classes of differentially accumulated metabolites. MetFamily [5] groups features with available MS/MS information according to common fragments or neutral losses and allows a survey of regulated compound classes.

Conclusions: A workflow for automated annotation of known and clustering of unknown metabolites

Advances in processing large metabolomics data sets, the growing wealth of publicly available databases and the development of customized tools for metabolite annotation largely facilitates the batch annotation of previously known and unknown metabolites of biological relevance.

Novel Aspect:
A comprehensive approach to annotate metabolites of biological relevance.

References

Targeted metabolomic analysis of urine for improving the diagnosis of asthma and COPD

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Introduction: Asthma and COPD are chronic inflammatory conditions of the respiratory airways. Their differential diagnosis can be hampered by overlapping clinical presentations. Currently, physicians rely on patient history and trials of therapy. Therefore, there is a need for new diagnostic tests. A recent untargeted exploratory 1H-NMR metabolomic study [1] suggested 50 urinary metabolites as candidate biomarkers for the differentiation of asthma and COPD.

Methods: The 50 metabolites were divided into 4 groups. Groups 1 and 2 (37 targets) contain amine and carboxylic acid-containing metabolites. Two LC-MS/MS methods for their quantification was developed using differential isotope labelling (DIL) with dansyl chloride and dimethylaminophenacyl bromide, respectively. Group 3 contains 7 metabolites of diverse structures. Therefore, HILIC-MS/MS method was developed. All methods were fully validated for clinical testing.

Results: All methods were fully validated according to the FDA and European Medicines agency (EMA) guidelines[2, 3]. While almost all of the validation criteria were still applicable, in some situations, the guidelines were insufficient to drive the validation process. Accordingly, uncommon analytical or statistical approaches were adopted. In addition, the use of DIL with 2 mass unit difference between the derivatized analyte and its internal standards necessitates the optimization of the analytical methods to account for isotopic interferences. Non-blinded patients’ samples with asthma and COPD were analyzed and the data was processed using partial least square discriminant analysis. A set of fifteen metabolites was found to differentiate asthma from COPD with high accuracy ($R^2 0.935 \ Q^2 0.864$). The constructed model was then tested using a blinded subset of subjects, where an accuracy of diagnosis of 80% for asthma was achieved. However, COPD had modest accuracy; examination of clinical information suggested misdiagnosis for some of these patients.

Conclusions: Novel validated analytical methods were developed for clinical application. Promising clinical results were obtained which may improve the management of airway diseases. Of the 15 metabolites identified by statistical analysis from the mass spectrometry data, eight metabolites were common, as critical for disease diagnosis, to the previously reported 1H-NMR study [1]. Therefore, this work also validates the diagnostic importance of these metabolites.

Novel aspects: New LC-MS targeted methods are developed, providing insights into targeted metabolomics method validation. 15 urine biomarkers are identified for respiratory illnesses.

A method for group 4 metabolites is currently under development.

INFORMED UNTARGETED METABOLOMICS USING LC-MS/MS

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Keywords: untargeted metabolomics, data-dependent acquisition, LC-MS/MS, Orbitrap, library

Introduction:
Metabolite identification is the main challenge in untargeted LC-MS based metabolomics. Although there are libraries available, they suffer some limitation. Therefore an LC-MS² standards library of high resolution and accurate mass spectra was generated. Another aspect is the inevitable bias in every metabolomics study, due to the diversity of the metabolome. To assess this bias the LC-MS² standards library can be used (Informed unTargeted Metabolomics).

Methods:
An automated flow injection/t-SIM/ddMS² method was developed to generate various MS² spectra of more than 600 synthetic standards of metabolites (MS² library). Chromatographic data was acquired under different conditions (LC library). Using a hybrid quadrupole-orbitrap mass spectrometer (Q-Exactive™, Thermo scientific™) allows high-resolution and accurate-mass detection. Data analysis was performed by using FreeStyle, Library Manager and Tracefinder.

Results:
We developed a method to acquire MS² spectra at different collision energies without the need for manual injection. Data-dependent acquisition proved superior to other scan modes as it offers high-quality MS1 and MS² spectra. Due to flow injection, several different MS² spectra could be acquired for every metabolite. Using this flow injection/t-SIM/ddMS² method, we generated about 4000 MS² spectra for over 500 metabolites. LC data was acquired for 295 metabolites in 139 unique conditions. Chemical properties of the metabolites will be included in the LC-MS² library as well. To further expand the library, more compounds, LC conditions and also extraction methods will be tested. By utilizing a certain extraction and LC method, an analysis is shifted to a subset of metabolites, that can be estimated by comparing features of metabolites already detected using this workflow and documented in the library. Informed unTargeted Metabolomics can be adjusted to cover the highest number of metabolites possible or to analyze specific metabolites of interest.

Conclusions:
For unambiguous metabolite identification high mass accuracy, high-quality MS² spectra and retention times under specific chromatographic conditions are essential – information that is included in the LC-MS² standards library together with chemical properties of the metabolites. Informed unTargeted Metabolomics uses this information to assess or determine the bias that goes along with the impossibility to cover the whole metabolome with a single workflow.

Novel Aspect:
Using a novel flow injection/SIM/ddMS² method on a hybrid quadrupole-orbitrap instrument we developed a LC-MS² library, that can be used for Informed unTargeted Metabolomics.
AN INVESTIGATION OF BIOMARKERS IN THE SERUM OF DEVELOPMENTAL DISORDERS USING AN INTELLECTUAL DISABILITY MODEL MOUSE, WHICH SHOW IMPAIRED SOCIAL BEHAVIOURS

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Keywords: Developmental Disorder Model, Motopsin Knockout (KO) Mice, Biomarkers, Serum, High Resolution LC/MS

Introduction:
Our aim is identifying biomarkers of developmental disorders by a liquid chromatography/mass spectrometry (LC/MS). In the present study, serum samples of motopsin KO mice were used for the exhaustive analysis of biological metabolites-amino acids. We demonstrated the exhaustive analysis of mouse serum using a reversed-phase separation mode and an electrospray ionization method of the LC/MS, and compared metabolites between motopsin KO and wild-type (WT) mice.

Methods:
Acetonitrile and 1% acetic acid, as well as 15N-glutamic acid as an internal standard, were added to serum samples of motopsin KO and WT mice, and the solution was centrifuged. After the solid-phase extraction of the supernatant using a ODS cartridge, the eluent was injected to the Intrada Amino Acid column (Imtakt). The high resolution LC/MS system is composed by Accela (LC) and Orbitrap Exactive (Thermo Fisher Scientific).

Results and Discussion:
Various kinds of amino acids, neurotransmitters, and their metabolites were simultaneously separated and detected by the LC/MS method using standard reference materials. To analyze the effects of genotype and sex, we performed two-way analysis of variance to compare serum concentrations of those substances between KO (male, n=5; female, n=3) and WT mice (male, n=4; female, n=5). Serum concentrations of Glu, Gly, and Asn were significantly higher in KO mice than WT mice (p=0.016, p=0.028, p = 0.010, respectively), whereas that of DOPAC was lower in KO mice than WT mice (p =0.003). Interestingly, post hoc analyses revealed that such difference was significant in males, but not females. Asp, Ser, dopamine, and DOPAC showed higher concentration in males than females (p=0.006, p= 0.044, p=0.000, p=0.000, respectively). Glu and Gly are neurotransmitters and Asn affects the metabolism control of the cell function of the nervous system and the brain tissue. DOPAC is a metabolite of the neurotransmitter dopamine and is degraded to form HVA. Interestingly, the current result is consistent with our previous research demonstrating significantly higher concentration of Glu in saliva of autism children compared to typically developing children. According to the above results, possible biomarkers of developmental disorders are suggested. It will be necessary to increase the number of subjects for identifying biomarkers with statistical reliability.

This work was supported in part by JSPS KAKENHI Grant No.17K18650.

Novel Aspect:
We demonstrated a possible strategy to find biomarkers of a developmental disorder based on a comparative LC/MS analysis of serum samples between motopsin KO and WT mice.

References:
Electrochemical oxidation is a rapid, relatively inexpensive approach to simulate natural metabolic redox events and potential subsequent reactions in vitro [1,2]. This makes it possible to study biologically relevant oxidative and reductive processes in an organism and thus, to replace animal studies within the meaning of the directive 2010/63/EU [3]. In this case, coupling of an electrochemical and a mass spectrometric system should simulate phase I and phase II metabolism of bile acids.

Methods:
Electrochemical oxidation was performed using a ROXY™ EC system (equipped with a µ-Prep cell consisting of a BDD working electrode, a titanium counter electrode and a Pd/H2 reference electrode). A solution containing 100 µM CA/CDCA was injected into the EC-system. First scan mode was applied (0 V to 3 V in 0.3 V steps). Afterwards a constant potential was used. Detection of oxidation products was performed with an ESI-MS ion trap mass analyzer in positive ion mode.

Results:
First, the applicability of an electrochemical oxidation for transforming bile acids was evaluated. Simulation of endogenous metabolism of primary bile acids was successful. It was shown that it is possible to oxidize cholic acid and chenodeoxycholic acid under various conditions. Best parameters were acidic pH values and a constant potential (DC-mode). It was also shown that switching to pulse-mode (as mentioned in literature [4]) did not increase the yield. Moreover, it was possible to generate mg-amounts of the oxidized products with a SynthesisCell™ and a general comparison to mass spectra of commercially available bile acids standards.

Conclusions:
Electrochemical oxidation using a ROXY™ EC system coupled to a mass spectrometric system is suitable to simulate phase I and phase II metabolism of primary bile acids. It is possible to investigate biosynthetic pathways without using animals or animal tissues.

Novel Aspect:
Applicability of an electrochemical oxidation of cholic acid and chenodeoxycholic acid using a ROXY™ EC system was successfully demonstrated.

References:
TARGETED ON-LINE BREATH ANALYSIS SUPPORTS THE HYPOTHESIS OF ALTERED COLLAGEN TURNOVER IN IDIOPATHIC PULMONARY FIBROSIS

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Keywords: on-line breath analysis, idiopathic pulmonary fibrosis, biomarker, secondary electrospray ionization – mass spectrometry

Introduction:
On-line breath analysis is a powerful technique to obtain insights into the metabolism of a person. Idiopathic pulmonary fibrosis (IPF) is a chronic and poorly understood lung disease. Following a recent study that reports increased levels of collagen related amino acids in lung tissue of IPF patients using GC-MS profiling[1], we hypothesized that these altered amino acid levels might be mirrored in exhaled breath, which would allow for a non-invasive screening of IPF.

Methods:
Breath analysis was performed on-line using secondary electrospray ionization-high resolution mass spectrometry (SESI-HRMS). For all previously reported target compounds where a signal was detected at the accurate mass in real-time, UHPLC-MS measurements of exhaled breath condensate (EBC) were performed, to confirm their identity. Their classification performance was estimated using 1 million leave-one-out cross-validations.

Results:
On-line breath spectra were recorded for 21 IPF patients and 21 healthy controls, matched in terms of age, gender and smoking history. Within these, we could detect robust signals for proline, 4-hydroxyproline, alanine, valine, leucine/iso-leucine, allysine, phenylalanine and pyroglutamic acid, most of which could also be confirmed in EBC. Six of the eight compounds showed significantly increased signal levels (p<0.05) in exhaled breath of IPF patients. Additionally, we observed a strong signal correlation across subjects, and those amino acids with a higher abundance in collagen or elastin showed the most pronounced effect. This is consistent with altered collagen and elastin turnover being the underlying metabolic processes. Using the signals of all detected amino acids, we were able to obtain a cross-validated area under the receiver operating characteristic curve of 0.86.

Conclusions:
Using targeted on-line breath analysis with SESI-HRMS, we could detect increased amino acid levels in IPF patients, which allowed for a good discrimination from healthy controls. This is consistent with previous metabolomic findings from lung biopsies, strongly reducing the probability of false discoveries. However, we were able to capture this information in a non-invasive and rapid fashion, underlining the strength of real-time breath analysis.

Novel Aspect:
Our results suggest that on-line breath analysis using SESI-HRMS might be a valuable tool for screening of IPF, thereby reducing the time of diagnosis as well as medical costs.

References:
Introduction:
Diagnostic of human brain tumors has developed greatly in the recent few years. Proper classification of tumor type plays significant role in prediction of the response to a given treatment, but histology often fails in this regards and genotyping is used instead. However, several mutations can affect therapy efficiency and identification of molecular biomarkers could be useful complement to current testing.

Methods:
In the current study, described approach was applied to human brain tumors. To provide minimal tissue damage ca. 0.2 mm diameters probe had been used to extract metabolites from brain tumor sample. For chromatographic analysis two types of stationary phase had been used: pentafluorophenyl (PFP) and underivatize silica (HILIC). The samples were analyzed on Q-Exactive Focus mass spectrometer and Compound Discoverer 2.1 were used for data processing and statistical analysis.

Results:
Metabolomics studies with SPME-LC-HRMS showed that there are several compounds which discriminate tumors of different origin as well as gliomas of different malignancy. Identification of these features revealed involvement of creatine, hypotaurine, cystathionine, etc. in cancerogenesis of gliomas, which was already reported in the literature based on cell line study. It was investigated, that higher concentration of other from those above mentioned naturally produced in the body aminoacids and its homologues indicates the presence of high grade of malignancy glioma in examined patient. Moreover in current study it has been shown that there are several endogenous metabolites that differentiate high grade of malignancy glioma in patients with genetic mutations (IDH1, IDH2, metylation, deletion 1p19q) from those without that mutation. Comparison of the data obtained from genotyping and metabolomics profiling (phenotyping) exhibited very good correspondence.

Conclusions
According to performed experiments in the current study, the use of SPME fibers for extraction and separation via liquid chromatography coupled to high resolution mass spectrometer provided an appropriate workflow for comprehensive brain tumor metabolomics analysis. The simplicity of the approach when compared to routinely used diagnostic methods indicates a great potential of the method to serve as an intra-operative tool in the future.

Novel Aspect:
The experimental results obtained using low invasive sample preparation method coupled with LC-MS provide new insight into the diagnostic options of human brain tumors.

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Introduction: How fast is carbon transferred from leaves to roots and exuded?
Plants interact with their belowground environment by the secretion of compounds, a process called exudation [1]. Little is known about this exudation process, exudate biosynthesis and upstream metabolic dynamics. To resolve metabolite origins, we used 13CO2 to track labelled carbon in metabolites from fixation by photosynthesis via transport into roots down to exudation with respect to time dependent dynamics covering light and dark phases.

Methods: 13C labelling and spatio-temporal metabolite profiling of Arabidopsis thaliana
Arabidopsis thaliana plants were pulse labelled in a 13CO2 atmosphere. Aerial plant parts, roots and exudates were collected 3, 6, 9, 20 and 23 hours after labelling and analyzed for primary and secondary metabolites using GC-MS and HPLC-MS. Metabolite labelling was quantified by the investigation of shifts in the isotope pattern in the MS. An XCMS based analysis pipeline was developed for the detection and quantification of isotopes in GC-MS and LC-MS data.

Results: Arabidopsis thaliana - Labelling dynamics of metabolites
In GC-MS, up to 14000 peaks were detected and clustered in isotopes, resulting in more than 1000 isotope patterns for approximately 350 peak groups (metabolites). Fast labelling was observed in mono- and disaccharides, amino acids and organic acids. Tissue distinction and time-scale harvesting demonstrated that labelling often followed a time dependent delay from leaves (early labelling) to roots to exudates (late labelling); however, spatio-temporal labelling dynamics were still metabolite specific. Especially in saccharides, interesting labelling patterns were observed during the dark phase.
In LC-MS, up to 15 000 peaks were clustered in about 500 peak groups that contained more than 1500 isotope patterns. Labelling was observed in many metabolites, e.g. glucosinolates and related compounds in leaves, roots and exudates, providing the opportunity to compare labelling between tissues and metabolites. Co-labelling dynamics pointed to common biosynthetic pathways and metabolite classes and facilitated metabolite identification.

Conclusions: 13C tracking in metabolites from leaves to exudates: Biosynthetic connections
This dataset provides insight into the dynamics between 13C fixation in the aboveground plant, transportation into the roots and exudation of metabolites. The comparison and correlation of labelling patterns revealed biosynthetic connections and consequently facilitated metabolite identification (e.g. fatty acid amines). Time dependent sampling resolved changes in metabolite labelling after switches from light to dark phase and back to light.

Novel Aspect: The first MS based study of 13C tracking through leaves, roots and exudates in Arabidopsis
We applied MS based 13C labelling analysis of Arabidopsis plant tissues and exudates using GC-MS and LC-MS for a comprehensive profiling of primary and secondary metabolites.

References
Metabolomic Approach to Investigate Metabolite Alterations Attributed to a 25-Hydroxyvitamin D in Healthy Korean Adults

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Keywords: 25-hydroxyvitamin D [25(OH)D], UHPLC-QTOF/MS, untargeted metabolomics analysis

Introduction: Vitamin D deficiency is prevalent in Korean adults, affecting up to 80% of them [1]. Vitamin D deficiency, which impairs bone mineralization, may induce many diseases including chronic autoimmune, infectious diseases, cancer, cardiovascular disease, and diabetes. This appears to be associated with levels of the main metabolite, 25(OH)D [2]. We examined the association between serum levels of 25(OH)D and metabolite alterations in healthy Korean adults.

Methods: Forty-five healthy Korean adults were divided into two groups based on serum levels of 25(OH)D (23.0 and 10.4 ng/mL on average for high (n=21) and low (n=24) group, respectively). Demographic, anthropometric, and blood profile parameters were measured. Metabolic profiling was performed using UHPLC-QTOF/MS and multivariate statistical analysis. The candidate metabolites were tentatively identified through the search of web databases.

Results: No significant differences were observed in demographic, anthropometric, and blood profile parameters between two groups. For untargeted metabolomic analysis, 47,499 spectral features were detected from UHPLC-QTOF/MS data using XCMS software. Score plots, obtained from principal component analysis and orthogonal projections to latent structures-discriminant analysis (OPLS-DA), showed a tendency that two groups were separated. R2 (goodness of fit) and Q2 (predictability) were 0.654 and 0.125, respectively. Metabolites which had the variable importance in projection value ≥ 1.0 and p-value ≥ 0.05 were identified using web databases such as Human Metabolome database (HMDB) and ChemSpider. These results suggest that serum levels of 25(OH)D are related to metabolic alterations in healthy Korean adults. And we will further study to identify putative metabolites and assess their association with synthetic mechanism of 25(OH)D.

Conclusions: This research suggests that serum levels of 25(OH)D have influence on metabolic alterations in healthy Korean adults. However, further research with larger sample sizes and other clinical trial designs (e.g., administration of 25(OH)D to osteoporotic or elderly populations) is required before solid conclusion can be reached.

Novel Aspect: First untargeted metabolomic approach to investigate the influence of serum levels of 25(OH)D on metabolic alterations in healthy Korean adults

References
Introduction:
Peptidomics, an analytical workflow for systematic identification of short, native and endogenous peptides, has been widely used to study peptides in a wide variety of biological matrices. The workflow of peptidomics is largely diverted from proteomics, including LC-MS/MS and spectrum matching algorithm. However, sensitive and reliable identifications for endogenous peptides are still difficult even with recent mass spectrometry instruments.

Methods:
To overcome the difficulties in the conventional peptidomics technologies, we have developed an analytical framework dedicated to the exploration of naturally occurring endogenous peptides. We created a analytical workflow combining tandem mass spectrometry and a series of dedicated informatics modules that enables highly sensitive exploratory search for endogenous peptides.

Results:
One of the modules creates potential forms of peptides based on user-defined structural properties such as substructures, motifs, potential cleavage sites, post-translational modification and other molecular properties. The predicted forms of peptides can be used as a target sequence dataset of MS/MS peptide search in a top-down fashion. This approach effectively reduces the search space of MS/MS peptide search, therefore increasing the sensitivity of identification. Moreover, in-silico fragmentation spectra of predicted peptides are generated and their spectral similarities are searched against the experimental fragment spectra acquired from sample. This allows “fuzzy” matching to identify peptides with unexpected post-translational modifications or amino acid substitution with high-sensitivity.

Conclusions:
Our analytical framework is built in a modular together with other informatics, data analysis tools, and therefore is flexible and applicable to any kind of peptides in a wide variety of matrices. We discuss the effectiveness and flexibility of our framework by demonstrating identifications of novel endogenous peptides.

Novel Aspect:
A novel analytical workflow to improve identification of endogenous peptides by combining tandem mass spectrometry and a series of dedicated informatics tools.

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Towards the deciphering of the mechanism of action of an experimental drug against Chagas disease, using multi-omics platform

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Keywords: metabolomics, proteomics, lipidomics, NMR, mass spectrometry

Trypanosoma cruzi (T. cruzi), a kinetoplastid protozoan parasite, is the etiologic agent of Chagas disease. The disease affects millions of people and represents a major burden for the socio-economic development of affected countries (mainly Latin America). The mobility of patients infected with Chagas disease makes it a global public health burden. So far, no vaccine exists and current drugs suffer from significant drawbacks, which limit their use in disease endemic areas. In the foreseeable future, the very few drugs currently in clinical development do not offer new alternatives to patients. Thus, there is a need to identify and develop novel drug candidates with new mechanisms of action to overcome the current situation and eventually feed the Chagas clinical trials pipeline.

The objectives of our study were to identify metabolites and proteins markers of the efficacy of an experimental drug, S205, and to determine the related metabolic pathways to formulate hypotheses on its unknown mode of action. To this aim, we focused on uninfected and T. cruzi infected rat myoblast cells, in presence or not of the drug targeting intracellular amastigotes form of the parasite (intracellular phase in the life-cycle of trypanosomes). The drug effect on cells was analyzed after 24h of exposition. We analyzed polar extracellular metabolites by 600 MHz NMR and intracellular lipids and proteins were determined by LC-HRMS using a Q-Exactive (Thermo) and a Q-TOF (Bruker Maxis HD), respectively.

Through our profiling approach, we identified potential markers for infection and drug mode of action. Indeed, the GAPDH (glyceraldehyde-3-phosphate dehydrogenase) protein of T. cruzi was identified as a potential target of the drug S205 by proteomics and metabolomics has shown that the drug affects the energy metabolism and hence, through the impact on Acyl-CoA synthesis, the formation of lipids. Thus, S205 seems to act through a specific target and both technologies applied (proteomics and metabolomics) complement each other in the description of the mechanism.

We have shown the relevance of proteomics and metabolomics approaches to contribute to the elucidation of drug mechanism of action as a complement to classical Methods: The methods are applicable even to complex systems such as mammal cells infected with parasites.
Introduction
Nostoc is a cosmopolitan genus of cyanobacteria found in soils and in symbiotic associations and a prolific producer of bioactive natural products. Nodularin (NOD) is a cyclic nonribosomal pentapeptide, hepatotoxic protein phosphatase inhibitor and tumor promoter. Spumigins are small linear nonribosomal peptide protease inhibitors. Anabaenopeptins are cyclic nonribosomal hexapeptides. Namalides are a cyclic tetrapeptide desstructurally as anabaenopeptin.

Methods
LC-MS analyses were performed with an Agilent 1100 Series LC/MSD Ion Trap XCT Plus System (Agilent Technologies, Palo Alto, CA, USA). High accuracy mass was measured by UPLC-ESI-QTOF mass spectrometer Synapt G2 Si HDMS (Waters Corp.).

Results
In this study we analyzed non-ribosomal peptidomes with LC-MS methods from 18 cyanobacterial genera that had been collected from Brazilian saline-alkaline lakes of the Nhecolândia, Pantanal wetland area. Our objective was to discover novel bioactive compounds. We found a cyanobacterium strain Nostoc sp. CENA543 which produced 18 different peptide variants from which 13 were novel. Peptides belong to four different peptide families namely nodularins[1], pseudospumigins[1], anabaenopeptins[2] and namalides[2]. Pseudospumigins constitute a novel trypsin inhibiting peptide family. Pseudospumigin A inhibited trypsin (IC50 4.5 μM after 1 h). Hepatotoxic nodularin was produced at exceptional high amounts, at same levels as many Nodulariaspumigena strains isolated from blooms from different geographical locations produces nodularin. Together our results demonstrate that the nonribosomal peptidome of Nostoc sp. CENA543 analyzed at gene and metabolite level is strikingly similar to N. spumigena but contains also clear differences.

Conclusions
Natural products are an important source of antimicrobial, antifungal, anticancer, immunosuppressant and other bioactive molecules. We demonstrate that Nostoc sp. CENA543 produces one phosphatase inhibitor peptide group and three protease inhibitor peptide groupspseudospumigins, anabaenopeptins and namalides with 13 novel peptide structures.

Novel Aspect
Complete genome contains biosynthetic gene cluster only for anabaenopeptin so namalides are a module skipping products from the anabaenopeptin biosynthetic pathway.

References
METABOLOMIC APPROACH FOR THE ESTIMATION OF AGE OF BLOODSTAIN

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Keywords: Bloodstain, Elapsed time, Forensic, HPLC Q-TOF, Metabolite

Introduction:
Bloodstain is created by the blood left at the crime scene, and it has a lot of information including genetic information.[1] It is very meaningful because it can identify the time of the attack at the incident. Although various methods have been studied to estimate the age of bloodstain, it has not been applied to forensic field.[2-6] In this study, we identify the metabolites that have changed as time passed to discover markers that we can determine the age of bloodstain.

Methods:
The bloodstains were prepared using filter paper over a 21-day period. Metabolites were extracted from bloodstain and analyzed using high-performance liquid chromatography-mass spectrometry (HPLC Q-TOF). We performed multivariate analysis, Venn diagrams and partial least squares discriminant analysis (PLS-DA).

Results:
We chose 62 candidate molecular features and analyzed partial least squares discriminant analysis (PLS-DA) that the group have been classified with an accuracy of 75.0%, and R2 and Q2 were 0.7513 and 0.6998, each. Ten metabolites were identified matching of METLIN database. Ten of Two metabolites, vitamin D3/cholecalciferol and 1-palmitoyl-2-glutaryl phosphatidylcholine (PGPC), are up-regulated as time passed, whereas ergothioneine is decreased.

Conclusions:
We identified several candidate metabolites that are up- or down-regulated over time so that we are able to measure the age of the bloodstain.

Novel Aspect:
In this study, we aimed to discover metabolites markers so that we can determine the elapsed time of bloodstain.

References:
405 - METABOLOMIC ANALYSIS OF HUMAN PLASMA TO CHARACTERIZE METABOLITE CHANGES ASSOCIATED WITH EXPOSURE TO PERSISTENT ORGANIC POLLUTANTS

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Keywords: persistent organic pollutants, organochlorine pesticides, polychlorinated biphenyls, metabolic syndrome, UHPLC-QTOF/MS, untargeted metabolomics

Introduction: Humans are widely exposed to persistent organic pollutants (POPs) such as organochlorine pesticides (OCPs) and polychlorinated biphenyls (PCBs). Because of their high lipophilicity, POPs are known to bioaccumulate mainly in adipose tissue and to be resistant to biodegradation [1]. Low-dose exposure to OCPs and PCBs is related to metabolic syndrome [2]. Here, we investigated alterations of endogenous metabolites in human plasma caused by OCPs and PCBs exposure.

Methods: A total of 203 subjects (age ≥ 60) participated in this research and serum POPs levels were measured. All subjects were categorized into quartile based on serum concentration levels of OCPs or PCBs. Plasma samples were analyzed using ultra-high performance liquid chromatography (UHPLC)-quadrupole time-of-flight (QTOF)/mass spectrometry (MS), then multivariate statistical analysis was performed. The metabolite features of variable importance in projection value (VIP) ≥ 1.0 were compared with web databases.

Results: A total of 37 POPs, 4 OCPs and 6 PCBs were selected and considered in this study. Through the untargeted metabolic profiling, the 32,301 ion peaks were detected. Score plots obtained from principal component analysis (PCA) and partial least squares-discriminant analysis (PLS-DA) accounted for the relationship among variables, especially Q1 and Q4. The PLS-DA results showed a tendency to divide that Q1 group is separated from Q4 groups by OCPs concentration, not by PCB's. The 4,491 ion peaks were acquired (VIP ≥ 1.0), and considered to contribute separation of two groups. Thirty three compounds were obtained which had a possibility to consider the accurate mass fragmentation pattern. These results suggest that serum OCPs levels can cause metabolic alterations in human. And we will further study to identify putative metabolites that contribute to metabolic changes caused by OCPs exposure.

Conclusions: Through this study, we will show UHPLC-QTOF/MS-based metabolomics research can be a useful tool to evaluate the effects of POPs exposure by characterizing the metabolic alterations in general population by serum OCPs levels and figuring out the relation of POPs exposure and metabolic syndrome.

Novel Aspect: The large scale untargeted metabolomics approach with human plasma to characterize metabolic alterations associated POPs exposure and metabolic disease.

References
A RAPID MICROBORE METABOLIC PROFILING (RAMMP) ANALYTICAL PLATFORM FOR DISCOVERY METABOLOMICS AND LIPIDOMICS

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Keywords: Metabolomics, Lipidomics, Rapid Phenotyping, UPLC-QToF.

Introduction:
Metabolic phenotyping has been employed in large epidemiological studies in the effort to discovery more about human health and disease [1 & 2]. UPLC and hybrid mass spectrometers have been essential tools in analyzing the matrices chosen for these studies [3]. However, long acquisition times per sample (15 – 30 mins) have meant large batches can take days to analyse [2]. Here we have developed a suite of rapid profiling methods reducing acquisition time to ~3 mins per sample.

Methods:
Rodent urine and human plasma extracts (1:4 v/v IPA) were analysed using Waters Acquity I-class UPLC system coupled to a Synapt G2-Si QTof capable of ion mobility separation. Urinary metabolites were chromatographically resolved through HILIC separation with lipids by reversed-phase, both utilizing 5.0 cm X 1 mm i.d. columns. Mobile phases consisted of water, acetonitrile for HILIC and the addition of IPA for lipid, both with ammonium formate.

Results:
Standard LC-MS methods for metabolite and lipid profiling using 2.1 mm i.d. columns were geometrically scaled down, reducing column i.d., cutting analysis times, injection volumes and mobile phase flow rate ultimately increasing the linear velocity. Each method scaling resulted in a reduced overall acquisition time to approximately 1/3 of the original method. Compound standard mixtures were used to confirm that chromatographic performance and analyte retention was not compromised with the method scaling. The relative retention time of 3 marker compounds between the standard HILIC separation and the rapid method were comparable, demonstrating a preservation of the HILIC mechanism. When analyzing biological matrices, the potential peak capacity of the assay was 28, however, enabling the ion mobility function, this increased to 51 with the overall number of compound ions detected doubled. The inclusion of ion mobility separation and resulting CCS values enabled an additional confirmatory database search for compound identification.

Conclusions
A set of rapid LC-MS methods have been successfully employed in profiling polar and non-polar molecules in rodent urine and human plasma from breast cancer patients. A reduction in analytical run time has enabled large batch data acquisition to be reduced by 60%, eliminating the need for multiple sample batches and the risk of inconsistent results. The inclusion of ion mobility has provided an additional dimension to the data and improved compound identification.

Novel Aspect: (Limit of 150 characters without spaces)
Rapid 3 minute long discovery phenotyping assays in conjunction with high resolution accurate mass and ion mobility spectroscopy.

References:
RAPID AND SIMULTANEOUS DETERMINATION OF 23 AMINO ACIDS IN NON-INVASIVE AND INVASIVE BIOLOGICAL SAMPLES FROM OBESITY PATIENTS BY LIQUID CHROMATOGRAPHY-TANDEM MASS SPECTROMETRY AFTER DERIVATIZATION

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Keywords: exhaled breath condensate (EBC) samples, amino acids, derivatization step, LC-MS/MS, bariatric treatment

Introduction
The analysis of free amino acids (AAs) in biological samples is important for diagnosing the health of individuals, because their concentrations are known to vary with various diseases [1-4]. In this study, reliable and high-throughput analytical method for AAs analysis in different biological matrices was proposed and applied to assess their concentration in noninvasively collected breath condensate samples obtained from patients before bariatric treatment.

Methods
A robust and sensitive method for identification and quantification of free amino acids in exhaled breath condensate, plasma, saliva and urine samples, based on liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS) has been developed. Due to the presence of highly polar groups (-NH₂, -OH, -COOH) and their non-volatile nature, amino acids need to be derivatized with methyl chloroformate before LC-MS/MS analysis.

Results
To evaluate the efficiency of the present method, the method was applied to human exhaled breath condensate, urine, plasma and saliva samples collected from obesity patients qualified for surgical bariatric treatment. Adequate preoperative preparation and postoperative care of patients requires profiling of amino acids to control of diet and search potential biomarker of obesity-related diseases. In our developed method, the separation of 23 of physiological amino acids was achieved in 18 min. It was observed that arginine, glutamine, serine, proline, hydroxyproline, glycine, threonine, alanine, proline, methionine, aspartic acid, histidine, lysine, norvaline, glutamic acid, tryptophan, leucine, phenylalanine, isoleucine, asparagine, cystine, cysteine and tyrosine levels are higher in plasma and saliva with reference to breath condensate samples. Only valine concentration are comparable in both saliva and EBC samples.

Conclusions
Methyl chloroformate derivatization followed by LC/MS/MS analysis provides an effective platform for quantitative analysis of amino acids in complex biological samples such as plasma, urine, saliva and exhaled breath condensate samples. The proposed method is rapid, sensitive and can easily be performed in aqueous media. Further it does not require any pre-purification, cleanup and/or lyophilization steps before analysis. The method developed has wide applications for the routine analysis of amino acids in non-invasive breath samples, without the need for blood collection.

Novel Aspect:
A sensitive and direct analytical method has been proposed for the first time for simultaneous determination of twenty-free amino acids in non-invasive EBC samples.
References

Comparative Evaluation of Different Ginseng Berry Species Based on UPLC-QTOF/MS and HR-MAS NMR Metabolite Profiles

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Keywords: ginseng berry, ginsenoside, UPLC-QTOF/MS, HR-MAS NMR

Introduction
Many studies have previously reported the pharmacological activities of ginsenosides from Panax ginseng C.A. Meyer. The interests in therapeutic potential of ginsenosides also have increased. Ginsenosides are distributed in several parts of P. ginseng, including not only root but also leaf and berry. Different parts of P. ginseng may contain distinct composition of ginsenosides and pharmacological activities [1]. Recent studies have indicated the utility of ginseng berry as a good ingredient for various ginsenosides [2,3]. Thus, in the herbal market and food industry, it is critical to evaluate and control the quality of ginseng berry. The profiling of ginsenosides and primary metabolites will be applicable for this purpose.

Methods
The ginseng berry samples were obtained from four different P. ginseng species (i.e. Sunone (SW), Gumpoong (GP), Yunpoong (YP), Gopoong (GoP). From the extract mixture, ginsenosides were separated by using UPLC system with ODS column. Exact mass measurements based on QTOF/MS were also effective for the reliable identification of individual ginsenosides. Also, different species samples of P. ginseng berry were analyzed through global metabolite profiling by HR-MAS NMR spectroscopy.

Results
In the negative ion mode of MS, ginsenosides were detected as [M+COOH]– ions. As a result, 35 ginsenosides were profiled from the four ginseng berries. The raw data of many metabolites including ginsenosides from four ginseng berries were subjected into the principal component analysis (PCA). In the PCA score plot, the four ginseng berries were well separated. The PCA loading plot also represented several ginsenosides as the major variables to discriminate the four ginseng berries. 16 primary metabolites of P. ginseng berry were identified by 1H NMR spectra. The PLS-DA clearly distinguished P. ginseng berry extract according to the four ginseng berries.

Conclusions
These results demonstrated that contents of ginsenosides differ depending on the species of ginseng berry, and UPLC-QTOF/MS and NMR based profiling method can be a good tool to evaluate the metabolites contents of ginseng berry. This method will help the quality control and effective use of ginseng berry in the food industry.

Novel Aspect
This study indicated that the proposed method based on the UPLC-QTOF/MS and NMR can be an effective tool for characteristics of Korean ginseng varieties.

References
Introduction:
Bloodstains obtained from crime site provide important information for scene reconstruction. Various researches have been conducted to obtain information of the crime by analyzing bloodstain components. However, the components vary with bloodstain volume and other unpredictable factors. In this study, we identified internal standard metabolites that having stable volume even long after its deposition for standardize bloodstains.

Methods:
Blood was collected from six subjects. Bloodstains were kept until following time points: day 0, day 7, day 14, day 21 and day 28. The metabolite profiling was conducted by using a liquid chromatography-tandem mass spectrometry (LC-MS/MS). Coefficient of variation (CV) of molecular features was calculated for each criterion: time point, subject, all data. Fold change and statistical analysis were performed to select suitable candidates for internal standard.

Results:
Five molecular features with average of CVs ≤ 5% were selected as candidates for internal standard. Partial least squares discriminant analysis (PLS-DA) and principal component analysis (PCA) shows well grouping of time condition. This results indicated the candidates unrelated to elasped time. Fold change value of those candidates’ abundances was calculated to each time points versus day 0. The stigmasterol had the most stable pattern and the L-methionine remained stable until day 14 and after day 21. Therefore, two metabolites of candidates were suitable for internal standard.

Conclusions:
In this study, we selected the candidates of internal standard to obtain the information of initial volume of bloodstain for more accurate analysis. Further studies should be conducted to validate the candidates using more samples and conditions associated with the real field situation. This study provides the basic data to obtain absolute quantitative information and proposed the possibility of bloodstain standardization method using the internal standard.

Novel Aspect:
The internal standard metabolites will contribute to establish standardization of bloodstains and help to perform accurate bloodstain analysis in the field.

References:
959 - AN INFUSION “SHOTGUN” APPROACH FOR HIGH-THROUGHPUT UNTARGETED METABOLOMICS

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Keywords: MSMS all, metabolomics, high resolution, DIA

Introduction:
Metabolomics studies the complexity and large variety of various chemical compounds. Mass spectrometry-based untargeted metabolomics requires approaches to collect data in an unbiased fashion. Current LC approaches are biased to the column chemistry and metabolites which can be retained on such chemistries. The main goal of researchers in the untargeted metabolomics field is to analyze a large number of samples and obtain the most information in the shortest time with limited sample preparation. Here we evaluate the direct infusion or “shotgun” approach to such analyses by using a technique, Infusion MS/MSALL Acquisition, for untargeted metabolomics. This data-independent technique allows the MS/MS of all possible candidates, improve identifications significantly, allows to have quantitative data and enables retrospective analysis of the data.

Methods:
Data were acquired using a TripleTOF® 6600 System (SCIEX) coupled to a high flow liquid chromatography system (Shimadzu) and employing the Infusion MSMSALL Acquisition to collect one survey scan and then MS/MS of every single precursor within a given mass range. Mass ranges were evaluated from 50 to 900 in varied sizes to optimize the number of MS/MS and time needed to collect the data. Data were collected on a small case study of urine extract and statistical analyses was performed.

Data were processed using MasterView™ Software and the accurate mass metabolomics spectral library for fast metabolite identification and confirmation. Statistical analysis was completed in MarkerView™ Software and any quantitation results were generated by MultiQuant™ Software.

Results:
By optimizing for mass range, MSMS ALL method was developed to capture all relevant metabolites per ionization mode (positive and negative mode). Metabolites are extracted from several biological samples and directly infused by using the developed method. The Total Ion chromatogram shows the presence of a large number of separate peaks. The extracted ion chromatograms show that most peaks represent, as expected, different metabolite classes, which are characteristic for each sample. Preliminary, by using the fragment filter tool, metabolites classes occurring in the different samples, were identified on the basis of their diagnostic fragment peaks and/or neutral loss. Statistical analysis is successively performed to differentiate samples and highlight rapidly marker compound in each sample. Using the high quality of MS/MS spectra thanks to SWATH acquisition, identification of marker and occurring metabolites is obtained together with the relative amount.

“Shotgun” approach for Metabolomics results a powerful and rapid tool to obtain huge and complementary data of numerous samples even if metabolites present only at very low levels in biological samples.

Post-acquisition data processing software, allows the user to have rapidly structural information and/or identification and quantification of metabolites occurring in complex samples to derive important biological conclusions.

Conclusions:
By using Infusion MSMSALL with SWATH acquisition, a rapid metabolic fingerprint of each biological sample can be captured. The optimized method is able to capture all relevant metabolites per ionization mode (positive and
negative mode). Verifying the method through a simple biological extract case study highlighted the metabolites responsible for differentiating the different sample groups.

Novel Aspect:
An Infusion “Shotgun” Approach for High Throughput Untargeted Metabolomics
Introduction:
Data independent acquisition (DIA) workflows are well adopted in quantitative discovery proteomics, but still not commonly used in discovery metabolomics. Data dependent acquisition (DDA) techniques are heavily employed in the field of metabolomics and workflows on mass spectrometers have been adapted so that as much data as possible can be captured. Researchers were limited by the speed of their QTOF mass spectrometers meaning a multiple injection workflow. Also, the stochastic nature of data dependent workflows often means MSMS of low abundant metabolites are often missed. Here, we describe how DIA enables the identification of a higher number of metabolites for untargeted metabolomics workflows compared to traditional DDA approaches thus enabling a broader profile of the metabolome.

Methods:
Urine and plasma were processed according to standard extraction protocols. Urine was diluted with water at a ratio of 1:4 (v/v) and centrifuged for prior analysis, whilst plasma was extracted 1:4 (v/v) with ice-cold methanol allowing for protein precipitation. Separation was performed on reverse phase chromatography.

DDA and DIA specific settings were chosen and evaluated. The data were acquired on a QqTOF mass analyzer. For the DDA acquisition, we selected the top 5, 10, 15, 20 and 25 precursor ions for MSMS. For DIA, we applied 15, 20 and 30 mass windows with either fixed window (fw) or variable window (vw) widths. Results were evaluated by the highest number of identifications and coverage of metabolites in plasma and urine extracts.

Results:
At the DDA level, the data demonstrate a significant improvement of metabolite coverage at the MSMS level when comparing the top5 to the top25 DDA method. We show over 100% increase of metabolite coverage in plasma extracts by increasing the number of selected precursor ions for DDA acquisition from top5 to top25. This result highlights the capability of the QTOF mass analyzer for fast MSMS acquisition, which allows for the fragmentation of a large number of precursors in a single DDA cycle, leading to a larger number of metabolites identified.

In the second part of this study, we evaluated the DIA strategy with various fixed (fw) and variable window (vw) sizes with similar cycle time in a plasma extract. Increasing the number of fixed windows resulted in ~30% gain in metabolite coverage. Using the variable window method resulted in a ~70% gain in metabolite coverage.

Conclusions
Data Independent Acquisition allows to quickly obtain a comprehensive identification and quantification of the metabolites in your sample since is able to acquire full scan (MS1) and MS/MS of every single metabolite in a single injection. Variable Window Calculator can be used to optimize Q1 isolation window pattern to achieve the right balance of metabolite coverage and specificity.
Novel Aspect:
Data Independent Acquisition Improves Metabolite Coverage over Traditional Data Dependent Techniques for Untargeted Metabolomics.
Keywords: Infrared ion spectroscopy, metabolite identification

Introduction:
Small molecule identification is a key component of many areas of the (bio)analytical sciences, including metabolomics and drug development. Here, we demonstrate how infrared (IR) ion spectroscopy is being used for small molecule identification by metabolomics labs. We present technological developments that enable separation by (ultra) high pressure liquid chromatography followed by structural identification by IR spectroscopy of the mass-selected ions.

Methods:
The experimental setup is based on a modified commercial ion trap mass spectrometer in combination with the tunable infrared radiation of the FELIX infrared free electron laser. Resonant absorption of multiple infrared photons leads to dissociation. Relating the extent of dissociation as a function of the laser frequency gives the infrared spectrum of any mass-selected ion that can finally be matched to reference (or calculated) IR spectra.

Results:
The majority of clinical metabolomics laboratories rely on mass spectrometry to identify the presence of abnormal levels of specific small molecules in common body fluids that correlate with diseases, drug treatments, and environmental factors. In a first example, we focus on the differentiation of N-acetylmannosamine, a recently discovered biomarker for NANS-deficiency (a new inborn error of metabolism in sialic acid metabolism, from other possible N-acetylhexosamines found at the same m/z. This differentiation is not possible using standard LC-MS protocols available in most metabolomics laboratories. Here, we demonstrate the use of IR ion spectroscopy to cleanly distinguish these three N-acetylhexosamines directly from urine and cerebrospinal fluid (CSF). Finally, we show how IR ion spectroscopy has been used to partially identify a previously detected but yet unidentified biomarker of antiquitin deficiency. This peak “X” is thought to be associated with intellectual disability that persists despite current treatment strategies.

Conclusions:
The results presented here show how IR ion spectroscopy compares very favorably against alternative techniques, such as NMR spectroscopy, for small molecule identification. It maintains the sensitivity (low nanomolar) and (mass) selectivity inherent to mass spectrometry while providing orthogonal identification on the basis of retention time, mass-to-charge ratio, MS/MS fragmentation patterns and the IR spectral fingerprint.

Novel Aspect:
Metabolite identification using combined liquid chromatography-infrared ion spectroscopy

INTRODUCTION:
The metabolome is a highly dynamic network of small molecules that represent not only the end-product of cellular metabolism, but also regulate and catalyze key biological processes. Here, we integrate Trapped ion mobility spectrometry (TIMS) and “Parallel Accumulation – Serial Fragmentation” (PASEF) [1, 2] to multiply the speed and sensitivity of MS2 acquisition in untargeted liquid chromatography – mass spectrometry-based lipidomics and metabolomics.

METHODS:
High-flow and nano-flow liquid chromatography was coupled online to a hybrid TIMS quadrupole time-of-flight mass spectrometer (Bruker timsTOF Pro) via electrospray ionization. Complex lipid and metabolite extracts from tissue and plasma sample were measured in PASEF mode. Raw LC-TIMS-MS data were processed with MetaboScape (Bruker) using on four-dimensional feature recognition and ion mobility-enhanced compound identification from spectral libraries.

RESULTS:
IMS can separate even isobaric ions within ~100 ms, compatible with LC and TOF time scales. In TIMS this happens by the opposing forces of an electric field and the drag of a gas flow. In PASEF, the quadrupole switches rapidly within a single TIMS scan to capture as many precursors as possible for fragmentation without diminishing sensitivity. We found that in crude metabolite extracts, we could fragment up to 17 precursors in 100 ms TIMS scans, which increased the total number of data-dependent MS2 scans by more than an order of magnitude. This extreme sequencing speed can be used in part to increase spectral quality for low-abundance precursors by re-sequencing. In terms of its physical principle, TIMS closely resembles conventional drift-tube IMS. This allows straightforward determination of collisional cross sections (CCS). We measured CCS values for a set of metabolites comprising vitamins, hormones, and lipids, which were in good agreement with literature values and highly reproducible with median coefficients of variation below 1%.

CONCLUSIONS:
TIMS literally adds a novel dimension to conventional metabolomics LC-MS workflows that is only beginning to be explored. The speed of PASEF enables very high sample throughput. We anticipate that the accurate and precise measurement of CCS values in conjunction with highly sensitive PASEF acquisition will allow deeper metabolomics and lipidomics characterization of, for example, clinical samples.

NOVEL ASPECT:
Robust high-speed and high-sensitivity metabolomics with online PASEF

REFERENCES:
Introduction:
Identification of metabolites from biological matrices is necessary for studying potential disease biomarkers and to further understand their biological implications. Liquid chromatography–tandem mass spectrometry (LC-MS/MS) analysis is an essential tool for identification and quantitation of metabolites in complex sample matrices. Here, we present HILIC method using a µflow LC-MRM to evaluate the assay performance on a colon cancer study.

Methods:
Metabolite extracts from urine, plasma and an MDCK cell line samples were analyzed with both analytical and µflow rate on SCIEX ExionLC™ AD HPLC and M3 MicroLC system. HILIC separation was performed using Luna- NH 2 columns (Phenomenex). MRM analysis with pos/neg polarity switching was completed on a QTRAP® 6500+ system. A total of 363 positive and negative ion mode MRM’s for a total 312 unique polar metabolites were monitored.

Results:
While µflow LC in general has become increasingly popular, it has not been readily employed in metabolomics because the typically used sample solvent does not allow for injecting larger volumes of samples without sacrificing HILIC chromatographic resolution. However, by simply reconstituting the sample in an organic solvent (95% acetonitrile, pH9), we could inject up to 5 µL sample, while maintaining good peak shape. Using this single injection workflow we identified 263 unique metabolites out of 312 from all three matrices, covering all major metabolic pathways. This µflow HILIC-MRM method on average provided 10 times more sensitivity compared to similar high flow methods. The µflow HILIC LC-MRM method improved the S/N ratio by up to 60 times for some polar metabolites such as uridine 5’-diphosphate, hippuric acid and cytidine. Excellent chromatographic separation of improved sensitivity and resulted in higher number of detections with a 50% improvement in MDCK cell line, 35% in urine and 11% in plasma when compared to high flow Methods:

Conclusions
For the colon cancer study the previously classified healthy and diseased (Cancer) samples were clearly differentiated by data acquired using this microflow method and data analysis with the multivariate data analysis techniques in MarkerView™ Software. This method identifies the metabolites responsible for the differentiation of the sample grouping (healthy vs cancer), which is clearly being driven by 14 metabolites profile changing due to these conditions.

Novel Aspect:
A single microflow targeted metabolomics method allowing detection of over 300 polar metabolites across multiple biochemical pathways.
Introduction:
Diversity in metabolite polarity and electrospray ionization efficiency challenge the development of a single method. Chemical derivatization can significantly improve chromatographic retention time and MS response. We propose a workflow including an automated fast parallel derivatization of amines, phenols, aldehydes, alcohols and ketones followed by QUAL/QUANT SWATH/MS analysis for broad metabolite coverage.

Methods:
Dansyl-chloride (DanCl), dansyl-hydrazine (Dan-N2H3) and their 13C label analogs were used as derivatization agents. Samples (49 analyte mix and urine) were derivatized with 12C reagents while standards were derivatized with 13C labeled regents using PAL RTC autosampler (CTC Analytics). The two fractions were mixed and injected onto a column-switching LC system. MS acquisition was performed on a TTOF6600 (Sciex) using SWATH acquisition.

Results:
Polar metabolites are converted to more hydrophobic products (DanCl and Dan-N2H3 derivatives), enabling them to be separated on reverse phase liquid chromatography. The presence of a basic p-amino group on both reagents increase the electrospray response factor by a factor of 10 to 200. Primary alcohols, phenols, primary and secondary amines are derivatized with DanCl and Dan-N2H3 transforms ketones and aldehydes to hydrazones. The automated parallel derivatization enables reproducible derivatizations in a single workflow including light labeling of sample and heavy 13C labeling of metabolite mix in 15 minutes during the LC analysis of the previous sample. Collision induced dissociation generates fragments specific for the analyte, and for the light and heavy derivatized analytes and for tags (XIC m/z=171 and m/z=173). LC-SWATH/MS which collects all precursors and all fragments allowed the screening and the relative and absolute quantification (n=45) of metabolites in urine.

Conclusions:
Labelled reagents enable to generate adequate standards for amines, phenols, aldehydes, alcohols and ketones. Automation was found to be key for reproducibility and is performed on-line prior LC analysis. In a batch, sample preparation and sample analysis are overlaid resulting in a significant gain of time. Dansyl-chloride and dansyl-hydrazine were found to be ideal for light/heavy labeling, improved LC retention, improved MS response and MS/MS tag

Novel Aspect:
Automated parallel heavy II light derivatization of sample II standard mix using dansyl-chloride and dansyl-hydrazide for improved metabolites coverage with LC-SWATH/MS.
Introduction:
Metabolomics is a powerful tool to gain new insights contributing to the identification of complex molecular mechanisms in human and animal cells. Our aim is to understand how metabolism is rearranged during the development of age-related diseases. In particular, we focused our attention on the role of mitochondria that represents the energy-generating hubs of the cell in the context of two pathologies related to age: the peripheral neuropathy and type 2 diabetes.

Methods:
We used ESI-LC-MS/MS metabolomics of more than 500 metabolites involved in energy metabolism to investigate changes in the sciatic nerve of an experimental model of peripheral neuropathy. The same analyses were also used to characterized a novel mitochondrial regulator in vitro and in humans. In these latter cases, we also determined the rates of metabolic reactions by culturing cells with uniformly 13C-labeled nutrients (glucose, palmitate and glutamine).

Results:
Mice lacking the master regulator of de novo lipogenesis Sterol Regulatory Element Binding Factor (SREBF-1cKO) developed peripheral neuropathy. Metabolomics indicated that SREBF-1cKO peripheral nerves experienced decreased fatty acid synthesis and glycolytic flux, but increased fatty acid catabolism and mitochondrial function. These alterations were the result of local accumulation of two endogenous PPARα ligands, specifically identified by mass spectrometry [1].
We have identified Zinc finger CCCH-type containing 10 (Zc3h10) as novel mitochondrial regulator. Depletion of Zc3h10 results in mitochondrial dysfunction and blunted Krebs cycle flux. We have also identified a loss-of-function mutation of Zc3h10 in humans (Tyr105 to Cys105) that is associated with increased body mass index and hyperglycemia. Isolated peripheral blood mononuclear cells from Cys105 homozygotes display reduced oxygen consumption rate and decreased levels of some Krebs cycle metabolites, which all together derive in mitochondrial dysfunction [2].

Conclusions:
Collectively, our results contributed to elucidate metabolic pathways modulated by two specific proteins, namely SREBF-1c and Zc3h10. Specifically, we identified changed energetic metabolites when both proteins were experimentally knock-down or functionally altered. Further, stressing the physiologic relevance of our findings, we demonstrated the key role of SREBP-1c and Zc3h10 in peripheral neuropathy and mitochondrial dysfunction development.

Novel Aspect:
Steady-state and dynamic metabolomic analyses are powerful analytical methods to unravel metabolic re-wiring in the transition from physiology to pathophysiology.

References
HIGH-THROUGHPUT LC-MS/MS-BASED CHIRAL METABOLIC PROFILING FOCUSING ON AMINO ACIDS AND RELATED METABOLITES

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Keywords: d-amino acid, chiral metabolomics, enantioseparation, LC-MS/MS

Introduction:
Metabolomics has been an evolving science with a wide range of applications in various fields. However, previous studies have rarely focused on metabolite chirality. We previously developed analytical methods for the simultaneous analysis of eighteen chiral proteinogenic amino acids[1, 2]. In this study, to achieve metabolic profiling of chiral amino acids and related metabolites, we developed a high-throughput method using LC-MS/MS.

Methods:
LC-MS/MS analysis was performed using the Nexera HPLC System (Shimadzu Corporation, Kyoto, Japan) connected to an LCMS-8060 (Shimadzu) with dual ion source of electrospray ionization and atmospheric pressure chemical ionization in MRM mode. Chromatographic separation was achieved with CROWNPAK CR-(-)/CR- (+) (3.0 mm i.d., 150 mm, 5 µm) and CHIRALPAK ZWIX(-) (4.0 mm i.d., 150 mm, 3 µm) (Daicel CPI, Osaka, Japan).

Results:
The combination of two types of chiral columns (with binaphthyl-based crown ether and cinchona alkaloid-derived zwitterionic stationary phases) enabled the analysis of 115 chiral and non-chiral metabolites. By finely optimizing MS/MS parameters, the method allowed the highly sensitive (0.001-50 nmol/mL) and wide dynamic range detection (100-50,000) of target analytes in a standard solution without derivatization. We applied the method to food samples (cheese), and successfully quantified trace levels of metabolites such as d-amino acids in samples. Additionally, we performed principal component analysis (PCA) on the metabolome data and obtained unique profiles that reflected metabolite chirality.

Conclusions:
This method highlights the highly sensitive detection of trace amounts of targeted metabolites without the need for derivatization. Moreover, we demonstrated the usefulness of our system by simultaneously analyzing both chiral and non-chiral metabolites in samples. According to the profiling, some d- and l-amino acids showed different behaviors in the PCA plot, which could not be observed by conventional methods that rarely consider separation of enantiomers.

Novel Aspect:
We anticipate that this method will enable comprehensive profiling to provide novel insights into the discovery of molecular markers utilized in various fields.

References:
1242 - TARGETED METABOLOMICS APPLIED TO HEART FAILURE PATIENTS – A PILOT STUDY

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Keywords: Targeted metabolomics, heart failure, reduced ejection fraction (HFrEF), preserved ejection fraction (HFpEF)

Introduction:
Targeted metabolomics was performed at AstraZeneca on a pilot study with plasma samples from heart failure (HF) patients in the 4D heart failure PREFERENCES project for improvement of heart failure management and research in Stockholm, initiated by the Karolinska Institute and Stockholm County. The patients were divided into three groups: HF with reduced ejection fraction (HFrEF, n=13), HF with preserved ejection fraction (HFpEF, n=22) and a HF control (n=41) group. All 76 patients had coronary artery bypass surgery performed.

Methods:
The effective analysis of polarionic metabolites by LC-MS, represents an analytical challenge for metabolic profiling. Ion-pair-chromatography (IPC) coupled to negative-electrospray-ionization tandem-mass spectrometry (1) was used to profile 100+ endogenous metabolic intermediates in human plasma. A complementary method based on hydrophilic interaction chromatography (HILIC) positive-electrospray-ionization tandem-mass spectrometry was developed covering another 70+ metabolites. By combining these two platforms, metabolites representing amino acids, phosphorylated sugars, citric acid cycle intermediates, glycolytic intermediates, acylcarnitines, lysophospholipids, and nucleosides were recovered.

Results: There was no clear metabolite phenotype separation between normal, HFpEF and HFrEF patients based on targeted metabolomics but two metabolites were differentially abundant between HFrEF patients and controls and survived adjustment for multiple testing. There was also significant pair-wise separation between groups for more than 15 metabolites.

Conclusions:
In conclusion, targeted metabolomics was successfully applied to the heart failure pilot study. Results were in-line with published data, confirming the capability of the platform to support future work on an expanded cohort aiming at identifying dysregulated metabolic pathways to advance the understanding of heart failure pathophysiology in search for new treatments and biomarkers.

Novel Aspect: The application of combined targeted metabolomic platforms on samples from heart failure patients with preserved or reduced ejection fraction.

References:
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Keywords: tandem mass spectrometry, in vivo labelling, auxin, metabolism, Arabidopsis thaliana

Introduction:
The phytohormone auxin (indole-3-acetic acid; IAA) plays a fundamental role in various processes of plant growth and development. Crucial for auxin action is the interplay between its transport, biosynthesis, degradation and conjugation. The use of heavy isotope labelling of intermediates in conjunction with mass spectrometry analysis has proven to be a powerful tool for monitoring fluxes through IAA metabolic pathways.

Methods:
7-day-old seedlings of Arabidopsis thaliana were incubated with 10 µM [13C]indole. Seedlings were harvested after 1, 2, 4, 6 and 24 hours, then extracted and subsequently purified by solid phase extraction (SPE) as described by Novák et al. [1]. Incorporation of [13C]indole and its metabolism in planta were monitored by LC-MS/MS with multiple reaction monitoring transitions and quantified by isotopic dilution method with appropriate internal standards [2].

Results:
We performed a feeding experiment using 13C labelled indole in Arabidopsis wild type (Col-0) and several IAA biosynthetic and metabolic mutants to investigate auxin metabolism. Incorporation of the 13C from labelled precursor into most abundant IAA biosynthetic intermediates (tryptophan, indole-3-pyruvic acid (IPyA), indole-3-acetamide (IAM) and indole-3-acetonitrile (IAN)) as well as metabolites (IAA-aspartate (IAAsp), IAA-glutamate (IAGlu), 2-oxoindole-3-acetic acid (oxIAA), IAA-glucose and oxIAA-glucose) was monitored by mass spectrometry. Our results are in consistence with published role of particular enzyme in Arabidopsis metabolism[3]. Applied isolation procedure and LC-MS/MS method showed sufficient sensitivity for in vivo labelling studies. Therefore, our developed approach allows to monitor phytohormone metabolic pathways in extremely small samples (<1 mg), such as root tips, meristems or embryos.

Conclusions:
Obtained results provide us with important information about the rates of IAA biosynthesis and degradation and the relevance of individual IAA precursors, catabolites and conjugates. Combined with mutant line screening[4], the developed approach can improve our knowledge about auxin metabolic pathways in different plant species.

Novel Aspect:
Using heavy isotope labelling and mass spectrometry, we have developed a new functional bioanalytical approach for studying IAA biosynthesis and metabolism in Arabidopsis.

Acknowledgments
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References
Introduction:
Since breath analysis shows a high potential as a non-invasive diagnostic tool, many explorative case-control studies investigating different diseases have been carried out in the recent years. However, only very few results have been validated in a larger cohort later on. Here, we present a blinded validation study for the diagnosis of obstructive sleep apnoea (OSA) by analyzing exhaled breath with secondary electrospray ionization mass spectrometry (SESI-MS).

Methods:
Subjects with suspected OSA (n=150) were diagnosed by conventional in-laboratory respiratory polygraphy. In addition, they underwent exhaled breath analysis using SESI-MS. Metabolic breath patterns were analyzed and the diagnosis of OSA was predicted for the subjects using a panel of biomarkers found to be characteristic for the presence of OSA in our previous pilot study [1]. The person analyzing the data was blinded regarding clinical diagnosis.

Results:
Between the pilot study and the validation study, some technical improvements on our SESI-MS setup were made. We upgraded to a commercial SESI source making the data challenging to compare. In a preliminary data analysis of 130 subjects measured so far, we were able to render the old and the new dataset comparable using empirical Bayes methods[2]. Thus, the data from the pilot study can be used for training the classification model for predicting the validation cohort.

Conclusions:
A positive outcome of this study will strengthen real-time breath analysis by SESI-MS as a diagnostic tool tremendously and bring it a step closer to its application in clinical routine.

Novel Aspect:
This is the first validation study for any biomarkers found in exhaled breath using secondary electrospray ionization mass spectrometry.

References:
Introduction:
Complete coverage of a metabolome of interest can be challenging due to the diversity of small molecules. Here, we integrated reverse phase (RP) and hydrophilic interaction chromatography (HILIC) to maximize throughput and metabolome coverage. This dual separation system was coupled to an Orbitrap™ mass spectrometer and used for untargeted metabolomic analysis of beer samples, identifying compounds important in discriminating flavor and indicating spoilage.

Methods:
Sixteen beers were purchased from retail stores. For each beer, samples were collected at time of purchase and after storage at room temperature or 4°C for 6 and 18 weeks. A Thermo Scientific™ Vanquish™ UHPLC system equipped with an autosampler with two injection units, was coupled to a Thermo Scientific™ Q Exactive™ HF mass spectrometer. Data were processed with Thermo Scientific™ Compound Discoverer software for unknown identification and differential analysis.

Results:
We developed a dual liquid chromatography/mass spectrometry (LC/MS) system that combines RP and HILIC separation and we evaluated it for its reproducibility and separation capabilities of complex samples. Small polar metabolites from beer, like malate and succinate, were not retained during RP, but showed good reproducibility of retention times and MS signal during HILIC separation. Hydrophobic metabolites, such as apigenin and isoxanthohumol, were reproducibly retained only during RP. By combining the two complementary separation techniques, we were able to reproducibly analyze a larger number of metabolites. Two independent UHPLC pumps were incorporated into the system to allow independent control of the two columns (RP and HILIC). This enabled the use of different solvents, additives and pH ranges and resulted in broader metabolite coverage. By equilibrating one column, while separation was carried out on the other, analysis time was decreased by 30% and the overall throughput of the method increased.

Conclusions:
The increased throughput of the dual LC/MS system described above, did not compromise reproducibility or compound detection. It was successfully employed for the differential analysis of beer and provided robust indicators of flavor profiles and degradation. One can envision extending its application to any metabolomics application, where increased throughput is needed.

Novel Aspect:
Innovative integration of RP and HILIC separations leads to improved metabolome coverage while simultaneously increasing throughput of untargeted metabolomic workflow.
Introduction:
Compound identification is a bottleneck in untargeted metabolomics, hindering biological interpretation of results. Here, we describe a data-informed workflow that maximizes the number of metabolites interrogated by MS/MS and MSn, while minimizing the acquisition of uninformative spectra. This workflow was used to analyze human plasma resulting in high confidence identifications, deeper metabolome coverage and enhanced biological knowledge generation.

Methods:
Human plasma was purchased from NIST. Metabolites were extracted with methanol and injected on a Thermo Scientific™ Hypersil GOLD™ column. Instrumentation included a Thermo Scientific™ Vanquish™ UHPLC system and a Thermo Scientific™Orbitrap Tribrid™ Mass Spectrometer with modified instrument control and data acquisition software. Data were analyzed using Thermo Scientific™ Mass Frontier software and Thermo Scientific™ Compound Discoverer™ software.

Results:
During data-dependent MS/MS, ions are selected based on abundance, without any knowledge of biological relevance or type of ion. In a typical DDA experiment, we determined, that >40% of MS/MS spectra could be attributed to background ions. By enabling the automatic generation and implementation of a background exclusion list based on real-time feature detection in LC-MS data, background ion MS2 spectra were practically eliminated (<0.1%), allowing for the analysis of more true sample components. Small molecules form different types of adducts and cluster ions during electrospray ionization. Highly abundant compounds may prevent the fragmentation of metabolites of lower abundance. By populating the inclusion list with the preferred ion for each metabolite, more compounds can be sampled by MS/MS and MSn in a single run. Additionally, by automatically updating inter-run inclusion and exclusion lists during analysis, we can ensure that compounds not selected for MS/MS and MSn will be prioritized during a subsequent injection.

Conclusions:
The combination of MSn and automatically generated inter-run inclusion and exclusion lists resulted in fragmentation of more unique metabolites and a greater number of metabolites confidently annotated. Application of this innovative workflow addresses the identification bottleneck of untargeted metabolomics studies and enables confident biological interpretation of the results.

Novel Aspect:
Automated workflow for information-rich fragmentation data acquisition, designed to minimize irrelevant spectra and maximize metabolome coverage.
NON-TARGET METABOLOMIC ANALYSIS OF PEPERONI DI SENISE PEPPERS IGP (CAPSICUM ANNUUM L.) AND EVALUATION OF THEIR NUTRACEUTICAL PROPERTIES

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Keywords: Capsicum Annuum L., Peperoni di Senise, FT-ICR-MS, Phytochemical Screening, hypoglycemic activity

Introduction: Peperoni di Senise peppers (Capsicum Annuum L.), a typical food product cultivated in Basilicata (Southern Italy), protected with a PGI quality mark, are known for their unique taste. A Mass Spectrometry-based phytochemical screening was performed to promote their functional food properties.

Methods: Direct injection negative-ion ESI-UltraHigh Resolution Mass Spectrometry was used to obtain a metabolic fingerprint of the sample. High-resolution mass spectra were acquired on a Bruker (BrukerDaltonik GmbH, Bremen, Germany) solariX XR Fourier transform ion cyclotron resonance mass spectrometer (FT-ICR-MS) equipped with a 7T superconducting magnet and an ESI source. Moreover, antioxidant activity of the extracts was evaluated.

Results: UltraHigh Resolution Mass Spectrometry data obtained from analysis of the extract of Peperoni di Senise peppers were used to perform a rapid analysis of metabolome by converting accurate m/z values in putative elemental formulas in order to better understand the chemical composition of the sample. Molecular formula maps were obtained by making Van Krevelen diagrams, that lead to a direct visualization of different classes of metabolites. As expected, the most abundant compounds were polyphenols and carotenoids. A significant antioxidant activity of the extracts was evaluated also.

Conclusions: The results of the present study shed some light on metabolic composition of Peperoni di Senise peppers and highlighted their health-promoting properties.

Novel Aspect: This study improved the knowledge of metabolites occurring in Peperoni di Senise fruits and it can be used for comparative studies with other types of Capsicum Annuum L. fruits.

References

559 - DETERMINATION OF METABOLIZATION OF NEW FLUORESCENTLY LABELED AUXIN-LIKE COMPOUNDS IN PLANT MATERIAL USING UHPLC-MS/MS

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Keywords: UPHLC/MSMS, auxin, fluorescence, in planta stability

Introduction:
Auxin is an indole-derived plant hormone which significantly contributes to plant growth and development [1]. Using a fluorescent label to visualize auxin natural distribution in different physiological processes will provide new insights into auxin biology [2]. Here we present one-step purification and quantification methods used to determine the chemical stability of the new fluorescent derivatives and their potential in vivo metabolization in plant system.

Methods:
The purification procedure based on liquid-liquid extraction was developed using hexane:water:methanol (1:1:1) as an extraction solution. After isolation step, an Acquity UPLC® I-Class System coupled to a triple quadrupole mass spectrometer XevoTM TQ-S MS(Waters) was employed for detection of the analytes. Concentrations of all compounds were then calculated by an isotopic dilution method using the stable isotope labelled standards by deuterium and 13C.

Results:
Applying the presented method, time-dependent uptake and metabolization dynamics of two new fluorescent auxin derivatives in roots of Arabidopsis thaliana seedlings was determined. After the treatment of 5-d old plants by 10 µM exogenous application of tested compounds, both fluorescent conjugates showed similar metabolization rate in vivoyielding 20-28 pmol/50 roots of free auxin in 3 h (5 % of their total content). Furthermore, the detected amount of free auxin analoguein short-term treatment (30 min) was close to the detection limit of the UHPLC-MS/MS method (in range 5.8-9.5 pmol/50 roots representing just 2% of the total content of fluorescent analogues). Despite a slight metabolization on the background, the evidences of biological activity of the whole fluorescent compounds were provided in various auxin-related bioassays.

Conclusions:
A new detection method was developed to determine stability of two fluorescent analogues of plant hormone auxin in planta. Our analytical approach helped to reveal the time-dependent metabolization of new fluorescent auxin derivatives in plant tissues. Furthermore, the detected amount of free auxin analoguewas proven not to be sufficient to cause the biological response.

Novel Aspect:
A developed purification method is applicable for determination of a stability of various plant hormone derivatives and presents a useful analytical tool for plant research.

References
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Metabolomic approaches to investigate the role of the mitochondrial regulator Zc3h10 in adipocytes.

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Keywords:
Zc3h10; LC-MS/MS; metabolism; mitochondria; adipocytes;

Introduction:
Mitochondria play a crucial role in many cellular processes and they are essentials organelles for the health of the cell. Beyond their contribution to energy production, they are key regulators of tissue development and cell differentiation. We recently isolated the new mitochondrial regulator zinc finger CCCH-type containing 10 (Zc3h10) [1] and here, we validated its role during adipocytes differentiation.

Methods:
C3H/10T1/2 cell line (mesenchymal stem cells) can be differentiated into white adipocytes using a specific adipogenic cocktail. Quantification of different metabolites was performed with a liquid chromatography/tandem mass spectrometry (LC-MS/MS) on an API-4000 triple quadrupole mass spectrometer coupled with a HPLC system using a C18 column for amino acids and cyanophase LUNA column for metabolites.

Results:
Zc3h10 protein levels increases during C3H/10T1/2 differentiation. Zc3h10 silencing significantly affects adipocyte differentiation and mitochondrial activity. To demonstrate that Zc3h10 plays a role in energy metabolism we evaluated the intracellular levels of by-products belonging to the main metabolic pathways (i.e. glycolysis, tricarboxylic acid (TCA) cycle and pentose phosphate pathway (PPP), amino acids) using LC-MS/MS. Steady-state metabolomics indicated that lack of Zc3h10 led to decreased AMP, ADP, ATP and NADH levels. We also observed reduced levels of acetyl-CoA, α-ketoglutarate (α-KG), citrate, and succinyl-CoA and increased levels of glutamate. We also used metabolic tracers ([U-13C6]-glucose, [U-13C16]-palmitate or [U-13C5]-glutamine) to confirm that the flow of energy substrates into the TCA is affected by Zc3h10 silencing.

Conclusions:
Our results indicate that Zc3h10 expression increases during murine white adipocyte differentiation. Further, Zc3h10 silencing in white preadipocytes and adipocytes deeply impaired mitochondrial function, decreased adipogenic potential and altered metabolic profile.

Novel Aspect:
These data annotate Zc3h10 as a new regulator of mitochondrial function and cell differentiation in adipocytes.

References:


Preferred presentation: poster
349 - LC-MS/MS QUANTIFICATION OF THIOL-CONTAINING METABOLITES WITHIN THE DE NOVO GLUTATHIONE SYNTHESIS PATHWAY OVER THE LIFESPAN OF CAENORHABDITIS ELEGANS

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Keywords: glutathione, thiol, quantification, LC-MS/MS, aging

Introduction:
A consistent underlying index of aging is declining cellular levels of the tripeptide glutathione (GSH). This could be due to either limited cysteine availability or a decrease in the activity of the rate-limiting enzyme glutamate cysteine ligase (GCL). Insight into the availability of cysteine levels and the regulatory control of GCL will inform the development of therapeutic strategies aimed to overcome age-related dysregulation of the GSH synthesis pathway.

Methods:
We have developed a rapid and sensitive LC-MS/MS method that can simultaneously quantify each thiol in the de novo GSH synthesis pathway (Cys, γ-GC and GSH together with GSSG) using a relatively unexplored thiol-derivatizing reagent, 4-(dimethylaminoethylaminosulfonyl)-7-chloro-2,1,3-benzoxadiazole (DAABD-Cl). This method was applied to a nematode model to determine if the dysregulation in GSH homeostasis is due to cysteine availability or GCL activity.

Results:
The nematode, Caenorhabditis elegans, which has a median lifespan of approximately 17 days, presents a potentially useful model to investigate the age-related decline in GSH homeostasis, due to the presence of the metabolites and orthologues of the mammalian GSH synthesis pathway enzymes.

Work to date has validated the LC-MS/MS method as having the dynamic range, sensitivity and selectivity to be suitable for the analysis of age-dependent changes of all the thiol and disulfide levels in nematode tissue samples. Using this method, thiol and disulfide levels were monitored in aged-matched wild-type cohorts. It was observed that γ-GC and GSH levels significantly declined over the first 10 days of adulthood. The GSH:GSSG molar ratio followed an age-related oxidizing shift, declining from 115:1 in day 1 old worms to 25:1 in day 10 old worms. Cysteine levels did not appear to show any age-dependent trends, indicating that factors other than the availability of cysteine are perhaps causing this age-related decline in GSH synthesis.

Conclusions:
The age-associated decline of the GSH pool is likely due to a progressive lowering of GCL activity. The corresponding decline in the GSH/GSSG ratio may be a function of a decline in the activity of the GSH recycling enzyme, glutathione reductase, or a depletion of its NADPH cofactor pool. Future work will expand the assay and quantify other key thiol metabolites to further our mechanistic understanding of the aging process in relation to glutathione homeostasis.

Novel Aspect:
We developed a strategy using LC-MS/MS coupled with derivatization for the quantitative measurement of labile low molecular weight thiols in biological tissue.
INTEGRATION OF TANDEM MASS SPECTROMETRY MOLECULAR NETWORKING AND GAS-PHASE FRAGMENTATION REACTIONS FOR STRUCTURAL ANALYSIS OF FLAVONOID GLYCOCONJUGATES

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Keywords: Molecular Networking (GNPS), Dereplication, O-glycosyl-flavonoids, Gas-phase Fragmentation

Introduction:
Gluconjugated flavonoids (GF) are important natural products in plants [1]. Their identification in the different isomeric forms, stereochemistry and positions still represent a great challenge. HPLC-MS/MS combined with bioinformatics proved to be efficient in analyzing and organizing large datasets of MS2. For this work, we integrate the Molecular Network (MS2) and the fragmentation patterns to the GF annotation in Chrysobalanaceae species.

Methods:
Our approach uses massive molecular networks that embeds chemical - glycosylation type and position, nature of their aglycones, and the structure/linkage information of their glycan moieties -, and biological information layers to highlight the flavonoid composition through structural-based organization. We exemplify this workflow by identifying the glycoconjugated flavonoids present in six Chrysobalanaceae species analyzed using LC-ESI-QToF-MS.

Results:
The combination of Molecular Network capabilities, gas-phase fragmentation patterns with detailed taxonomical knowledge on flavonoid biochemistry allowed a rapid screening and annotation of several glyconjugate flavonoids including those with different glycosylation type and position, nature of their aglycones and the structure/linkage information of their glycan moieties. According to the literature, more than fifty flavonoids have been described, so far, from Chrysobalanaceae species, with a predominance of flavonol glycosides [2]. Based on the systematic inspection of all MN nodes related to the flavonoid groups, sixty-four metabolites were identified in the Chrysobalanaceae plant species, with a predominance of flavonol 3-O-glycosides. This is also the first report of methoxyl, O-di- and tri-O-glycosyl flavonols for this family.

Conclusions:
The application of this strategy using LC-MS in automatic data-dependent acquisition (DDA) capability combined to molecular networking allowed the visualization and data comparison not only for known compounds but also provided information for the determination of correlated analogues of glycoconjugated flavonoids described for the first time in the Chrysobalanaceae family.

Novel Aspect:
This is first time molecular networking, fragmentation pattern and chemotaxonomic informationis combined to annotate glycoconjugate flavonoids considering glycosylation type and position, aglycones and linkage information of their glycan moieties

References:
Keywords: colorectal cancer, pattern recognition, metabolomics workflow, data processing methods

Introduction:
As mentioned in recent research in USA about 50000 deaths have happened from colorectal cancer (CRC), also authors postulate the need to create a method for screening CRC in the early stages [1]. The comprehensive workflow for pattern recognition of three group CRC patients based on UPLC-MS with fully comprehensive algorithm for optimization methods of preprocessing and data acquisition were reported here.

Methods:
All morning fasting urine samples (on an empty stomach, immediately after sleep) of control group volunteers (8 pcs.), colorectal cancer patients before surgical operation (20 pcs.) and after surgical operation (12 pcs.) were collected for this research.
The HPLC separation was conducted on a C18 column. The separation was carried out in a gradient elution mode with MS detection in a positive ion (TIC mode). “Dilute and shoot” technique was used for sample preparation.

Results:
Three types of signal drift correction were used for reduction unwanted variations [2]: total ion current, quantile, median and without any method. ANOVA was applied, to test correction Methods: Two methods for metabolites concentration normalization were examined [3]: mass spectrometry total useful signal, creatinine concentration and without normalization. Log transformation and three type of scaling methods (auto, mean, Pareto and without scaling) were tested.
So, the dataset was applied to assess all possible 48 permutations of normalization, correction, transformation and scaling followed by PLS-DA and sPLS-DA. Analyzing sPLS-DA result, we found that using a log transformation, regardless of the application of any other data processing methods, the first 10 features with the maximum values of their loadings were the same for all permutations. After an extraction from the entire set of data set only the top features, in all cases samples groups were fully resolved from each other by PCA, sPLS-DA, PLS-DA, RF, dendrograms and heat map.

Conclusions
We propose the pragmatic decision procedure for selection and evaluation of preprocessing methods in metabolomics studies. This algorithm is based on the estimating of the influence each stage using multivariate and univariate statistical analysis. Extraction selected features from raw data set will led to appropriate pattern recognition if the model is built right.

Novel Aspect:
This LC-MS workflow with algorithm of evaluation, optimization and scheme for performing statistical analysis operations can be applied in other relatively short studies.

Reference
1264 - PHOSPHOLIPID REMOVAL BY SOLID PHASE EXTRACTION FOR UNTARGETED METABOLOMICS: IMPROVED SENSITIVITY FOR POLAR METABOLITES IN BRAIN

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Keywords: Untargeted metabolomics, HILIC, solid phase extraction, phospholipid

Introduction:
Untargeted UHPLC-ESI-HRMS metabolomics of polar brain tissue metabolites is challenging because brains contain large amounts of phospholipids. As phospholipids ionize easily, they cause signal suppression of polar metabolites and detector overloading at large injection volumes. Here we compare four solid phase extraction cartridges for phospholipid removal from brain extracts.

Methods:
Rat brain proteins were precipitated with methanol, and supernatants subjected to phospholipid removal by pass through SPE. Non-endcapped, octadecylfunctionalized silica (C18), zirconia coated silica (HybridSPE), and two types of hydrophilic-lipophilic balance polymer cartridges (HLB and PRiME HLB) were compared for the lipid removal. The analytes were separated by HILIC with BEH Amide column, and detected in negative ion mode with ESI-Q-TOF-HRMS.

Results:
Amount of phospholipids was significantly reduced with all sorbent materials, and subsequently, concentration and injection of larger volumes of sample was enabled. However, the removal was less efficient with PRiME HLB compared to the other cartridges. The C18 and HLB sorbents removed efficiently also other lipid types from the samples, such as fatty acids, sulfatides, and ceramides. This is expected based on their non-polar retention mechanisms. PRiME and HybridSPE showed variable removal efficiency for the lipid classes that do not contain the phosphate group. In addition to lipids, some analytes of interest were retained in the sorbents. For example, in the case of HybridSPE, lactic acid, glucose, the dipeptide neurotransmitter N-acetylaspartylglutamic acid, and its precursor N-acetylaspartate were not recovered from the samples, while the C18 sorbent retained GABA completely. Extraction RSDs were mainly below 20% for C18 and HLB, whereas PRiME and HybridSPE showed larger variance.

Conclusions:
All tested sorbent materials are able to reduce the amount of phospholipids in methanolic brain extracts with very simple pass through protocols and thus improve sensitivity towards polar metabolites. However, some metabolites are lost in the process. The HLB sorbent provided best combination of lipid removal efficiency, repeatability, and metabolite recovery for brain metabolite analyses.

Novel Aspect:
Systematic evaluation of solid phase extraction sorbents for phospholipid removal for the untargeted metabolomics of polar brain metabolites.
Coffee is one of the most appreciated beverages in the world. It has a unique flavor and aroma, resulting from a complex chemical composition of the beans and influenced by several factors [1]. The beverage quality is directly related to the chemical composition of the beans and studies about quality x chemical composition have been carried out for decades. However, they usually focus on a small number of metabolites, not covering the complex chemical composition of the beans.

Methods: Raw (unroasted) coffee beans with different beverage qualities were extracted comprehensively and exhaustively using a two-step solvent extraction and analyzed using a non-target method by liquid chromatography-data dependent high-resolution mass spectrometry (LC-HRMS ddMS2) in the Thermo QExactive Plus. The obtained files were converted to the mzXMLopen format and submitted to target and non-target metabolomic analysis as well as to molecular networking using the GNPS online platform [2].

Results: Sixty-four substances from different chemical classes including xanthines, chlorogenic acids, diterpenes, serotonin amides, among others, were identified using the m/z value with high accuracy (ppm error < 5ppm), fragmentation profile and isotopic pattern as the criterion for identification. The GNPS online platform allowed the creation of molecular networks containing molecular families according to the similarities in their chemical structure, helping in the better understanding of the chemical composition of the beans. By PCA and PLS-DA multivariate data analyses sucrose and the lipid fraction showed high correlation with beans of better quality, while substances in coffee wax were correlated to low quality coffees.

Conclusions: With this work it was possible to develop a comprehensive methodology based on metabolomics to study and better understand the chemical composition of coffee beans and its relation to the quality of the beverage. So, it may help coffee growers, cooperatives and other professionals in the coffee chain to produce better quality products.

Novel Aspect: We have developed a comprehensive metabolomic study identifying a large number of coffee compounds from the non-volatile fraction together with the creation of molecular networks based on tandem mass spectrometry data.

References:
Introduction
Cancer is one of the leading causes of morbidity and mortality worldwide and the number of new cases is expected to rise significantly in the future. Improvements in early detection and therapeutic evaluation are essential to decrease cancer mortality [1]. Metabolomics is increasingly applied for the identification of biomarkers for disease diagnosis, prognosis and risk prediction. The metabolic analysis in non-invasive biofluids such as urine may help to obtain a complete functional molecular picture of the biochemistry and ultimately the discovery of new cancer biomarkers [2].

Methods
The different properties of the huge diversity of chemical compounds mean that the complete metabolic analysis of urine is practically impossible with a single analytical method. Therefore, multiple analytical approaches have been used such as capillary electrophoresis-mass spectrometry (CE-MS), liquid chromatography-mass spectrometry (LC-MS) and gas chromatography-mass spectrometry (GC-MS).

Results
A urinary metabolic fingerprinting analysis has been performed in an animal model in order to detect potential biomarkers that could help in the early detection of prostate cancer, which is the second most frequent type of cancer in men [3]. The comparison between healthy and diseased mice was carried out analyzing urine samples from Wild-Type (WT) mice and Transgenic Adenocarcinoma of the Mouse Prostate (TRAMP) mice of C57/BL6 strain. The experimental design offers a wide range of metabolites with a significant chemical diversity, due to the different platforms applied: very polar (CE-MS) non-volatile (LC-MS) or volatile metabolites (GC-MS). This strategy produces large amounts of complex datasets that must be handled with powerful computational analysis. An unsupervised method, such as principal component analysis (PCA), reduces the complexity of the data obtaining plots of easier interpretation. This approach is useful to see whether the two groups (TRAMP and WT) can be discriminated by their characteristics.

Conclusions
To summarize, we have a preliminary study that permits the detection of a wide range of metabolites, some of which could be possible biomarkers because it allows the discrimination of the tumour progression. Those compounds are identified based on the capabilities of mass spectrometry.

Novel Aspect
The search for biomarkers as an alternative to the analysis of the prostate-specific antigen (PSA) which has been questioned due to a lack of specificity at this cancer diagnosis [4].

References
Xia, J., Broadhurst, D., Wilson,M., Wishart, D., Metabolomics,9,280-299 (2013)

For information please contact: scientific@imsc2018.it
Keywords: metabolomics, microbial metabolites, gut microbiota, cancer, organoids

Introduction:
Currently, colorectal cancer (CRC) is the second cause of death in EU[1]. In EU, the Czech Republic and Slovakia showed the highest rates. By contrast, Greece presented the lowest rates across Europe[2, 3]. This incidence is postulated to reflect risk factors associated to socioeconomic status, including poor dietary habits and obesity. However, emerging evidence also implicates gut microbiota as an important effector in the relationship among diet, human health and cancer[4].

Methods:
A novel integrative strategy to deepen understanding of CRC is vital. To accomplish this, a real-life scenario is needed. In this context, colon organoids/tumoroids will be established. Afterwards, an apple will be digested/fermented in vitro using a batch culture colonic model inoculated with feces from lean/obese healthy donors. In the end, such polyphenol metabolites will be tested in colon organoids/tumoroids and the mechanisms of action will be revealed by metabolomics and organoids assays.

Results:
In summary, the results from TRIANGLE through the integration of polyphenols (diet), gut microbiota and colon organoids (host), will be highly multidisciplinary, and will undoubtedly set the stage towards the identification of mechanisms contributing to CRC understanding and will ultimately pave the way to phytochemical treatment and prevention. First, it is still unknown how apple phytochemicals are affected by lean and obese microbiotas, and it must be understood in order to comprehend chemopreventive efficacy of polyphenols and to be able to give nutritional recommendations related to functional-group class therein. Secondly, tumor organoids (tumoroids) and organoids can bridge the gap between human 2D cancer cell lines and animal-based models. Lastly, metabolomics analysis and 3D cell assays of colon organoids/tumoroids will provide valuable new insights into the mechanisms by which nutrient-gene interaction influences colon stem cell niche and CRC, and will open up new possibilities for CRC understanding and prevention.

Conclusions
TRIANGLE will provide novel insights towards CRC prevention by (1) using a new in vitro model based on colon organoids, (2) considering both gut microbiota composition and microbial metabolites, and (3) applying novel techniques. By implementing this, colon organoids will clearly mimic human real-life scenario. Secondly, the manner how polyphenols are affected by microbiotas will reveal the type and quantity of polyphenol metabolites, which could be correlated to the chemoprevention of CRC.

Novel Aspect:
Metabolomics will provide a holistic signature in organoids and, together with organoid assay data can generate new hypotheses regarding underlying the mechanisms by which polyphenol-gene interaction influences CRC.

References
380 - INDOLOME ANALYSIS FOR NUTRACEUTICAL AND PHYSIOLOGICAL STUDIES.

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Indolome analysis for nutraceutical and physiological studies.

Keywords: isotope dilution, liquid chromatography, melatonin, tandem mass spectrometry, tryptophan ethyl ester

Introduction: Biotransformation products of tryptophan are gaining an increasing multi-faceted interest as nutritional supplements to cope with environmental and lifestyle related stressors. Their identification and measurement in nutritional and biological matrices mandates for flexible, yet accurate and precise analytical methods based on mass spectrometry and adapted to studies with stable isotopic labels. Fragmentation studies are necessary to fulfil this aim.

Methods: An API3000LTQ LC-MS system is used for extensive tandem MS fragmentation studies on 3tryptophan amino-acids, 4 tryptamines and 3 N-acetyl-tryptamines. A stable-isotope analogue of melatonin is custom synthesized. LC separation and quantification parameters are optimized for the detection by MRM of 8analytes and 3 internal standards in four different biological and food matrices.

Results: Spectroscopic studies highlight key fragmentation pathways for the identification and measurement of 8 target analytes. The study of the fragmentation pattern[1] allowed designing and synthesizing[2] an under-considered isotope-labelled form of melatonin [3],to improve quantification by ID-MS-MS, with respect to previously employed isotopologues. Furthermore, the discrimination of the critical pair of isobaric melatonin and isomeric tryptophan ethyl ester, that also yield very similar fragmentation, could be accomplished with a scan function that addresses a triplet of consecutive fragments, only a pair of which are specific for each compound. With this method, the levels of melatonin and of its metabolic pathway are measured in human fluids from several unique conditions, as well as from nutraceutical and pharmaceutical formulations and food matrices.

Conclusions Accurate fragmentation studies of biological indole compounds supply information to design a new internal standard for isotope dilution measurement of melatonin, and identification and quantification methods suitable for the measurement of the indolome in different natural matrices of interest for biological and technological applications.

Novel Aspect: An innovative internal standard to measure melatonin by ID-MS-MS and a method to discriminate isomers and isobars of melatonin are proposed.

References
The exposome, defined as all exposures to which an individual is subjected along life course, involves chemicals or metabolites that can be directly measured in biospecimens such as blood or urine. Hundreds of these chemicals or metabolites, derived from diet, drugs, pollutants and other environmental factors, can affect health and risk of diseases. High-resolution mass spectrometry (MS) techniques, combined with biostatistics and bioinformatics in metabolomic studies, are increasingly used to measure these chemicals and metabolites, in order to discover new biomarkers of exposure or new risk factors for diseases in exposome-wide association studies. These MS techniques are particularly adapted to the measurement of a large diversity of biomarkers of exposure and to assess complex exposures such as diet. Each food is indeed characterized by a specific chemical profile, with compounds often specific for a particular food or food group that are eventually absorbed during digestion. These compounds found in the systemic circulation constitute the ‘food exposome’. Examples will be presented on the identification of novel biomarkers of food intake in dietary intervention and cross-sectional epidemiological studies and on the study of their associations with cancers in recent case-control studies nested in large cohort studies, thus providing new evidence on the role of the diet in disease risk. The same metabolomic studies also provide new data on associations between exposures and the host metabolism shedding new light on mechanisms linking exposures to diseases as will be illustrated for diet, air pollution, overweight or birth weight.

Development of these studies also faces a number of challenges. These include a more comprehensive annotation of the exposome, the measurement of components of the exposome present at low concentrations, the development of quantitative, high throughput and robust methods (targeted metabolomics) for large epidemiological studies. Recent progress in this rapidly moving field will be presented. It should contribute to the identification of novel disease risk factors and to the improvement of our understanding of disease aetiology.
Introduction:
In this study, we present an automated, fast and effective way to identify different cell cultures based on their metabolic profile using laser assisted rapid evaporative ionization mass spectrometry (REIMS [1]). The aim of this study was to create an integrated workflow for the automatic analysis of cell line samples from well plates, and the building of a NCI60 cell line database based on the metabolic and lipid fingerprints of the samples.

Methods:
The technology is based on the direct analysis of aerosol generated by a laser sampling device through thermal ablation of the samples. OPOTEK Opolette IR 532 tunable laser was combined with 3 stages (Thorlab NRT) for 3 dimensional sample movements. This stage prototype was coupled to a XEVO G2-XS QTOF MS and used on a number of different liquid and dried cell line samples from the NCI60 lines. Different multivariate statistical models were used to characterize cell lines.

Results:
A total of 30 different cell lines were analyzed in both dried and liquid form in the mass-to-charge range of 50-2000. Number of parameters such as sample format (dried and liquid in different solvent, cell number per well), laser parameters (wavelength, pulse energy, frequency) and sampling methods (e.g.: aerosol intake) were optimized. The results showed that best signal was achieved with 2900 nm, 20Hz and a minimum of 3mJ energy was applied on 1 Million cells / well. As the acquired mass spectra featured partly different species in liquid form compared to the dried samples, thus a different database was built for the two sample format after lock mass correction, background subtraction and normalization. Testing different classification models created with numerous artificial intelligence algorithms (Support Vector Machines, Neural Networks, Random Forests and Principal Component Analysis followed by Linear Discriminant Analysis) showed us a > 98.0% correct classification rate based on phospholipids, plasmalogens and sphingolipids.

Conclusions:
The results demonstrate that our technology was capable of a rapid, fully automatic LA-REIMS based well plate reading and identification of different cancer cell lines. This method is suitable to analyze lipid and metabolic compounds of different cell lines. Our future plan is to finish the analysis of all cell lines within the NCI60 panel, and to identify the effect of perturbation of cell culturing environment on the metabolic and lipid profile of the cells.

Novel Aspect:
Automated high throughput well plate analysis of NCI60 cell line metabolic fingerprint by OPO laser based ambient MS technique.
A WELLNESS STUDY USING MICROFLOW TARGETED METABOLOMICS TO INVESTIGATE THE EFFECTS OF DIET AND EXERCISE ON THE METABOLOME

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Keywords: metabolomics, QTRAP®, microLC, dried blood spot, polar metabolites

Introduction:
LC-MS/MS is an essential tool for identification and quantitation of metabolites in complex samples to investigate the affected metabolic pathways in dietary assessment studies and precision medicine. Self-testing is on the rise as people are interested in monitoring their health. Here, we have analyzed dried blood spots (DBS) with microflow LC coupled to QTRAP® 6500+ system for a wellness study and monitored polar metabolites covering all major metabolic pathways.

Methods:
Metabolite extracts from a 6-mm dry blood spot disk at three metabolic states; fasted, fed and active were analyzed in 5 replicates on a M3 MicroLC system with HILIC separation at pH9 using Luna-NH2 columns (Phenomenex). MRM analysis performed in positive/negative polarity on a QTRAP® 6500+ system with IonDrive™ Turbo V source. 187 positive and 176 negative ion mode MRM’s were combined into a single experiment to monitor polar metabolites across metabolic pathways.

Results:
A total of 312 polar metabolites were monitored for this wellness study across the three metabolic states (fasted, fed and active). The PCA analysis of these groups in MarkerView™ Software clearly shows that these three groups are differentiated based on their metabolic profile. It is known that during exercise protein degradation occurs, releasing amino acids (AA) into the body including Tyr which is released from human skeletal muscle in the presence of insulin hence we observed the spike in Tyr concentrations in the samples after cycling activity was completed. This release of AAs from protein breakdown also releases branched chain AAs such as Ile and Leu, which are differentiating the active group. Technical replicate analyses of the DBS extracts show insignificant variation between sampling which does not interfere with ability to differentiate these metabolic conditions. The method provides accuracy and precision across each of the different DBS extracts. DBS is a minimally invasive way of collecting blood samples with low cost.

Conclusions:
The microflow targeted metabolomics analysis of DBS shows to be a rapid, sensitive, and accurate method for profiling polar metabolites in a variety of biofluids and could be utilized for monitoring these targeted polar metabolites in other wellness-based studies as the method successfully differentiated these three different conditions we monitored during this wellness study.

Novel Aspect: A sensitive microflow targeted metabolomics method allowing detection of over 300 polar metabolites across multiple biochemical pathways for studying wellness.

For information please contact: scientific@imsc2018.it
Fecal Metabolomics of a Mouse Model of Autism

Keywords: Metabolomics, MALDI, autism, biomarkers, FT-ICR

Introduction: (Limit of 400 characters)
Autism spectrum disorders (ASD) are a group of neurodevelopmental disorders lacking a clinical biomarker for diagnosis. In preliminary studies from our group, it was observed that the level of stercobilin, a metabolite produced in heme catabolism, is depleted in the fecal material of an animal model of autism. Here, we discuss creating an isotopologue standard for quantitation by MALDI FT-ICR MS.

Methods: (Limit of 400 characters)
Utilizing labelled stercobilin synthesized by adding 18-O water to our standard and heating it at 70°C for several hours, it is possible to create an isotopologue to quantify stercobilin levels. Fecal samples are chosen in pairs between the ASD model group and gender-matched controls. Stercobilin is then extracted and prepared for MS analysis. Analysis is completed by MALDI FT-ICR MS (n=5).

Results: (Limit 900 characters)
Although the initial 18-O isotopologue of stercobilin used was shown to be stable through 20 days of storage, back labelling was observed for longer storage. By incubating the sample a second time under the same conditions, labelling efficiency of stercobilin was comparable but more labelling was observed at the labile sites with a lower amount of unlabeled material. Once a new batch of isotopologue was prepared, a new calibration curve was generated to quantify stercobilin within the fecal samples. It is from this work that an observed depletion of stercobilin was observed in the fecal material in a mouse model of ASD. An average depletion of 45% has been observed with a p-value of less than 0.001 (n=14). A less dramatic depletion was also observed with stercobilinogen; more samples will need to be analyzed to determine the statistical significance of the results.

Conclusions (Limit of 400 characters)
The 45% depletion of stercobilin in the fecal material of an ASD mouse model relative to controls at a greater than 99% confidence level suggests that depletion of fecal stercobilin may serve as a potential ASD biomarker in humans. While less statistically significant, it appears that stercobilinogen, the precursor to stercobilin, is also depleted in the fecal samples of ASD mouse models.

Novel Aspect: (Limit of 150 characters)
Quantitative analysis of stercobilin, a putative autism biomarker, in mouse excrement utilizing isotopologue standards and MALDI FT-ICR MS.
965 - URINARY METABOLIC PROFILE OF NEWBORNS WITH TRANSPOSITION OF GREAT ARTERIES UNDERGOING CARDIAC SURGERY WITH CARDIOPULMONARY BYPASS

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Keywords: transposition of great arteries; cardiopulmonary bypass; untargeted metabolomics; newborns

Introduction:
Complete transposition of the great arteries (TGA) is a common cyanotic congenital heart defect. Surgical correction, with the use of cardiopulmonary bypass (CPB), should be performed within the first month of life. CPB-assisted open heart surgery results in brain hypoperfusion and in a powerful systemic inflammatory response and oxidative stress [1,2].

Methods:
Thirteen TGA newborns undergoing arterial switch operation with the use of CPB were recruited. Urine samples were collected before and after CPB and were analyzed using an untargeted metabolic approach based on UHPLC-high resolution mass spectrometry.

Results:
Since the post CPB metabolic spectra could be heavily “contaminated” by metabolites derived from administered drugs, we have constructed a library with drugs used during surgery and the relative metabolites retrieved from urine samples. This library was applied to our samples and a total of 2163 drug metabolites were correctly identified and excluded from the analysis. Afterward we detected over 14000 unique features in urine and 67 metabolites were significantly differentially abundant between pre and post CPB samples (p<0.02; FDR<0.10). Multivariate PCA classification model was established to differentiate between pre and post CPB urine samples.

Conclusions:
This is the first report on the metabolic response to cardiac surgery in TGA newborns. Using UHPLC-high resolution mass spectrometry we identified metabolites whose concentrations appeared to be associated with clinical outcome. Metabolic profiling could be useful for patient stratification and directing investigations of clinical interventions.

Novel Aspect:
We developed a high coverage metabolomic approach that has potential for biomarker discovery in CPB related damage.

References
Keywords: Parkinson’s disease, biomarker, sebum, volatilome, super-smeller

Introduction:
Parkinson’s disease (PD) is the 2nd most common age-related neurodegenerative disorder. The onset of symptoms such as tremor, slowed movements and muscle rigidity do not present in the early stages; however do form the basis of clinical diagnosis [1]. This often results in late diagnosis and treatment, highlighting an urgent need to identify a biomarker. Evidence from a super-smeller has shown there is a characteristic odour released from the skin of PD sufferers [2].

Methods:
Sebum was collected non-invasively using gauze swabs from the upper back of 63 participants; 20 control subjects and 43 PD patients. Dynamic headspace was measured directly from swabs using thermal desorption-gas chromatography-mass spectrometry (TD-GC-MS). For three samples the instrument was interfaced with an odour-port allowing our super-smeller to highlight regions of interest within the GC chromatogram.

Results:
Headspace analysis was performed in two groups; firstly a discovery cohort consisting of 30 subjects was used to build a classification model in which metabolites of interest were identified. Partial least squares discriminant analysis was performed and validated with 5-fold cross validation alongside 26 permutation tests. The variables (n=17) which contributed to classification were selected using variable importance in projections (VIP) scores, where VIP > 1. The volatile components of the second group, a validation cohort of 30 different subjects, were targeted for the presence or absence of these identified biomarkers. Out of 17 metabolites, 9 were found within the validation dataset and subsequent statistical testing was performed. Further to this, regions of interest were identified by superimposing olfactogram data with the corresponding TD-GC-MS chromatograms. A 2 minute window associated with a strong PD-like scent in all 3 samples correlated to the elution time of 3 differentially expressed volatiles identified in both cohorts.

Conclusions:
We demonstrate there is a characteristic volatile profile associated with the scent of Parkinson’s disease. Several potential volatile biomarkers have been found to be statistically significant in the differentiation between disease and non-disease subjects and also associated to the PD-scent detected by a super-smeller.

Novel Aspect:
A non-invasive method of sampling has enabled volatilome analysis of sebum produced from the skin of Parkinson’s disease sufferers and produced a panel of putative biomarkers.

References:
DeMaagd G., Philip A., Pharm. Ther., 40, 504-532 (2015)
Introduction:
Scientific knowledge of chemistry and medicinal properties of native Australian flora is sparse and only occasionally applied in practice. This is especially valid for Tasmania where the information on the Aboriginal use of herbal medicine was poorly documented. This project compares the chemistry of selected native Tasmanian plants (NTP) and their taxonomical medicinal “relatives” as a first step towards a chemical map of Tasmanian flora.

Methods:
Seven plants from three genera were included in this study. Each genus was represented by one (or two) NTP species and one non-native species. Extracts of plants were prepared using ultrasound aided extraction and subsequently analyzed by UHPLC coupled to an LTQ-Orbitrap. High resolution LCMS data were mined using multivariate statistics and targeted metabolomics approaches to compare chemical differences / similarities between the plants from the same genera.

Results:
Principal component analysis and agglomerative hierarchical clustering helped to visualize the chemical relations between the different plants. Interestingly, all native/non-native plant pairs (from the same genera) showed a high degree of chemical similarity. The greatest similarity was detected between the two Epilobium spp. By searching for compounds previously detected in their non-native counterparts, targeted data mining provided insight into the chemistry of the native plants. We determined, for example, that both Epilobium spp. were rich in polyphenolic compounds. Some of these compounds were previously found to have neuroprotective, anti-inflammatory, antioxidant, anti-proliferative, and other pharmacological properties [1]. This finding suggests that native Tasmanian Epilobium species may hold promise for medicinal uses.

Conclusions:
We demonstrate that high resolution LC-MS combined with multivariate statistics is a powerful analytical approach to explore the chemistry of previously unmapped plant species. This is the first comparative study of native Tasmanian & non-native medicinal plants from the same genera.

Novel Aspect:
Novel experimental design and analytical approach based on ultrasonic extraction, UHPLC & HRMS was used to create a proof-of-concept chemical map of native Tasmanian plants.

References
Introduction: (396/400 characters without spaces)
Crohn’s disease is a chronic inflammation of the gastrointestinal tract where the etiology remains largely unknown. LC-MS profiling of serum metabolites has become a powerful tool for biomarker detection. Here, an untargeted metabolomics approach using high resolution accurate mass (HRAM) Orbitrap™ technology with dynamic acquisition for automatic data dependent MS/MS and multistage fragmentation was used to analyze serum from healthy and disease donors.

Methods: (399/400 characters without spaces)
Metabolites were extracted using cold methanol from 18 serum samples consisting of healthy donors and disease donors with or without infliximab treatment. Supernatant was dried and re-suspended in acidified water and separated with a C18 column. Samples were analyzed by an Orbitrap Tribrid™ mass spectrometer with modified acquisition software. Differential analysis and compound annotation were applied using Thermo Scientific™ Compound Discoverer™ software.

Results: (892/900 characters without spaces)
In this study, we explored the use of an intelligent LC-MSn untargeted metabolomics workflow for the identification of potential discriminant markers of Crohn’s disease. We analyzed serum samples from three different populations. Accurate m/z measurements determined molecular formula and putative annotation of thousands of metabolites by searching against ChemSpider. To increase annotation confidence, MS/MS data was searched against the mzCloud library. Multistage fragmentation (MSn) enabled structural elucidation of unknowns. The modified instrument control software automated inter-run inclusion and exclusion lists, facilitating MSn acquisition for more unique metabolites.

Differential analysis detected metabolite perturbations in the urea cycle and catabolism of amino acids in serum from disease donors when compared to that of healthy donors; these metabolic changes are often associated with inflammation. Some of those inflammation driven changes were minimized when disease donors were treated with infliximab.

Conclusions (359/400 characters without spaces)
The combination of an untargeted metabolomics profiling approach with intelligent high resolution MSn acquisition described here, addresses the identification bottleneck in untargeted metabolomics. This strategy presents
promising and facile workflow for the discovery and identification of novel disease biomarkers that could lead to further biochemical insights in disease progression and treatment outcome.

Novel Aspect: (146/150 characters without spaces)
Combination of HRAM Orbitrap technology with automated MSn acquisition strategy for untargeted metabolomics enables detection of Crohn’s disease metabolic signature.
MONOAMINE MAPPING BY MASS SPECTROMETRY IDENTIFIED BRAIN NUCLEI REGULATING ANXIETY IN A SEROTONIN DEFICIENCY MODEL

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Keywords: Imaging MS, on tissue derivatization, neuroscience, monoamines

Introduction:
In the brain, monoamines, dopamine (DA), norepinephrine (NE), and serotonin (5-hydroxytryptamine, 5-HT), act as neuromodulators, diffusing from the nuclei to affect slow-acting receptors of many neurons. Levels of monoamines are important not only for cognitive functions, including memory and learning, but also mood, appetite, and sleep. However, the relationship between actual monoamines' concentrations within the nuclei and behavioral output remains unclear. Here, utilizing imaging mass spectrometry (IMS), we generate a 3-dimensional mouse whole brain atlas of serotonin (5HT), dopamine (DA) and norepinephrine (NE), consisted of 30 coronal slice images.

Methods:
After euthanasia, the mice brains were isolated, immediately frozen and sliced into 8um sections. SHTd4 solution was sprayed on them as an internal standard with an automated sprayer(SunCollect). To perform on-tissue derivatization of monoamines, DPP solution and then DHB solution were manually sprayed. Imaging were performed by a MALDI-FT-ICR MS (Bruker Daltonics). Three dimensional images was constructed using SCiLS lab software.

Results:
Using the atlas, we unexpectedly found several brain nuclei co-contain 5HT and a catecholamine (DA or NE) at high levels; among these, paraventricular nucleus of the thalamus (PVT) was 2nd 5HT rich nuclei in the brain. This novel observation suggests that 5HTergic system was integrated into catecholaminergic system by sharing several major nuclei.

Moreover, we attempted to reveal which 5HT-rich nuclei regulates mice behavior. Here, we used acute tryptophan deficient model (ATD) which exhibits decreased total brain 5HT followed by anxiety behavior. By generating and comparing two 3D monoamine-atlas of control and ATD-model brains, we successfully visualized specific reduction of 5-HT in PVT, which correlates anxiety-like behavior.

Furthermore, we show that the abnormal behavior and brain 5HT level were recovered by injecting [13C][15N]Trp into the ATD model. By visualizing [13C][15N]Trp conversion into [13C][15N]5HT, it is demonstrated that several brain nuclei including PVT have surprisingly high turnover of de novo 5-HT synthesis.

Conclusions:
These results proved usefulness of brain-monoamine mapping by imaging MS to study relationship between monoamine concentrations within small brain nuclei and animal behavior.

Novel Aspect:
3D brain atlas of monoamines created by imaging MS was utilized to study relationship between monoamine concentrations and animal behavior.
Introduction:
High-resolution LC-MS is an important platform for metabolite detection and quantitation. However, for untargeted Metabolomics rapid, unambiguous and universal compound identification is still challenging. In this work, we report the construction of a library for relevant endogenous metabolite and its successful application to human biofluids.

Methods:
Standards were obtained from the Human Metabolome Database (HMDB). They were injected into an Intensity Solo C18 column via an Elute LC system and detected by a QTOF-MS (impact QTOF, all Bruker Daltonics) for acquiring MS/MS library spectra and the retention time determination (RT). For the analysis of biofluids the same setup was used following a standard operating protocol (SOP) for consistency.

Results:
In this study a library from over 800 endogenous metabolites was created. It contains MS/MS spectra of 635 compounds acquired in positive mode and 474 negative mode spectra. Up to 5 collision energy levels were applied for each standard giving more than 6000 MS/MS spectra in total. For each metabolite, library fragment spectra were manually curated by confirming each fragment via a molecular formula. For unambiguous identification we determined the RT of each standard. Automatic metabolite identification was performed in the MetaboScape software based on matching of multiple parameters: precursor mass accuracy and isotopic pattern, RT, and MS/MS spectrum quality.

Conclusions
The established SOP and library was applied analyze to biofluids, e.g. plasma and urine, and metabolite identification results will be presented. Finally, we have examined the portability of this library for different instrumental conditions and for different labs.

Novel Aspect:
Development of a high-confidence and rapid metabolite identification method by combining a high-resolution MS/MS library and RT information.
Introduction: (Limit of 400 characters without spaces)
The challenge of metabolic profiling requires the use of analytical platforms that maximize the peak capacity. Previous studies have shown that the hyphenation of highly orthogonal methods such as hydrophilic interaction liquid chromatography (HILIC) with field asymmetric waveform ion mobility spectrometry (FAIMS) and mass spectrometry (MS) increased the number of peaks detected [1,2].

Methods:
The developed workflow included one-step sample preparation by protein precipitation with acetonitrile which was followed by a 13 minutes chromatographic run on a HILIC column. The elute from the column was ionized by positive ion ESI and separated by FAIMS with the compensation field (CF) scanned from -0.5 to 3.5 Td (DF 240 Td) in 1 second at an MS scan rate of 20 scans per second, resulting in the acquisition of 19 MS across each CF scan, with one scan for re-initialization.

Results:
The described workflow was applied to the analysis of fresh and aged (72 hours) urine samples. The method has shown good reproducibility for LC retention times (%RSD 0.13-0.87) and peak areas (%RSD 4.46-11.31). Principal component analysis (PCA) demonstrated a clear separation of fresh and aged urine at different CFs. Several biomarkers (up- and down-regulated) were found and identified for aging of urine by the presented method. The highest change in abundance was observed form/z 121.072 (urea dimer) which increased in concentration with the age of the sample.

Conclusions
The combination of HILIC with fast scanning FAIMS and MS was applied to the analysis of urine samples. This rapid method including a simple sample preparation shows the potential for untargeted metabolomics and biomarker discovery.

Novel Aspect:
For the first time, the hyphenation of FAIMS with LC-MS was applied to untargeted metabolomic studies of urine samples.

References
INTRODUCTION

Plasma samples are commonly used for biomarker screening by LC-MS. However, pre-analytical variability in processing blood can lead to spurious results [1]. Our previous experience with thymosin beta-4 as biomarker interest us in the problem of residual platelets in plasma [2]. Proteomics-based markers however may not be conveniently incorporated into a metabolomics-only workflow, hence a panel of metabolic markers for sample quality control is ideal.

METHODS

A lipidomic-focused screen for markers that is consistently higher in platelet rich compared to platelet poor plasma was conducted. Plasma was extracted using a single-phase method [3], then analyzed on a UPLC-QTOF system. Lipid identification and shortlisting was done using Lipid Data Analyzer 2.6.2 (LDA) [4].

A systematic literature search was also conducted to identify other reported pre-analytical sample quality issues and markers proposed for them.

RESULTS

Molecular features 774.54 m/z and 746.51 m/z are the most consistently elevated in platelet rich plasma compared to platelet poor plasma. Based on tandem ms spectrum matching on LDA, these had been tentatively identified as plasmalogen phosphatidylethanolamine (P-PE) 36:4 and P-PE 38:4 respectively. Further validation are underway.

Common sample quality issues identified in the literature are: inappropriate use/non-use of anticoagulant, pre-centrifugation time delay, freeze-thaw cycles, hemolysis, sample handling, and sample storage. Markers for markers pre-centrifugation delays are the best studied: Sphingadienine-1-phosphate [5], ornithine/arginine ratio [6], ascorbic acid/lactic acid [7], cytokine levels [8], gamma-glutamyl-transferase and lactate dehydrogenase [9] have been proposed as markers. Gamma-glutamyl-transferase and lactate dehydrogenase [7] also marks for excessive freeze-thaw cycles. Hemolysis can be detected by hemoglobin or discoloration.

CONCLUSIONS

P-PE 36:4 and P-PE 38:4 are candidate markers for residual platelets in plasma samples. Because P-PE is a major constituent of cell membrane, we suspect it may be “housekeeping” in platelet membrane. We intend to further characterize whether these markers can normalize for residual platelet content in plasma. Through validating the existing plasma quality markers in the literature, we hope to put together a robust LC-MS panel for assessing plasma sample quality.
Novel Aspect:
Standardized protocols can reduce variability, however compliance may be infeasible. A LC-MS panel for plasma quality check would be transformative for biomarker screening.

References


Introduction:
We studied the metabolic adaptation of the bacterium Acinetobacter baylyi ADP1 (ADP1) after a biotic stress that consisted in shifting the carbon source from succinate to quinate (1). Quinate metabolism in ADP1 has been investigated for decades (2). Yet, we spotted unexpected and unknown metabolites in quinate-grown cells (1). These orphan metabolites (OM) may participate in new pathways and represent entry points into the dark part of bacterial metabolism.

Methods:
100 µg of a first metabolite of m/z = 211 (M211) was purified by preparative HPLC and its structure was analyzed by NMR. The interpretation of the 1H, COZY, HSQC-HMBC (1H-13C), ROESY, 13C and 1H-15N spectra, recorded on a 600 Hz NMR fitted with a cryoprobe.

Results:
NMR analyses led us to conclude that the molecule was a (3-aminopropyl)-amine-hydroxybenzoic acid. Because of the small quantity of purified material, NMR data acquired did not allow us to unambiguously conclude on the position of the alcohol and carboxylic acid groups on the benzene ring of the molecule. To overcome this uncertainty, the compounds corresponding to the different six possible structures were chemically synthesized (Synthenova.com). Comparison of the NMR signalson one hand and high resolution (LTQ-Orbitrap Elite) LC/MS/MS data on the other hand between the purified metabolite and the reference standards led us to assign the structure of the compound of interest as 3 (3-aminopropyl) amine 4-hydroxybenzoic acid. This is a previously undescribed compound that is missing from all interviewed databases.

Conclusions
The identification of a second metabolite (M193) is under way. These structural elucidations are a first step in deciphering pathways that take place during quinate metabolism. We would next identify genes involved in their synthesis. We are using the complete library of deletion mutants of ADP1 (3) for determining their molecular phenotype (2600 metabolomes) and seek mutants in which OM are absent. In such mutants, the deleted genes thus participate in MO synthesis.

Novel Aspect:
We solved the structure of a novel metabolite and investigate the genes involved in its biosynthesis for deciphering previously unreported metabolic pathways.

References


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Introduction:

Thirdhand tobacco smoke (THS) is a novel and poorly understood pathway of tobacco exposure produced by the accumulation of secondhand smoke (SHS) on environmental surfaces that ages with time, becoming progressively more toxic[1]. Although the emerging evidence of THS harm, the molecular mechanisms of THS exposure are still unclear. Here, we present a multiplatform metabolomics approach for the characterization of metabolic disorders in liver of mice exposed to THS.

Methods:

C57BL/6 mice were exposed for 24 weeks to THS, under conditions that mimic exposure of humans in homes of smokers. Aqueous and lipid liver extracts were analyzed by 1H NMR and UHPLC-MS. AMIX and Chenomix programs were used to process NMR data and XCMS to LC-MS. Statistics and pathway enrichment were performed using the Metaboanalyst platform tools. Mass spectrometry images of liver were acquired by laser desorption-ionization MS, and processed with rMSI software[2].

Results:

Analysis of the lipid extracts by NMR confirmed that THS exposure increases the levels of triglycerides, polyunsaturated and monounsaturated fatty acids, which can lead to the development of fatty liver disease, as previously described[3]. UHPLC-MS analysis of the extracts allowed the identification of more than 30 triglycerides and 10 phosphocholine elevated in liver of THS-exposed mice. MS images showed lipid accumulation and increased lipid droplets due to THS-exposure. Statistically relevant ions in the images were further identified as 14 triglycerides. Untargeted metabolomics analysis of the aqueous extracts allowed the identification of 48 statistically significant metabolites. Pathways analysis showed that 19 of these metabolites are involved in the glutamate, glutathione and nicotine/nicotinamide metabolism pathways, which are related to nucleic acid and protein synthesis among others[4],[5],[6]. Furthermore, the dysregulation of these pathways confirms the oxidative stress nature of THS exposure health effects[7].

Conclusions:

The combination of three different analytical platforms allowed the identification of more than 80 metabolites relevant for the health assessment of THS exposure. NMR provided reliable identifications and quantifications and UHPLC-MS was key for the identification of many dysregulated metabolites and the identification of lipids.
whereas MSI allowed us to enrich the biological interpretation by the mapping of lipids in control and THS-exposed livers.

**Novel Aspect:**

First metabolomics characterization of dysregulations in liver from THS-exposed mice, by MS and NMR multiplatform approach, including extracts analysis and tissue MS images.

**Bibliography:**

56 - SIMULTANEOUS QUANTITATIVE ANALYSIS METHOD FOR HYDROXYPROLINE AND 4-HYDROXYGLUTAMIC ACID USING ISOTOPOologue-SRM TO CLARIFY HYDROXYPROLINE METABOLISM.

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Keywords: 4-Hydroxyglutamic acid, HILIC, isotopologue-SRM, Hydroxyproline metabolism

Introduction:
The main component of kidney stones is calcium oxalate. Oxalic acid is synthesized from hydroxyproline (HP) in vivo in addition to those from diet. We examined intermediate metabolites on HP metabolic pathway to clarify HP metabolism. 4-hydroxyglutamic acid (4HG) was found to be a marker of HP metabolism, and an simultaneous analytisis method for HP and 4HG was established.

Methods:
Culture media were acidified and deproteinized with acetonitrile and then centrifuged. The supernatant were injected into LC-MS/MS operated in ESI-positive mode. Chromatographic separation was achieved with Acquity BEH Amide column. HP and 4HG were quantified using 2H3-HP and 2H3-Lys as internal standards.

Results:
Chromatographic separation of HP from other amino acids such as Met, Leu, Ile and Asp was achieved. The range of calibration for HP and 4HG were from 0.5 to 1000 microM and from 0.05 to 100 microM, respectively. Isotopologue-SRM (iSRM)[1] enabled to quantitate HP and 4HG simultaneously improving the linearity of calibration. 4HG concentration was elevated after adding HP to culture medium of human hepatocytes.

Conclusions
Although 4HG analysis method with derivatization have already been reported[2], the developed method is highly sensitive in addition to excellent operability not requiring derivatization. This method is considered useful for the study of HP metabolism.

Novel Aspect:
Simultaneous quantitation of metabolite at lower concentration and substrate at higher concentration using iSRM.

References
AN UNTARGETED METABOLOMICS APPROACH TO GO BEYOND ASPIRIN CANONICAL EFFECT

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Keywords: metabolomics, untargeted analysis, aspirin, urine profile

Introduction: Pharmacometabolomics aims to highlight metabolic signatures after drug exposure. Although the canonical cyclooxygenase-1-mediated effect of Acetylsalicylic acid (ASA) is well defined, a substantial variability in drug response exists and the mechanisms at the basis of this variability are still poorly understood [1-2]. Applying an untargeted metabolomics approach, we aimed to reveal novel information about biochemical pathways modified by ASA treatment.

Methods: In this study we have developed an untargeted liquid chromatography-mass spectrometry method to define the urine metabolic profile of healthy subjects (n=7) before and 7 days after 100 mg once-daily ASA treatment.

Results: Through this untargeted method, we detected 2007 features: among them, 64 metabolites significantly differed (p<0.05) after ASA assumption. Pathway analysis, performed on identified metabolites, revealed low levels of those metabolites involved in histidine, alanine, aspartate and glutamate and purine metabolisms, after ASA treatment. In addition, we observed the decrease of several short- and medium-chain acylcarnitines.

Conclusions: The data here reported reveal unusual pathways affected by ASA treatment, in particular, the decrease in urine acylcarnitines levels indicates an increase in fatty acid \(\beta\)-oxidation process. This observation may suggest non-canonical use of ASA in clinical situations characterized by energy depletion.

Novel Aspect:
This study demonstrate the pivotal role of the untargeted metabolomics approach in exploring new clinical application of drugs.

References
Scientists across many disciplines are looking at metabolomics to answer challenging questions. Quantitative studies have focused on the steady-state metabolite levels where experimental variability can mask the true degree of metabolic regulation. Herein, we evaluate the merits of high resolution approaches to the measurement of the flux heavy isotopes in a metabolic pathway shows great potential to elucidate the regulation / kinetics of metabolic pathways.

Methods: 
MDCK cells were cultured under sterile conditions. A control and 13C labeled time course were created (0,1,2,5,10, and 20 min). Controls was fed with normal media and flux samples were fed with stable isotope labeled 13C6-glucose media. Samples were washed 2x with 37C PBS lysed in icecold 70% MeOH. Cell supernatant was injected onto weak anion exchange chromatography. Acquisition was performed on TripleTOF 6600 in positive and negative mode withDDA or DIA.

Preliminary Data: 
Samples were analyzed by multiple means to create a library of observed metabolites. Careful creation of control samples (unlabeled) was important for the discovery of metabolites as well as the confirmation of species by mass accuracy, isotope ratio and MS/MS fragment pattern. Multivariate analysis (PCA and PLSDA) of perturbed samples was particularly useful for identifying species that were undergoing significant flux of heavy atoms into a pathway. Major differences in the degree of kinetic flux were apparent between labeled controls and treatments that received pathway inhibitors.

Simple unit resolution data dependent MS/MS, which is the most traditional style of LC/MS data collection, was employed for basic confirmation of metabolites. In addition, DIA and some targeted MS2modes were also employed to evaluate their utility for kinetic measurements. The results support that the traditional DDA workflows were adequate for identifying and doing some basic quantitation of metabolites.

Conclusions: 
The stochastic nature of data dependent MS2 data collection limits the use of MS2 to identification only. Measurement of flux kinetics and identification of the locale of heavy atom incorporation is made possible by using targeted "scheduled" analyses as well DIA. 
This work demonstrates the combining high levels of resolution and scan speed at the MS and MS2 level. Level of performance can generate information rich targeted and DIA datasets for metabolomics flux studies.

Novel Aspect: 
The use of data independent acquisition (vs. traditional DDA) to elucidate the structure and kinetics of metabolomics flux.
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Keywords: targeted metabolomics, standardization, quantitation, reproducibility

Introduction: Type 2 Diabetes, T2D, is a metabolic disorder characterized by decreased insulin sensitivity and abnormal hepatic glucose production. Metabolomics creates new capabilities for understanding metabolic disorders as it enables gaining insights into physiological and pathophysiological processes. We employed a standardized, quantitative method for targeted metabolic profiling of 408 metabolites in serum samples from T2D study subjects and healthy controls.

Methods: Sample preparation was performed with the AbsoluteIDQ® p400 HR Kit (Biocrates Life Sciences, AG). Briefly, 10 ul of plasma samples were pipetted onto the 96-well plate containing the internal standards. Following derivatization, metabolites and internal standards were extracted and diluted for subsequent analysis. Mass spectrometric analysis was performed on a Vanquish™ UPLC system coupled to a Thermo Scientific™ Q Exactive™ HF Orbitrap mass spectrometer.

Results: We aim to monitor changes in metabolite concentrations in serum samples obtained from T2D study subjects and healthy controls. We have identified several metabolites that were significantly altered between the two study groups (p values < 9.97E-04). For instance, we observed increased concentrations of acylcarnitine AC(2:0), cholesterol ester CE(22:6), diglycerides DG(41:1), DG(36:4), sphingomyelins SM (36:1), SM(42:3), and phosphatidylcholines PC(38:6), PC(40:6) in the T2D serum samples versus the healthy controls. The concentration of serotonin, triclecyride TG(52:6), and phosphatidylcholines PC(40:4) were lower in the T2D serum samples. Acetylcarnitine AC(2:0), phosphatidylcholines PC(38:6), PC (36:6) and PC(40:6), serotonin and Ile, have been previously reported to be associated with higher likelihood of T2D, obesity, or they have shown differential changes in response to oral glucose tolerance test[1-4] in agreement with our findings. In terms of the method reproducibility, for 90% of all metabolites the % RSD was < 20%.

Conclusions: By employing a standardized and quantitative analytical method and high resolution accurate mass (HRAM) mass spectrometry we were able to monitor metabolite changes in serum samples obtained from T2D study subjects and healthy controls. Overall, our results demonstrate that the method provides standardized and accurate quantitation for monitoring and quantifying changes between different study groups, while, providing high reproducibility

Novel Aspect: Combining a standardized analytical method with high resolution mass spectrometry for targeted metabolic profiling of T2D serum samples

References
Keywords: Lithium ion, Fat-soluble vitamins, Simultaneous analysis

Introduction:
In drug development, it is frequently demanded to investigate the fat-soluble vitamins when malabsorption of the fat-soluble vitamins is suspected as a side effect of treatment. Despite the need for a high sensitive analysis with a limited sample volume especially in a non-clinical study, a quantification of the fat-soluble vitamins using LC-ESI-MS/MS has been difficult because of the low ionization efficiency and fragmentation reaction. The aim of the work was to develop a high sensitive simultaneous analysis of the fat-soluble vitamins in a biological sample using a post column infusion of a lithium ion.

Methods:
Lithium ion was added post-column and the fat-soluble vitamins were analyzed by the multiple reactions monitoring (MRM) method. The transition of the MRM method used was m/z 286 (M)+· to 255, 300 (M)+· to 255, 384 (M)+· to 118, 400 (M)+· to 118, 423 (M+Li)+· to 387, 431 (M+H)+· to 165 and 451 (M+H)+· to 187 for vitamin A, retinoic acid, vitamin D3, 25OH-vitamin D3, 1,25(OH)2-vitamin D3, vitamin E and vitamin K1, respectively.

Results:
When vitamins A and D3 were ionized in the presence of the lithium ion, the (M)+· as well as (M+Li)+· was detected as a precursor ion. Furthermore, the MS/MS spectrums pattern obtained from (M)+· or (M+Li)+· significantly differed from the MS/MS spectrums pattern obtained from the (M+H)+·. Surprisingly, when the standard sample of the fat-soluble vitamins was measured using a MRM method in the presence of the lithium ion, the intensity and S/N ratio of the peak of the vitamins A and D3 were much higher than those measured without lithium ion. The magnitude of their increase was 5 to 10 times.

Conclusions:
In a simultaneous analysis of the fat-soluble vitamins, the ionization efficiency and fragmentation pattern were improved using a post column infusion of a lithium ion. The sensitivity and S/N ratio increased because the product ion from (M)+· or (M+Li)+· significantly differed from the product ion from (M+H)+·.

Novel Aspect: (Limit of 150 characters)
A quantitative fat-soluble vitamins analysis with improved sensitivity and specificity in the presence of the lithium ion and detecting (M)+·.
Human Metabolite Identification by Searching a High Quality and Comprehensive Tandem Mass Spectral Library

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Introduction:
LC/MS/MS is a routine technique for compound identification in metabolomic studies. However, accurately identifying metabolites from millions of mass spectra is critical for data analysis. We applied an extended high quality and comprehensive reference tandem mass spectral library with >17,000 compounds, >141,000 precursors, and >671,000 spectra to identify human metabolites from LC/MS/MS data.

Methods:
Mass spectra (MS2, MS3, MS4) of each authentic compound were acquired in positive and negative modes at different collision energies for major ions including isotopic precursors and in-source fragment ions with Orbitrap Elite and Lumos instruments (HCD, FT-IT, IT) for extending a tandem mass spectral library. Human metabolites were identified by searching this library with MS Search program 2.3.

Results:
The extended tandem mass spectral library [1, 2] contains 671,473 spectra, 141,927 precursor ions from 17,631 compounds. Of these, 4,005 are human metabolites. This library has been examined and applied in metabolite identification in human plasma, urine, and milk samples that were run on an Orbitrap Fusion Lumos HCD mass spectrometer. For example, 325 metabolites and 154 in-source fragments from these metabolites by various neutral losses (e.g. [M+H-NH3]+ from tryptophan) were identified in a urine sample by searching the library. Over 700 human metabolites were identified with positive and negative precursor ions (e.g. [M+H]+, [M+Na]+, [M+2H]2+, [M-H]-). The in-source fragments were also identified in ~50% of the identified metabolites. Although the structures of the in-source fragments can be easily deduced from the original compounds, these sub-structures may not exist in the sample.

Conclusions:
The high-quality reference tandem mass spectral library (17,631 compounds, 141,927 precursor ions, and 671,473 spectra) can be used for comprehensive metabolite identification. The spectra of in-source fragments can identify common fragmentation artifacts and high duplicate “unknown” spectra, confirm metabolite identification and help identify metabolites that are not in the library.

Novel Aspect:
A high-quality reference tandem mass spectral library, including 4,005 human metabolites, facilitates accurate identification.

References:
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Keywords: In vivo real-time monitoring, Metabolites, PESI/MS/MS, Real-time metabolomics

Introduction:
Recent improvements in ambient ionization techniques have achieved real-time monitoring of biogenic compounds in organisms. Here, we developed a novel analytical system for in vivo real-time monitoring of metabolites in a living mouse brain [1]. It consists of a manipulation system and a probe electrospray ionization unit, which uses an extremely thin solid needle (tip dia.: 700 nm) for direct sampling and ionization, coupled to a tandem mass spectrometer [1-3].

Methods:
The system consists of a LCMS-8040 equipped with PESI (Shimadzu) and the manipulation system (Shimadzu). To validate the system, a mouse was anesthetized and ca. 4 mm-dia. hole was drilled at the center of the skull to expose the intact brain surface. A special sample cup was fixed onto the skull, and 8 metabolites were analyzed by the system. A CB1 agonist, which also acts as an inhibitor of TCA-cycle activity, or vehicle were administered 3 min after the analysis started.

Results:
Eight metabolites including TCA-cycle intermediates were successfully monitored in real time in a living mouse brain for 3 hours. Glutamic acid levels immediately decreased after the analysis started for both CB1 agonist-administered and vehicle mice. For CB1 agonist-administered mouse, glucose rapidly decreased 30 min into the analysis. At the same time, citric/isocitric acid increased remarkably. ADP and TCA-cycle intermediates, succinic, fumaric and malic acids, maintained its level or weakly increased, while α-ketoglutaric acid level slightly deceased. This state continued for about 30 min, then returned to their initial levels. During these dynamics observations, glucose and citric/isocitric acids seem to fluctuate coordinately. On the other hand, these phenomena were not observed in the vehicle mouse. Additional experiments demonstrated that the extracellular glutamic acid changes monitored by microdialysis showed the opposite profile, suggesting that the present system may be capturing intracellular metabolic changes.

Conclusions:
We developed a novel in vivo real-time monitoring system for a living mouse brain by combinational use of PESI/MS/MS and a manipulation unit. We applied the system to real-time monitoring of metabolites in a living mouse brain, successfully capturing the metabolic dynamics in mouse brain over 1 hour with 20-second intervals. The systems is highly expected to be expanded to real-time metabolomics in the near future.

Novel Aspect:
The newly-developed analytical system achieved in vivo real-time monitoring of the metabolites in a living mouse brain.

References
Introduction:
Antimicrobial resistance (AMR) is one of the most severe problems affecting public health and safety. It is crucial to understand the mechanism of antimicrobial resistance from molecular level. Herein, we presented an innovative TiO2 enhanced LDI method, which is able to recognize different types of bacteria at both species and strains level based on metabolites profiling by MVA analyses.

Methods:
The workflow mainly involves sample preparation, data acquisition and MVA analyses. Briefly, P25 TiO2 was utilized as LDI matrix. And then, a step by step procedure was applied with formic acid and 1-butanol acting as lysis solution together with salicylic acid and dopamine serving as charging additives in positive and negative mode respectively. Mass peaks were then aligned using an open source R package “MALDIquant” and normalized by total peak areas.

Results:
This TiO2-LDI MS based metabolomics method can easily type four species of bacteria, including E. coli, K. pneumoniae, P. aeruginosa and S. aureus, based on PCA and PLS-DA models. The robustness for bacteria taxonomy was demonstrated by iterative validation using 48 species of clinical bacteria. It is worth noting that this method also achieved fast bacterial AMR recognition, which possesses promising clinical diagnostic values. A multivariate ROC curve was obtained based on all obtained 11 potential biomarkers by Monte-Carlo cross validation (MCCV) with an AUC of 0.955. Pseudo-unknown bacteria test further confirmed AMR recognition capacity of our method.

In conclusion, pathway and metabolites network analysis were also performed to find responsible metabolites and mechanisms for AMR formation of ESBL+ E. coli at molecular level. Cysteine and methionine metabolism, purine metabolism, arginine and proline metabolism, glyoxylate and dicarboxylate metabolism, as well as peptidoglycan biosynthesis were found in disorder in ESBL+ E. coli.

Conclusions:
This research presented an innovative TiO2LDI-MS method with good performance in the applications of bacteria identification, rapid AMR recognition and metabolomics analysis, which may provide an alternative to traditional LC-MS and/or NMR based metabolic analysis strategies.

Novel Aspect:
This method presented a new metabolomics method based on LDI-TOF technique, which solved some crucial problems involving complexed background interferences, peaks alignment and normalization.

References
Introduction:
A fast and straightforward lipid extraction method from plasma is introduced in this study. Lipids are one of the major components of biological cell membranes, and they participate in many biochemical functions and metabolic processes, such as intercellular signaling, secretion, and energy storage [1]. The aim of our study was to develop a lipid extraction using a spin column with superabsorbent polymer beads (mSAPs). Although our previous study about fast lipid extraction method using SAPs has been reported [2], it is still challenging to realize quantitative analysis. Thus, a newly developed a simple and reliable lipid extraction method using a spin column filled with SAPs was built to shorten the time of extraction. We evaluated the extraction efficiency and the reproducibility of this method using liquid chromatography-mass spectrometry (LC/MS) analysis to compare properties of our method with the modified Folch method. The modified SAP method was applied to lipid extracts from the plasma sample, demonstrating that it can be powerfully utilized for high-speed (<10 min) preparation for lipid extraction.

Methods:
The ten microliter of plasma was dropped into the spin column with SAP beads and left for 30 s to complete absorption of aqueous solutions. Then, mixture of organic solvents, MTBE:MeOH, 2:1 (v/v) were loaded to the SAP beads, and the samples were incubated for 2−3 min. For obtaining extracts, the spin columns are followed by centrifugation at 6,000 rpm for 2 min. The organic phase was collected and dried with nitrogen gas. The extracted lipids were dissolved in 200 μL of IPA:ACN:DW (65:30:5 v/v) for storage at 4 °C before MS analysis. Then, they were analyzed using ultra-performance liquid chromatography−mass spectrometry (UPLC/MS).

Results:
There is an urgent requirement of a quantitative lipid extraction method for plasma sample especially in the area of clinical research. For the conventional lipid extraction, arduous supernatant transfer and large volume organic solvents need to be repeated twice. Hence, we have to adopt a spin column with SAPs. After its improvement, time-consuming reduces about 95 % for lipid extraction. For the qualitative lipid profiling after extraction using the modified SAPs method, plasma samples were analyzed using LC-ESI/MS. The results of the LC/MS chromatograms and MS/MS spectra indicated that the modified SAPs method successfully extracted lipids from plasma. Also, we found that LOD of the modified SAPs method (0.035pmol) was approximately about seven times lower than that of the modified Folch method (0.243pmol), indicating that modified SAPs method is more sensitive than the modified Folch method in positive-ion mode. For the possibility tests of the lipid extraction using the spin column with SAPs, lipid extracts in another biological sample such as CSF were analyzed for lipid profiling. These results similarly corresponded with modified Folch Methods:

Conclusions:
Generally, conventional lipid extraction demands multiple steps, including drying, incubation, partitioning, and centrifugation, which results in tedious labor and low performance. However, the extraction of lipids using the spin column-based SAP is much simpler and faster because it eliminates the complicated processes. Therefore, we firmly
conclude that the modified SAPs method is a very easy, fast, and quantitative method that is especially important due to its use of small amounts of samples from complex biological specimens, such as plasma/serum for clinical use.

Novel Aspect:
A newly high-speed and quantitative lipid extraction method was developed; this method has extensive application prosperity in the clinical field.

References
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Keywords: Atopic dermatitis; skin tape strips; sphingolipids; lysophosphatidylcholines

Introduction: Allergic skin diseases severely impair the quality of life of affected patients. Atopic Dermatitis (AD) is linked to a damaged skin barrier function that can precede clinical AD manifestations by many months. There are no effective tools to predict the development of AD or to characterize the efficacy of applied therapy. Therefore, novel approaches to advance mechanistic understanding of AD as well as clinical management of AD and related skin diseases are needed.

Methods: A novel protocol for simultaneous assessment of lipids and filaggrin breakdown products in skin stratum corneum using tape stripping and liquid chromatography tandem mass spectrometry was employed. Targeted analyses with scheduled MRM algorithm were employed for precise quantitation of analytes. The hallmark of our approach is in the ability to normalize targeted mass spectrometric data per sample total protein content that was never done before.

Results: Lesional and non-lesional stratum corneum of 30 AD subjects was compared with 25 healthy individuals. In addition to the known redistribution of ceramide molecular species in AD, our study provided novel findings. Thus, the levels of sphingomyelins, and especially those with short chain fatty acids, increased several fold in atopic lesional skin. Most strikingly, lysophosphatidylcholines demonstrated a profound redistribution towards the prevalence of short chain species not only in lesional but also in non-lesional skin of AD subjects. RNAseq analysis performed on stratum corneum from same subjects revealed the downregulation of the expression of fatty acid elongases ELOVL3 and ELOVL6 that may explain the observed global redistribution of the long chain and short chain lipids. The modeling of the effect of type 2 cytokines on sphingolipids using human differentiated keratinocytes in vitro along with stable isotope pulse labeling confirmed the ability of type 2 cytokines IL-4 and IL-13 to provoke similar changes in sphingolipids.

Conclusions: Our ongoing studies further validate the power of the developed methodology and the value of lysophosphatidylcholines as biomarkers of atopic disease. Furthermore, skin tape stripping in conjunction with our unique processing protocol and targeted mass spectrometric methodology opens novel possibilities in clinical research by unveiling novel biomarkers of skin diseases and by allowing clinical studies in the skin using minimally invasive methodology.

Novel Aspect: Previously unrecognized association of sphingomyelins and lysophosphatidylcholines in skin stratum corneum with atopic dermatitis.
Introduction
A new computational tool for the prediction of mass spectra based on quantumchemical calculations is being developed, called Quantum Chemical MassSpectrometry for Materials Science (QCMS2). It was benchmarked by predictingthe EI fragmentation pathways of anumber of organics witha variety of functionalities. Themain features in the mass spectra were correctly reproduced and new fragmentation routes proposed by QCMS2 were confirmed by MS/MS measurements [1].

Methods
QCMS2 is based on DFT/B3LYP/6-311+G* calculations and selects fragmentation pathways based on the relativevalues of bond orders, and reaction (for bond cleavages) and transition-state energies (for rearrangements).Known peptide-specific fragmentation mechanisms such as the Mobile Proton Model for His, confirmed by quantumchemical calculations, have been implemented into the method [2].

Results
QCMS2 has been applied to predict the fragmentations of tripeptides in ESI/MALDI CID MS, focusing on the influence of inter-side-chain(ISC) interactions on initial protonation and subsequent fragmentation. The fragmentations of non-cyclic tripeptides consisting of His as the central amino acid (AA) have been studied and a number of observations illustrate the importance of strong ISC interactions between the AA; as a result, QCMS2 outperforms common tools such as PEAKS and Prospector in the assignment of tripeptide mass spectra [3].

Given the more recent interest in lipidomics QCMS2 is currently being used to predict the fragmentations of lipids. Common tools such as LipidXplorer and LipidBlast generally perform poorly due to the limitations of the models (e.g., the absence of McLafferty rearrangements). The results obtained for compounds such as sphingosylphosphorylcholine will be presented: fragmentation pathways in both positive- and negative-ion mode ESI/MALDI CID MS have been calculated and compared to the experimental spectra.

Conclusions
QCMS2 reproduces the main features in the mass spectra of tripeptides, i.e., the traditional backbone cleavages resulting in a-, b2- and y1-ions, but, significantly, also predicts fragments resulting from ISC interactions. Likewise, it provides superior assignments of lipid spectra by taking a considerably larger number of fragmentation pathways into account. QCMS2 surpasses common tools in terms of insight into fragmentation and assignment of spectra.

Novel Aspect
QCMS2 assigns up to twice the number of signals in peptide/lipid spectra assigned by common mass spectral assignment software, and generates detailed fragment structures.

References
Introduction
DEHP is a phthalate used in many consumer products. It is metabolized in the liver to MEHP, MEOHP and MEHHP. These compounds are known to alter organ lipid metabolism acting as metabolic disruptors [1]. Since the liver is a key organ in whole body metabolism and waste handling, the aim was to quantify DEHP metabolites in liver by UHPLC/MS-QTOF and relate them to alteration in organ lipid profile [2,3].

Methods
We evaluated the effect of 28 days exposure to DEHP on hepatic lipidomic profile. We studied 3 groups of rats (n=4 each, 2F/2M) exposed to low, medium and high dose vs non exposed. Lipids and DEHP metabolites were extracted from liver tissues (10-20mg) and analyzed with ZORBAX Eclipse Plus C18 and ZORBAX SB Phenyl 2.1x100mm 1.8µm columns respectively. Concentrations were quantified using labeled internal standards added before the preparation.

Results
Hepatic concentrations of unconjugated DEHP metabolites (that reflects exposition) were increased after exposure. Male rats had significantly higher hepatic accumulation of both DEHP metabolites and lipids. Different lipid composition was found in rats exposed to DEHP vs non exposed: Ceramide CER(18:1/24:0) and CER(18:0/24:1) known to be involved in apoptotic pathway, were significantly higher (p<0.05) in exposed rats indicating tendency to hepatic damage, while CER(18:2/20:0) was lower than in non-exposed rats (p<0.05). These ceramides were synthesized more in proportion to total hepatic accumulation of DEHP metabolites. After DEHP exposure we also observed decreased hepatic phosphocholine PC(32:2) and phosphoethanolamine PE(38:2) (p<0.05) and increased saturated triacylglycerols (TAGs) and diacylglycerols (DAGs), compounds known to be lipotoxic.

Conclusions
Rats exposed to DEHP showed a different hepatic lipid composition compared control rats, in particular in lipids involved in lipotoxicity and apoptosis.

Novel Aspect
We set up a new method by UHPLC/MS-QTOF to quantify DEHP metabolites and evaluate their metabolic effects from small amounts of livers of rats exposed to different doses of DEHP.

References
A QUANTUM CHEMICAL TOOL FOR THE IDENTIFICATION OF FRAGMENTATION PATHWAYS: ADDITIONAL INSIGHT INTO LIPID-SPECIFIC FRAGMENTATION MECHANISMS

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Keywords: Quantum Chemical Mass Spectrometry (QCMS2), Density Functional Theory (DFT), Tandem Mass Spectrometry, Tripeptides, Lipids

Introduction
A new computational tool for the prediction of mass spectra based on quantum chemical calculations is being developed, called Quantum Chemical Mass Spectrometry for Materials Science (QCMS2). It was benchmarked by predicting the EI fragmentation pathways of a number of organics with a variety of functionalities. The main features in the mass spectra were correctly reproduced and new fragmentation routes proposed by QCMS2 were confirmed by MS/MS experiments [1].

Methods
QCMS2 is based on DFT/B3LYP/6-311+G* calculations and selects fragmentation pathways based on the relative values of bond order, reaction energies (for bond cleavages) and transition state energies (for rearrangements). Knowledge of peptide-specific fragmentation mechanisms such as the Mobile Proton Model (MPM) for His, confirmed by quantum chemical calculations, have been implemented into the method and the code omits the breaking of irrelevant bonds [2].

Results
QCMS2 was applied to predict the fragmentation patterns of tripeptides focusing on the fragmentation of the side chain and the influence of inter-side-chain (ISC) interactions on the fragmentation. Therefore, it is able to predict not only the traditional backbone cleavages but also side chain fragmentations and, as a result, convincingly outperforms common tools such as PEAKS [3].

Currently, QCMS2 is being used in the prediction of the fragmentations of lipids. Since the interest in lipidomics is more recent, there is not much known about their fragmentation and, therefore, the shortcomings of their methods are more pronounced than with peptides: none of the available methods can be used for all eight lipid classes and major fragmentation pathways such as McLafferty rearrangements are missing. The results of a number of sphingolipids including sphingosylphosphorylcholine will be presented: fragmentation pathways in both positive- and negative-ion mode ESI CID MS/MS have been calculated and compared to the experimental spectra.

Conclusions
QCMS2 correctly reproduces the main features in the mass spectra of lipids. Furthermore, QCMS2 associates, in contrast to literature, multiple (new) fragmentation pathways with the most prominent fragment ion, m/z 184 corresponding to phosphocholine in positive ion mode. In addition, QCMS2 predicts considerably more fragments in negative ion mode. Therefore, QCMS2 represents a considerable leap forward with respect to the available tools.

Novel Aspect
QCMS2 assigns a significantly higher percentage of signals in lipid mass spectra than the common tools, and provides detailed insight into the fragmentation behaviour.

References
Introduction:
Microalgae Nannochloropsis salina provides high lipid yields and, therefore, it can be used as feedstock for bio-fuel production. Thesolvent extraction of apolar lipids (mainly triglycerides-TAGs) from microalgae must be optimized for the transformation of these lipids into a bio-fuel through hydrotreating. The entire process optimization needs a detailed characterization of the microalgae extract (algal oil). A lipidomic[1] approach must be developed.

Methods:
Algal oils were separated by solid phase extraction (SPE) using an amino based cartridge, obtaining two fractions: neutral compounds were eluted using a solvent mixture of chloroform and 2-propanol, polar compounds were obtained eluting with methanol. The fraction were then analyzed by flow injection with Electrospray ion source (ESI+) on a 7T-FTICR Mass spectrometer. The mass spectra were then elaborated[2] and the main peaks were attributed to a molecular formula.

Results:
The algal oil was separated by SPE in two fractions which were dried and weighted. Starting from about 12mg of algae ethanol extract, about 9.1mg were collected as neutral lipids (74%), 1.6mg as polar lipids (13%). Therefore, a fraction of about 13% was lost in the sample loading step or bonded to the solid phase of the cartridge. The Apolar fraction was first analyzed by GCHT-FID for the quantitation of the main apolar lipid compounds. The starting extract and the two fractions were then analyzed by direct flow ESI+ FTICR MS for the semiquantitative determination of the main species (TAGs, Phospholipids and Glycolipids) mainly ionized as adducts with sodium. Thanks to the high resolution and mass accuracy of the FTICR mass spectrometer, the main species can be separated and most of the peaks can be attributed to a molecular formula. Tandem mass spectrometry allowed fatty acid identification for the main species.

The lipidomic approach developed provides a molecular fingerprint of the algal oil that is extremely useful for the optimization of the whole process.

Conclusions:
A detailed characterization of algal oil was performed employing a lipidomic approach. Even if the SPE fractionation did not provide a total recovery, it allowed to obtain a preliminary quantification of apolar lipids (mainly TAGs) in the algal oil, by simple weighting of the extract. Finally, the detailed molecular characterization of lipid classes allowed the determination of the more suitable process conditions for the valorization of TAGs into a fuel-like mixture.

Novel Aspect:
A novel analytical approach was developed for the direct quantification of apolar and polar lipids obtained by microalgae.

References

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515 - ROBUST AND SENSITIVE LC-MS/MS BASED PLASMA LIPID PROFILING ON A THERMO SCIENTIFIC™ Q EXACTIVE™ HF-X MASS SPECTROMETER

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Keywords: Lipidomics, Q Exactive™ HF-X, Profile Analysis

Introduction:
Lipid profiling can be compromised by their extraction from biological samples, chromatographic separation, ionization and detection by mass spectrometry. The Thermo Scientific™ Q Exactive™ HF-X is a new Orbitrap based mass spectrometer with increased scan speed and sensitivity, which should both benefit lipid profiling experiments. In this study, we optimized a complete workflow for plasmalipidomics with improved sensitivity and quantitative performances.

Methods:
Human plasma samples were spiked with known concentrations of SPLASH™ Standard (Avanti) and extracted with varying sample amounts. Lipids were separated on a 15 cm Accucore™ C30 column and analyzed on the Q Exactive HF-X. Several instrument parameters were tested, including detection mass range, ion source conditions, ion transfer and data dependent parameters. LipidSearch™ software was used for identification and quantification of molecular lipid species.

Results:
Different plasma extraction volumes were tested in order to maximize the number of lipid species which could be detected and quantified with good precision. This evaluation was conducted by extracting the ion signals for the 14 spiked isotopic labeled lipid standards representing all major lipid classes present in plasma. Replicate extractions confirmed the reproducibility of the method with coefficient of variations below 20% for all standards. Further optimizations included the limitation of in-source fragmentation of labile lipids and improvement of MS1 detection across the studied lipid classes. Calibration curves were generated to determine the linearity of detection and limits of quantification. Finally, data dependent parameters were evaluated in order to maximize the number of high quality MS2 spectra generated in view of the higher scan rate and sensitivity of the instrument.

Conclusions:
This method optimization resulted in the confident identification and quantification of several hundreds of lipids from a human plasma sample. For Research Use Only.

Novel Aspect:
Development of a robust and sensitive mass spectrometry based workflow for large-scale plasma lipidomics profiling studies.
Introduction
There are about 150,000 different lipid molecular species present across the biological spectrum. ‘Shotgun’ lipidomics is an established non-targeted approach for broad-based lipidomic analysis but it can suffer from inherent ion suppression and ambiguous identification. Herein we introduce a targeted lipidomics workflow with the combination of HILIC LC separation and multiple reaction monitoring (MRM) mass acquisition. HILIC LC chemistry provides reproducible isomer separation based on lipid classes. The Scheduled MRM™ type data acquisition provides high sensitivity and wide coverage (over 1200 lipid species) for lipids screening and relative quantitation with 24 min run time. The method was extensively verified and is a robust solution for a targeted lipidomics workflow.

Methods
Standards and samplesLipids standards (SPLASH heavy isotopic labeled standards, 17:1 standards and regular light standards) and bovine heart extract were purchased from Avanti and SCIEX. They were diluted with 50%:50%:MeOH: dichloromethane containing 10mM ammonium acetate before injection. LC-MSBovine heart extract samples were subjected to LC-MS/MS analysis with a QTRAP® 6500+ mass spectrometer coupled with Exion HLPC system. A Waters XBridge Amide 3.5 µm, 4.6 x 150 mm column was used for HILIC based LC separation. A Scheduled MRM™ method was implemented to provide screening and relative quantification of over 1200 lipids with 24 min run time.

Preliminary Data
Isomer interference minimizationIsomer interference among different lipid classes is known as one of major challenges for LC-MS/MS method development for lipidomics analysis. Very commonly, one lipid molecule can have multiple isomers in other lipid classes sharing the identical precursor and product masses. This cross-class crosstalk could be troublesome because mass spectrometers cannot differentiate these isomers based on MRM transitions. To overcome this issue, a HILIC chromatographic method is implemented to provide good LC separation based on lipid classes. In order to confirm separation efficiency, lipids standards as natural lipids extracts distinguished by lipid class (one class per standard) were injected individually to confirm that there was no isomer crosstalk among different lipid classes. Retention time reproducibility improvementRetention time on a HILIC chromatography is very sensitive to changes of LC condition. A minor pH difference or organic composition change in the mobile phase might induce retention time shifts of analytes. To minimize retention time drift, extensive evaluation on mobile phase preparation was performed. A standard LC preparation protocol was developed to achieve optimal retention time reproducibility, which is especially critical for the Scheduled MRM™ method. Internal Standard SelectionLipid standards from 19 different classes, which are either heavy isotopic labeled lipids or odd chain lipids, served as internal standard. This method provided extensive lipid class coverages including, CE, CER, DCER, HCER, LCER, TAG, DAG, MAG, LPC, PC, LPE, PE, LPG, PG, LPI, PI, LPS and PS.
Novel Aspect
HILIC-MS/MS by QTRAP® system provides a reliable solution for targeted lipidomics workflow with maximum sensitivity, wide coverage and high throughput.
Introduction:
Gram-negative bacteria are responsible for a variety of foodborne diseases and healthcare-associated infections. The outer leaflet of their outer membrane is composed of lipopolysaccharides (LPSs) of diverse structure and composition. The beneficial (immune-stimulatory) and/or adverse (e.g., pro-inflammatory) effects of these molecules released from the bacteria depend mainly on the structure of the lipidA, the lipophilic anchor constituent of the LPS.

Methods:
The HPLC-MS/MS measurements were performed on an Infinity 1290 UHPLC system coupled to a 6530 Accurate Mass Q-TOF mass spectrometer (Agilent) equipped with a Jet Stream ESI ion source. The separation was achieved using a core-shell Kinetex™ C18 column. The collision energy dependence of the low-energy CID fragmentation pathways was followed both in negative- and positive-ion modes. LipidA samples obtained from Escherichia coli strains were used as “training sets”.

Results:
Recently, we have established a new RP-HPLC method combined with ESI-QTOF MS/MS for the simultaneous determination and structural characterization of complex and heterogeneous lipid A samples [1-3]. We have also investigated the effects of the application of different eluent additives on the separation and on the ionization. The fragmentation pathways of the adduct ions and of the protonated lipid-A molecules in the positive-ion mode and those of the deprotonated molecules in the negative-ion mode were explored and compared. Phosphorylation-site-specific diagnostic fragment ions were identified in the positive-ion mode. By a comprehensive analysis of the fragmentation pathways of numerous lipidA variants, a full structure elucidation method was proposed.

Conclusions:
The overall structural analysis of lipid A samples is of high importance, because this knowledge is essential to describe any structure-function relationship related to Gram-negative bacterial endotoxins. Energy-resolved tandem mass spectrometry proved to be a valuable method to mapping trends in fragmentation relative to the collision energy applied during the activation.

Novel Aspect:
We present an energy-resolved tandem mass spectrometry based strategy to characterize the microbial membrane lipidA structures.

References:

Acknowledgements:
The research was supported by the ÚNKP-17-4-III New National Excellence Program of the Ministry of Human Capacities, and the grant NKFIH K-125275.
Eicosanoids or omega-3 fatty acids metabolites are attractive targets to understand physiological roles. Comprehensive and quantitative monitoring methods using LC/MS has been developed in last decades. Here we report a novel method consisted of 300 MRM transitions packed in a 20 minutes’ chromatographic condition for 200 targets. From 30 µL human serum, 75 targets were assigned by the consistence of retention time or intensity ratio with authentic standards.

Methods
Authentic standards were purchased from Cayman Chemical (Ann Arbor, MI). Human serum and blood were obtained from Sigma-Aldrich Co. LLC. and from BioreclamationIVT, US, respectively. STRATA-X (Phenomenex, Torrance, CA) cartridge were used for solid phase extraction. An LC/MS system consisting of Nexera UHPLC system and LCMS-8060 (Shimadzu Corp.) was used. A Kinetex C8 column (2.1 x 150 mm, 2.6 µm) was used for target separation.

Results
A new method consisted of over 300 MRM transitions for 186 eicosanoids and related fatty acid metabolites and of 14 deuterium labeled analogs as internal standards. Expected retention times were confirmed by analyzing authentic standards with 20 minutes’ chromatographic condition using 0.1% acetic acid and acetonitrile as elution buffers. More than one MRM transitions were applied for over 100 targets. The most of MRM transitions were negative ion mode without 27 transition with positive ion mode. When 5 msec dwell time and 1 msec pause time were set, lower limit of quantifications were sub-pg for the majority of targets. The quantitative profiling of human serum resulted that 75 targets were assigned by the consistence of retention time within 3 seconds or that of the intensity ratio detected in multiple MRMs with corresponding authentic standards. Major eicosanoids PGE2 and PGD2 were not detected. An arachidonic acid metabolite 11-HETE was observed with the highest intensity close to saturation of the detector.

Conclusions
We have developed a new LC/MS method for comprehensive quantification of over 180 eicosanoids and related fatty acid metabolites. The method was applied to 30 µL of human serum sample to assign 75 targets including 30 arachidonic acid metabolites, 13 DHA metabolites, 9 EPA metabolites. The method will be detectable over 100 targets which covers wide range of fatty acid metabolites. We believe the method is useful to study physiological functions or biomarker discovery.

Novel Aspect
We successfully developed a comprehensive MRM panel of 186 eicosanoids and related fatty acid metabolites for high-sensitivity and quantitative LC-MS/MS analysis.

References
1294 - DUAL MASS SPECTROMETRY AS A TOOL TO IMPROVE ANNOTATION AND QUANTIFICATION IN TARGETED PLASMA LIPIDOMICS

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Keywords: Dual MS, lipidomics, glycerophosphocholines, sphingomyelins

Introduction
Lipidomics is rapidly developing as one of the most important areas in biomedical research[1-2]. New technologies and analytical approaches are advancing a field that is defining new guidelines and standards. High quality data, based on reliable quantification and clear identification of the reported lipid species, are required for the clinical translation of human plasma lipidomic studies[3-4].

Methods
As a tool to improve the quality of targeted lipidomics studies, we established a Dual MS platform by simultaneously coupling a reversed-phase liquid chromatography (RPLC) separation to a triple quadrupole (QqQ) and a quadrupole-time of flight (Q-ToF) mass spectrometers. In one single experiment, this platform allows to correctly identify, by high resolution MS/MS, the peaks that are quantified by MRM.

Results
Glycerophosphocholines (GPCs) and sphingomyelins (SMs) play important biological roles in various cellular functions and physiological processes and are highly abundant in plasma. Quantification of GPCs and SMs can be efficiently performed on QqQ mass spectrometers in targeted multiple reaction monitoring (MRM) mode. A series of issues encountered when aiming at the identification and accurate quantification of GPC and SM in MRM were solved with the Dual MS platform, including (i) resolving peaks of GPC polyunsaturated species, (ii) discriminating between plasmanyl-, plasmenyl- and odd chain GPCs and (iii) resolving the isotopic overlap between GPC and SM co-eluting species.

Conclusions
Our platform provides a higher level of confidence in the quantification and identification of phosphocholine-containing lipids in human plasma. Our work is aligned with several new initiatives in the lipidomic field with the aim to establish better methodologies and standard procedures to generate high quality lipidomic data.

Novel Aspect
This new methodology might have a great potential in comprehensive studies that want to achieve an acceptable specificity in the quantification of lipids.

References
Introduction: Microbial identification relies on bacterial culture followed by biochemical testing, which generally takes overnight. Protein analysis by MALDI-TOF MS has become a prominent player in clinical microbial diagnosis. Identification is nearly instantaneous, but requires overnight growth to produce the required pure colony. We recently published (1,2) a method for microbial identification direct from biofluid without the need for culture.

Methods: Bacterial membrane glycolipids were extracted as published (1) and reconstituted in chloroform/methanol/water (3: 1.5: 0.25, v/v/v) for LC-MS/MS analysis on a SYNAPT G2 (Waters). Signature ions from each ESKAPE species were selected and fragmented. Suitable transitions were determined for each ESKAPE pathogens. The existing MALDI library (1) was used to confirm LC-MS results after deconvolution and manual inspection used for polymicrobial analysis.

Results: Previously we published use of microbial lipids for identification of bacteria and fungi direct from specimen using the Bruker Biotyper platform (1, 2). Here we present development of a pseudo-MRM assay for the ESKAPE pathogens (1) on a SYNAPT MS. A new extraction protocol that takes less than one hour is also introduced. Individual glycolipid preparations were analyzed by direct infusion to generate mass spectra for each ESKAPE species. Signature lipid ions, which are those unique to a given microbe, were selected for tandem MS fragmentation and their unique transitions, attributable to loss of various fatty acids, noted. For example, precursor ions at m/z 1744, 1824, and 977 (z=2) were found to be unique to Enterobacter cloacae. After successfully generating tandem mass spectra for each individual ESKAPE pathogen, mixed samples containing multiple pathogens were tested to demonstrate the method could distinguish and detect each organism in a simulated polymicrobial sample and where appropriate antibiotic resistance (3).

Conclusions: ESKAPE pathogens, Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa, and Enterobacter spp., were targeted due to their high incidence of nosocomial infections and multidrug resistance. Here we present initial work to develop a rapid (< 1 hour) pseudo-MRM assay targeted at detection of these microbes that are sensitive and resistant to antibiotic therapy.

Novel Aspect: Rapid identification of ESKAPE pathogens by LC-MS/MS points toward a targeted clinical assay that may be carried out on triple quadrupole systems rather than MALDI-TOF MS.

References:

For information please contact: scientific@imsc2018.it
Introduction:
In biomarker research short analysis times are preferred, which are limited by problems like matrix effects and coelution of isobaric compounds. For this reason, run-times often exceed 30 or even 50 minutes. Our aim is to implement an untargeted method for high-throughput screening, reducing drifts in instrumental response, retention time shifts and improving sample stability.

Methods:
We developed a chromatographic method with a sub 2 micrometre C8 column and a binary gradient to cover the lipidome ranging from polar lipids to highly apolar triacylglycerides and cholesteryl esters in biological samples after liquid-liquid extraction. The chromatographic system was coupled with quadrupole-time-of-flight mass spectrometry for lipid identification.

Results:
The obtained chromatographic resolution allowed the separation of isobaric lipids and reduced ion suppression effects. Combined with high resolution mass spectrometry we could identify lipids from over 15 different lipid classes. The resulting acquisition time was 17 minutes per sample and polarity, simplifying the measurement of large sample numbers and improving system stability. Hence we achieved a coefficient of variation (CV) below 15% for the peak area of 220 lipids in human plasma at repeated extractions, with 205 of these having a CV below 10%. Additionally, the average CV of the retention times was 0.1%. We successfully applied the method on plasma, serum and cell culture samples as well as several different mouse tissue types. For the latter, we could show the importance of the sampling location of mouse kidney, heart and liver for the reproducibility of the lipid profile.

Conclusions:
The developed method can be applied for high-throughput lipidomics and biomarker discovery research in biological samples.

Novel Aspect:
LC-HRMS analysis of biological samples in 17 minutes with low matrix effects, adequate peak resolution, lipid identification and method reproducibility.
Introduction:

The refining of lipid-based oils is important to the food and pharmaceutical industries in the production of food supplements and excipients. ‘Bleaching’ refers to one step in the process where the oils are mixed with adsorbents to remove contaminants such as; lipid peroxides, pigments and soaps. This project uses high resolution mass spectrometry to study the bleaching of vegetable oils.

Methods:

Various techniques including reverse-phase UHPLC-MSn and MALDI MS were used to analyse crude and refined versions of sesame, olive and fish oils, as well as synthetic poly(ethylene glycol) (PEG) and polysorbate based products. A rapid method for processing spectra was built using software package Knime.

Results:

Refined versions of vegetable oils saw lower levels of lipid oxidation products and pigments as predicted from drops in peroxide value and removal of colour after processing. Refined oils also showed lower levels of soaps, metals and phospholipids. Compounds seeing the greatest removal saw a drop in abundance by factor 20.

Surprisingly, refined oils also showed lower concentrations of diglycerides. This was suggested to be due to presence of the glyceryl hydroxyl group.

Conclusions:

Combination of high resolution mass spectrometric methods with computer processing can provide a high throughput means of studying the removal of compounds during edible oil processing. Data confirmed removal of typical contaminants during oil processing. It also highlighted change of otherwise unknown compounds during bleaching.

Novel Aspect:

Use of high resolution mass spectrometric methods as a means of rapid study of bleaching processes in edible oil refinement.

References:

For information please contact: scientific@imsc2018.it
Introduction: (Limit of 400 characters)
Sebum is a complex mixture of lipids that are typically measured by using GC-FID. However, these methods are often unable to separate the individual components from each other which makes it difficult to definitively identify each of them. Conversely, GCxGC-HRTOFMS is a powerful tool for identifying analytes in complex mixtures. In this work, we measured lipids in sebum by using GCxGC-HRTOFMS.

Methods: (Limit of 400 characters)
The sample was collected onto square aluminum sheets wiped across a human forehead. The lipid compounds were then extracted from the aluminum sheets using a 50/50 volume solution of methanol/dichloromethane with sonication. Next, the extraction solution was centrifuged and concentrated. Afterwards, the final solution was analyzed using GCxGC-HRTOFMS.

Results: (Limit 900 characters)
A lot of lipid compounds such as free fatty acids, wax esters, diacylglycerols and triacylglycerols were observed, and these lipid compounds were separated by GCxGC perfectly. Obtained peaks were comprehensively identified using a combination of accurate mass measurements and library searches. As an example, the peaks detected at retention time 18.684 minute (1st column), 6.756 seconds (second column) was matched to oleic acid using a NIST library search. Furthermore, this mass spectrum showed a molecular ion that coincided with the calculated accurate mass of oleic acid (error of 1.4 mDa). The results for the other compounds present in the sebum sample will be presented in this work.

Conclusions (Limit of 400 characters)
Low boiling point fatty acids up to high boiling triglycerides were observed in a single sebum measurement. The cholesterol esters and triacylglycerols, which are difficult to separate by 1D GC, showed high chromatographic separation by GCxGC-HRTOF. Additionally, the peaks were comprehensively identified by using a combination of accurate mass measurements and library searches.

Novel Aspect: (Limit of 150 characters)
Analysis of a complex lipid profile in human sebum by using GCxGC-HRTOFMS.
NEW PEAK ALIGNMENT SOFTWARE: METABOALIGN IMPROVING ISSUES OF LC-MS/MS LIPIDOMICS ENABLES HIGH THROUGHPUT DATA ANALYSIS FOR THOUSANDS OF LIPIDS IN BIOLOGICAL MATERIALS.

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Keywords: ELOVL6, insulin resistance, targeted lipidomics, software, automatic-annotation

Introduction: Up to now many quantification softwares for targeted LC-MS/MS analysis using triple quadrupole mass spectrometer have been developed, but any of them could not be completely sufficient in peak alignment because of the difficulty to correct for any drift in retention time. Furthermore, it is difficult to identify the fatty acid composition of phospholipids (PLs) by simultaneous MS/MS assay using a thousand of polar head group MRM transitions because of the limitation of MS/MS cycle time in one assay. Therefore, we developed a new software for targeted lipidomics (TL) to improve these issues. Then we applied this software to lipidomics in liver of liver specific ELOVL6 knockout (LKO) mice, and found a lipid which was correlated with insulin sensitivity scores.

Methods: The liver tissues of LKO mice were homogenized in 1% acetic acid methanol solution on ice cold water. Then the whole lipid extraction and TL were performed by the method previously reported. TL and non-TL raw data were obtained by 4000QTRAP (Sciex) and QExactive HF (Thermo Fisher Scientific). The lipid identification data set including the information of PLs fatty acid analysis were made by Lipid Search (LS) software (Mitsui Knowledge Industry Co., Ltd.) from non-TL data. TL raw data analysis was performed by newly developed lipidomics software: MetaboAlign beta version with automatic peak annotation by means of merging with the LS identification data set.

Results: MetaboAlign was able to align peaks independent of peak shape and peak width. All Lyso-PL regio isomers of sn-1 form and sn-2 form could be separated, as for example LPCacyl (18:1) of sn-1 and sn-2 were separated accurately and partially LPCacyl (cis-Vaccenyl) and LPCacyl (Oleyl). LPCacyl (cis-Vaccenyl, sn-2) was correlated well with LPCacyl (16:1, sn-2). N-Acylethanolamine (18:1) (AEA) was separated into two peaks which were considered AEA (Oleyl) and AEA (cis-Vaccenyl). Liver PCs with a variety of fatty acids were separated by high resolution C18 reverse phase column, aligned, quantified, and identified about fifty PC species automatically by MetaboAlign. ELOVL6 is an enzyme which can elongate fatty acid chain from C16 to C18. LKO mice showed lower levels of C18 fatty-acyl PCs in liver than WT mice under high sucrose diet condition. Then we found a lipid_Y which was correlated with insulin sensitivity scores which might be involved in improvement of insulin resistance.

Conclusions: We successfully developed a new software MetaboAlign beta version for LC-MS/MS lipidomics to enable to align, quantify and identify thousands of lipid peaks. Both four isomers of LPCacyl (18:1) and two isomers of AEA (18:1) were aligned and quantified adequately. We found a lipid_Y which was correlated well with insulin sensitivity scores which might be involved in improvement of insulin resistance.

Novel Aspect: Successful development of a new software MetaboAlign beta version for LC-MS/MS lipidomics to enable to align, quantify and identify thousands of lipid peaks. Separation of four isomers of LPCacyl (18:1) and two isomers of AEA (18:1). Finding of a lipid_Y which was correlated with insulin sensitivity scores which might be involved in improvement of insulin resistance.

References

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RELATIVE QUANTIFICATION OF PHOSPHOLIPID SN-ISOMERS USING POSITIVE DOUBLY CHARGED LIPID-METAL ION COMPLEXES

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Sven Heiles, Simon Becher, Patrick Esch
Keywords: UVPD, FTMS, Lipidomics, Tandem MS

Introduction:
Phospholipids are ubiquitous in living organisms and are involved in a plethora of biochemical processes.[1] For glycerophospholipids assignment of double bond positions and sn fatty acid isomers is often not possible with routine tandem MS Methods: Especially untargeted assignment of sn isomers is challenging. Here, we present a simple tandem MS strategy that allows phospholipid sn isomer assignment by phospholipid metal ion complex fragmentation.

Methods:
All experiments were performed using a LTQ-FT Ultra or a Q Exactive (Thermo Fisher Scientific) mass spectrometer equipped with heated ESI sources. An in-house developed 213 nm UVPD setup [2], CID and HCD were used for tandem MS experiments. Lipid extracts were prepared according to Schwudke et al.[3] and phospholipase A2 digestion was performed as described by Ekroos et al.[4] Samples were mixed in alcohol/water and corresponding complexes were produced by ESI.

Results:
Abundant lipid-metal ion complexes (LM2+) complex formation was observed for phosphatidylcholines (PCs) and sphingomyelines (SMs) but not for other lipid classes. Upon LM2+ activation by CID, HCD or UVPD, lipid head group and fatty acid ester bond dissociation were detected. The relative abundances of the fragment ions depended on the metal ion identity. Only for iron(II) LM2+complexes, head group dissociation was suppressed and only fatty acid ester bond dissociation occurred. One singly charged ion containing the dissociated deprotonated fatty acid together with Fe2+ ([FA-H+Fe]+) and the complementary singly charged PC fragment ion were observed. For PCs containing two different fatty acid moieties, activation of LM2+ resulted in preferential dissociation of FAs located at sn2-position. PLA2 digestion revealed that fragment ions upon CID and UVPD are sn2-specific but for HCD sn-scrambling occurs. LM2+ ions were utilized in MS2 CID or UVPD experiments to quantify the relative abundance of PC sn-isomers in mouse organ lipid extracts.

Conclusions:
Abundant ion adduction of Fe(II) to neutral PCs in ESI mass spectrometry was observed and optimized. Tandem MS experiments of these LM2+ complexes allow to assign fatty acid moieties and to assign sn-isomer in one-step tandem MS experiments. Under low energy activation conditions, relative quantification of sn-isomers is possible and this method can readily be applied to complex lipid mixture in a shotgun approach and potentially be coupled to LC-MS experiments.

Novel Aspect:
Simple MS2 method to quantify the relative abundance of phosphatidylcholine sn-isomers in complex lipid mixtures using positive ion mode.

References
Keywords: lipids, bacteria, linear ion trap, high resolution mass spectrometry.
A.09 Life science-Lipidomics

Introduction:
Bacteria, regardless of whether they are beneficial or pathogenic all contain a large diversity of lipids with various structures and functions in the cells; and the structures are complex and difficult to define. Herein, we report LIT MSn method to identify the lipid structures from R. opacus, Cordyceps sinensis, P. gingivalis, L. monocytogenes and Corynebacterium stratum, which contain unique lipids different from those in eukaryotic and other prokaryotic cells.

Methods:
Cells were homogenized and lipids were extracted using a modified Bligh & Dyer method. Preparative HPLC experiments were carried out using a Thermo TSQ Vantage MS with Accela UPLC. Separation of lipids was achieved by a Supelco C-8 column. High resolution LIT MSn experiments were performed on a LTQ Orbitrap Velos. Peptidolipids collected from HPLC separation were dried, re-dissolved in CHCl3/CH3OH, and continually infused into the ESI source.

Results:
In addition to the lipids commonly seen for other bacteria, R. Opacus contains two novel families of peptidolipids; Cordyceps sinensis contains extremely rich linoleic acid containing phospholipids; P. Gingivalis and L. monocytogenes contain odd chain methyl branch iso-fatty acid containing phospholipids, with the former also contains dihydroceramides, including EPC, IPC, GPC, and GS-peptidolipid, and the latter also contains plasmalogen PG and lysyl-CL; C. stratum contains a novel glucuronosyl diacylglycerol family. All the fatty acyl substituents in EPC, IPC, and GPC belong to the β-OH fatty acid family, and the structure is readily identifiable by CID MS2.

Conclusions:
We found three new lipid families (peptidolipid, glycine-lipid, 18:1/16:0-glucuronosyl diacylglycerol) that have not been previously reported for these bacteria. ESI LIT CID/HCD MSn with high resolution mass spectrometry revealed the complexity of microbial lipid structures, including many isomers. The MSn capability of LIT adds additional dimensions in segregating ions, thereby, accelerating and simplifying data interpretation and structural characterization.

Novel Aspect:
LIT MSn with high resolution MS unravels the structural details of novel lipids including many minor isomers that would be very difficult to define using other analytical Methods:
1343 - JOINT APPLICATION OF ESI AND MALDI LIPIDOMIC PROFILES FOR CLASSIFICATION OF BRAIN TUMORS

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Keywords: brain tumors, lipidomics, molecular profiling, classification

Introduction:
Analysis of molecular profiles is one of the promising approaches to determine malignancy of brain tissues. Different ionisation methods could be used for molecular profiling: online extraction with ESI is better for fast intraoperative analysis, while MALDI more suitable for histological investigation. The purpose of this study is to quantify ability to construct a joint classifier of brain tumors, working with MALDI and direct spray MS profiles simultaneously.

Methods:
Set of molecular profiles of different brain tumor tissues was obtained by online extraction with ESI and MALDI Imaging experiments. Discriminating features for the malignancy level of tumor determination were identified individually for each ionisation method[1], and two brain tissue classifiers were created, followed by the comparison of selected features for each classifier.

Results:
The molecular profiles that were obtained by direct ionisation and MALDI show the high level of correlation between each other for the same type of tissue especially in a mass range of common lipids. MALDI brain tissue classifier and direct spray classifiers were able to determine features to quantify the ratio of malignancy, and a number of these features were presented in both classifiers. Since we use high-resolution mass analysers for measuring direct-spray molecular profiles, for some of the common features we predict the molecular origin of these ions.

Conclusions
It was demonstrated that the classifiers build on the molecular profiles from direct-extraction MS and MALDI MS contain signals of the same compounds so both types of data could be used for the creation of joint classifier. The analysis of features, used in the classifiers, will allow optimising the parameters of experiments to improve the efficiency of classification and visualization of the spatial structure of the samples.

Novel Aspect:
This work shows principal ability to use heterogeneous data to construct the classifier which works with online extraction and MALDI imaging experiments together.

References
INTRODUCTION

Quantification is a critical task in comprehensive lipidomics studies. Liquid chromatography-mass spectrometry (LC-MS)-based technique can provide a valuable tool for monitoring lipid changes as well as to identify specific biomarkers. A key limitation of this technique is the nonlinear responses due to stability, matrix effect, instrumental variations and others. However, those can be overcome by our novel method using 13C-labelled organism extracts (1).

METHODS

The lipidome profiles of five different microorganisms were compared to obtain the most coverage of human lipids leading to comprehensive compound-specific normalisation in quantitative mass spectrometry assays. An in vivo labelling strategy was used to produce a wide range of isotopically labelled lipid species from the selected microorganism grown on uniformly 13C-labelled glucose, which have similar physico-chemical properties of unlabelled lipids (2, 3).

RESULTS

Comparison of the lipid profiles of five different species including E. coli, Spirulina, S. cerevisiae CEN.PK 113-7D, S. cerevisiae BY4741, and P. pastorisNCYC 175 showed that there were marked differences in the relative amount of the identified lipid species as well to their labelling pattern. P. pastoris provides the highest number of common lipids with human metabolome database with good labelling efficacy. Therefore, P. pastoris was selected as a source of isotopically labelled internal standards (IS). Then, the lipidomic extract of P. pastoris used to normalise variations introduced during samples preparation, storage and LC-MS analysis. The labelled yeast extract was successfully used to correct variations in LC-MS response in long analysis time (230 injections over 3.5 days) and reduction in CV% of the identified lipids was achieved (from 8.4 to 4.9). Also, the extract was able to reduce batch to batch variations successfully (from 17.2 to 6.2) without the need for quality control samples (QC) and thus decrease the analysis time.

CONCLUSIONS

P. pastoris proved to be the ideal source for production of labelled IS with more coverage for higher eukaryotic lipids. Normalisation using the 13C-labelled extract could successfully correct for instrumental variation and batch to batch variations without the need for QC samples. These results show the high potential of the use of in vivo labelling strategy in quantitative measurement of a wide range of lipids especially in more complex matrices.

NOVEL ASPECT

To use labelled organism extracts as a source of IS to develop more reproducible, quantitative untargeted lipidomic analysis that can be applied to a variety of clinical studies

REFERENCES

Introduction:
Renal cell carcinoma (RCC) is the most common type of kidney cancer. The RCC incidence and the mortality rate have been increasing globally during the last decade. Up to date, there are still no recommended screening tests for early diagnosis. In our work, the matrix-assisted laser desorption/ionization coupled with Orbitrap mass spectrometry (MALDI-Orbitrap-MS) is used for the clinical study of patients with RCC and healthy volunteers.

Methods:
All samples were spiked before the extraction with the appropriate internal standard mixture. Lipidomic extracts of human plasma were prepared by modified Folch method, while reversed-phase solid phase extraction was applied for urine samples. The LTQ Orbitrap instrument was operated in the negative-ion mode over a mass range m/z 400 - 2000 and the mass resolution was set to 100,000. 9-aminoacridine was used as MALDI matrix.

Results:
First, lipid species are identified using high mass accuracy full scan and tandem mass spectra. Subsequently, the optimization and method validation for semiquantitative measurement is performed. The optimized methodology is finally used for the analysis of all studied body fluid samples. Each measurement is represented by one average MALDI-MS spectrum with thousands of m/z peaks. The automatic peak assignment is subsequently performed and particular m/z peaks of various intensities are matched with deprotonated molecules from a database created during the identification procedure using the Excel macro script for each sample. Multivariate data analysis methods, i.e., unsupervised principal component analysis and supervised orthogonal partial least square discriminant analysis, are further used for the visualization of differences between studied sample groups.

Conclusions:
Most upregulated and downregulated lipids in blood and urine samples of RCC patients are finally visualized using particular boxplots. Significant differences in lipid abundance between RCC patients and healthy volunteers proved potential of MALDI-Orbitrap-MS for early diagnostic of RCC, but further testing is still necessary. This work was supported by the Czech Science Foundation (project No. 18-12204S).

Novel Aspect:
Applicability of MALDI-Orbitrap-MS for the early diagnosis of renal cell carcinoma based on the lipidomic analysis of blood and urine samples.
764 - CHANGES OF SPECIFIC SPHINGOMYELINS DEPENDING ON THE SEVERITY OF NONALCOHOLIC FATTY LIVER DISEASE

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Keywords: Nonalcoholic fatty liver disease, lipidomics, sphingomyelin

Introduction:
Nonalcoholic fatty liver disease (NAFLD) is one of common causes of chronic liver disease that involve hepatic accumulation of triglycerides. The clinical-histologic spectrum of NAFLD extends from a nonalcoholic fatty liver (NAFL) to nonalcoholic steatohepatitis (NASH). NASH is divided from NAFL according to the presence of ballooning and lobular inflammation [1]. Although NAFLD is closely correlated with obesity, 5-8% of lean subjects also have NAFLD [2].

Methods:
Total 361 serum samples from patients with biopsy-proven NAFLD were classified in six groups on the basis of BMI (25 kg/m2) and liver histology (No NAFLD, NAFL and NASH). Lipidomics study for all serum samples were performed using ultra-performance liquid chromatography/quadrupole time-of-flight mass spectrometry (UPLC/QTOF MS).

Results:
The diverse lipid metabolites identified in serum samples showed a characteristic alterations depending on the severity of NAFLD between non-obese and obese groups. Most of all, specific sphingomyelins (SMs) had distinct increase patterns according to the stage of steatosis, lobular inflammation, and ballooning except for fibrosis only in non-obese NAFLD. Moreover, the receiver operator characteristic (ROC) analysis for determining the progression degrees of NAFLD produced higher the area under the curve (AUC) values in non-obese group than in obese.

Conclusions
These results presented that disease mechanism is different between non-obese and obese NAFLD, and these specific SMs might be used as markers to predict the severity and/or progression of NAFLD.

Novel Aspect:
Specific SMs were clearly associated with liver histology including steatosis, lobular inflammation, and ballooning in non-obese NAFLD patients.

References
A COMPREHENSIVE METHOD FOR TOTAL LIPID ANALYSIS IN BIOLOGICAL MATRICES BY HYDROPHILIC INTERACTION LIQUID CHROMATOGRAPHY ELECTROSPRAY IONIZATION MASS SPECTROMETRY HILIC/ESI-MS

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Keywords: Lipids, Extraction, liquid chromatography, Mass spectrometry, Ion suppression

Introduction: (Limit of 400 characters)
Lipid analyses are increasingly used for determining the development of several diseases. Because of their extremely heterogeneous structures, analysis of lipids is challenging. LC/ESI-MS, the major tool used to analyze complex lipidomes, is very sensitive towards the composition of lipid extract. Therefore, a complete analytical strategy is proposed in this work in order to efficiently extract, purify and analyze lipids in different biological matrices.

Methods: (Limit of 400 characters)
Lipids were extracted from rat brain and plasma samples with an optimized method using a mixture of solvents of increasing polarity. The efficiency of this method was compared to other reference Methods: Then, lipids of low relative abundance were purified by several methods (C18 solid-phase, phase partition [1] or Phree phospholipid removal columns). LC-MS (Triple quadrupole and Orbitrap FUSION) methods were developed for targeted and global analyses of lipids.

Results: (Limit 900 characters)
The optimized monophasic extraction method enabled a fast and efficient extraction of all lipids from the matrices. A better recovery of lysophospholipid and ganglioside (GG) classes, whereas similar recovery of other phospholipids (PL), ceramides, fatty acids and sterol, was obtained compared to other extraction Methods: The HILIC conditions allowed the separation of PL and GG classes, including acetylated forms, according to their increased polarity. Analyses conducted by LC-ESI-HRMS/MS enabled the identification of a large diversity of lipid molecular species. Quantification of lipids by MS tuned to specific acquisition modes showed high levels of phosphatidylcholine and phosphatidylethanolamine classes. Different purification methods of GG were evaluated in order to overcome some analytical challenges in LC/ESI-MS like ion suppression phenomenon [2] after co-elution with the more abundant PL of similar polarity. Therefore, an increase in quantification of all GG classes was observed after removing PL prior to analysis.

Conclusions (Limit of 400 characters)
Polar and complex lipids in biological samples usually present very low extraction yield with the most popular Folch-type procedures. In this work, we propose a monophasic extraction method adapted to extract lipids of a large range of polarity. A method in tandem MS was optimized and coupled to an HILIC system to separate, characterize and quantify all lipids. In this respect, powerful purification tools were used to avoid ion suppression effect for ESI-MS analysis.

Novel Aspect: (Limit of 150 characters)
Our complete analytical strategy offers an efficient extraction of both polar and non-polar lipids from complex biological matrices followed by an accurate LC-MS analysis.

References
Khoury S et al., Anal Bioanal Chem, 408, 1453-65 (2016)
Keywords: Lipidomics, Kendrick mass defect, High-resolution mass spectrometry, Liquid chromatography

Introduction:
Recent technical advances regarding liquid chromatography (LC) and HRMS enable the mapping of the lipidome of an organism with short data acquisition times. However, interpretation and evaluation of resulting multidimensional datasets are challenging and this is still the bottleneck regarding overall analysis times. Therefore, we present a novel adaption of Kendrick mass (KM) plot analysis.

Methods:
Separation of lipids by their respective head group was achieved via hydrophilic interaction liquid chromatography (HILIC) coupled to a HRMS instrument (Q Exactive). The resulting LC/HRMS datasets were processed to a list of chromatographically separated features by applying an optimized MZmine 2 workflow.[1] All features were plotted in a 3DKM plot for graphical lipid identification.

Results:
To demonstrate the novel adaption of KM plot analysis, occurring phospho-and glycolipid classes of the green alga Chlamydomonas reinhardtii have been separated using HILIC. Phospho-and glycolipid species have been identified by exact mass database matching and graphical 3DKM plot analysis according to [2]. LC-HRMS/MS datasets have been processed utilizing an optimized MZmine 2 workflow for feature list generation. Database matching was achieved by extending the already existing MZmine 2 module ‘glycerophospholipid search’ by adding missing phospho- and glycolipid classes. Furthermore, graphical analysis has been carried out by implementing a new visualization module in MZmine 2, which adds chromatographic characteristics, such as retention time, to a classic KMplot in form of a heat map. The developed visualization module is part of the MZmine 2 toolbox since version 2.31.

Conclusions:
The use of the novel adaption of the KM plot has accelerated the identification of the relevant lipid species in the green alga Chlamydomonas reinhardtii. 106 species were identified within the lipid classes: phosphatidylserine, phosphatidylethanolamine, phosphatidyglycerol, phosphatidylinositol, monogalactosyldiacylglycerol, digalactosyldiacylglycerol, and sulfoquinovosyldiacylglycerol.

Novel Aspect:
A tool for graphical analysis of HRMS/MS has been developed, using the Kendrick mass defect in combination with chromatographic characteristics.

References:
Introduction:
Polyglycerophospholipids include phosphatidylglycerol (PG), bis(monoacylglycerol)phosphate (BMP), Hemi BMP, and bis(diacylglycerol)phosphate (BDP) having two glycerols, and cardiolipin (CL), monolysocardiolipin (MLCL), and dilysocardiolipin (DLCL) having three glycerols [1]. These polyglycerophospholipids are generally synthesized from PG as a precursor and closely related to each other. Therefore lipidomic analysis of these lipids is important.

Methods:
Polyglycerophospholipids were methylated with (trimethylsilyl)diazomethane (TMSD) under an acidic condition. Deuterated reagents with DCl were used for relative quantitation by isotope-labeled methylation (ILM) method [2]. Efficacy of ILM for polyglycerophospholipids were evaluated using reversed-phase nanoflow UPLC-ESI-MS/MS.

Results:
The molecular structures of methylated polyglycerophospholipid standards were investigated with the CID experiments during nUPLC-ESI-MS/MS. The degree of methylation varied with the number of phosphate groups (1 for PG, BMP, Hemi BMP, and BDP, 2 for CL, MLCL, and DLCL). The efficiency of methylation for polyglycerophospholipids was found to be > 97% for most classes. Over-methylation in the free hydroxyl group of glycerol moieties were detected with less than 4.6%. PG and BMP which are geometrical isomers, and three isomers of BMP can be well resolved from each other after methylation by nUPLC-ESI-MS/MS. Furthermore, isomeric structures among Hemi BMP, MLCL, and DLCL can be well separated.

Conclusions:
In this study, analysis of polyglycerophospholipids were investigated with ILM method using TMSD. As a result, ILM method with nUPLC-ESI-MS/MS enhanced the capability of analyzing polyglycerophospholipids qualitatively and quantitatively. The developed method can be utilized to analyze urinary polyglycerophospholipids from neurodegenerative diseases such as Parkinson’s and Alzheimer’s diseases.

Novel Aspect:
Analysis of polyglycerophospholipids can be improved by using Isotope-labeled methylation (ILM) method in nanoflow UPLC-ESI-MS/MS.

References
PLASMA LIPIDOMIC PROFILING OF FIVE DIFFERENT TYPES OF CANCER BY NUPLC-ESI-MS/MS

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Keywords: cancer, biomarker candidate, lipids, nUPLC-ESI-MS/MS

Introduction:
Lipids are the substances that play key roles in the metabolism of human bodies and are known to be related to cancer genesis pathways [1]. In this study, plasma lipids from 5 different cancer types (liver, stomach, lung, colorectal, and thyroid cancer) were investigated in order to discover potential lipid biomarkers of each cancer.

Methods:
Lipids were extracted from patient’s plasma using the modified Folch method with MTBE/methanol [2], and the extracted lipids were analyzed by nUPLC-ESI-MS/MS. Due to the study that blood lipid metabolites are affected by hormones in women after menopause, only male plasma samples were used in this study.

Results:
As a result of analyzing the extracted lipids from patient’s plasma with liver, stomach, lung, colorectal, and thyroid cancers along with that of healthy controls, a total of 357 lipids structures (58 PCs, 39 PEs, 26 PGs, 37 PIs, 26 PAs, 31 sphingo, 150 glycerol) were identified. Based on identified lipids, a total of 241 lipids (55 PCs, 58 PEs, 19 PGs, 24 PIs, 17 PAs, 21 sphingo, 47 glycerol) were quantified. Lipids showing significant changes (high abundance, >2-fold change, and one-way analysis of variance (ANOVA) with Bonferroni post hoc test with p-value<0.05) in each cancer type were selected: 31 liver, 16 stomach, 23 lung, 24 colorectal, and 16 thyroid cancer. Statistical analyses of these selected lipids were performed with logistic regression and receiver operating characteristic (ROC) curve and the area under the curve values (AUC) of each cancer were more than 0.8 except for thyroid cancer.

Conclusions:
A total of 357 from patient’s plasma with liver, stomach, lung, colorectal, thyroid cancer, and controls, were identified by nUPLC-ESI-MS/MS and among them 241 lipids were quantified by multiple reaction monitoring (MRM). Selected lipids showing significant differences from healthy controls can be utilized to develop characteristic biomarkers of cancers in the future.

Novel Aspect:
Lipidomic profiles of five different types of cancer patient’s plasma were systematically examined by nUPLC-ESI-MS/MS with MRM along with statistical evaluations.

References
Introduction:
Liver is the primary organ for glucose metabolism and regulation; therefore it is interesting to elucidate the effects of hyperglycemia in cultured liver cells. HepG2 cell, the human hepatoma cell line, has been used extensively to study hyperglycemia in vitro as evidenced by several reports [1,2]. HepG2 cells treated with glucose exhibited an upregulation of the transcription of human apolipoprotein A-II gene which causes a rise in plasma triglyceride level and glucose intolerance, resulting in hyperglycemia [3]. High glucose exposed HepG2 cells exhibited inhibition of 50AMP-activated protein kinase (AMPK) which plays a role in cellular energy homeostasis, and increased accumulation of lipids which were prevented either by metformin or by expressing the constitutively active AMPKα [4]. The high concentration of glucose also leads to oxidative stress, which plays an important role in the glucose-induced cellular dysfunction in diabetes complications [5]. Oxidative stress is defined as an imbalance between free radical production and antioxidant defense in a biologic system. It has been reported that irregular cellular metabolism in diabetes leads to generation of free oxygen radicals and reduction of antioxidant capacity [6]. In cells, lipids have functions as structural membrane components, sources of energy, and signaling molecules. As hormones and cellular messengers, lipids are particularly involved in many intracellular and intercellular signaling processes [7]. Furthermore, lipids adjust membrane fluidity and support diverse cellular processes [8]. Thus, it is critical to determine the cellular lipid metabolism correlated with oxidative stress.

Recently, liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) has been used as an effective tool to analyze various lipids from biological samples [9]. An ultraperformance LC (UPLC) enables the rapid and effective separation of individual lipid species [10]. Multiple reaction monitoring (MRM) based on triple quadrupole (QqQ)/MS is also applicable for the sensitive analysis of target compounds [11]. Optimal lipidomics based on UPLC-QqQ/MS has been widely applied to shed light on many biological events [12]. In this study, UPLC-QqQ/MS-based lipidomics was used to elucidate the altered lipid metabolism of HepG2 cells affected by the oxidative stress. We also treated anti-oxidative compound and extracts to the high glucose induced HepG2 cells and assessed the altered levels of lipids. The detailed characterization of lipids in HepG2 cells having oxidative stresses may provide a good understanding of the lipid metabolism correlated with oxidative stress.

Methods:
MTT and Lactate dehydrogenase (LDH) assay
Cell viability was determined by the quantitative colorimetric assay using MTT reagent. The cytotoxicity was also measured by lactate dehydrogenase (LDH) assay using commercial LDH detection kit for detection the leakage of soluble cytoplasmic LDH into the extracellular medium.

Lipid extraction
First, the cell pellet was added to 990 µL chloroform/methanol (1:2, v/v) and 10 µL 1 µg/mL of lipid standards. The sample was incubated for 10 min on ice with vortexing for 30 s. After centrifugation (13,800 × g, 2 min at 4°C), 950 µL supernatant were transferred to a new Eppendorf tube. Second, the remaining pellet was resuspended in 750 µL chloroform/methanol/37% (1N) HCl (40:80:1, v/v/v) and incubated for 15 min at room temperature with vortexing. The tube was transferred to ice, and 250 µL cold chloroform and 450 µL cold 0.1 M HCl were added followed by 1 min of vortexing and centrifugation (6,500 × g, 2 min at 4°C). The lower organic phase was added to the tube, and subjected into LC/MS.

UPLC-QqQ/MS analysis
Lipid profiling was performed using the 6490 Accurate-Mass Triple Quadrupole LC-MS coupled to a 1200 series HPLC system (Agilent Technologies, Wilmington, DE, USA) with a Hypersil GOLD column (2.1 × 100 mm ID; 1.9 μm, Thermo Science). Solvent A consisted of an acetonitrile–methanol–water mixture (19:19:2) with 20 mmol/L ammonium formate and 0.1% (v/v) formic acid, and solvent B consisted of 2-propanol with 20 mmol/L ammonium formate and 0.1% (v/v) formic acid. The flow rate was 250 μL/min, and total run time was 30 min for each analysis. MRM conditions including transition and MS/MS collision energy were optimized to analyze target lipids in individual samples.

Data processing and multivariate analysis

LC/MS data were obtained and processed using Agilent Mass Hunter Workstation Data Acquisition software. The MRM data of target lipids, including m/z of precursor ions, m/z of product ions, and retention time (RT), were exported using Qualitative Analysis B.06.00 software (Agilent Technologies, USA). Next, the Skyline software package (MacCoss Laboratory, University of Washington, USA) was used as an in-house database to determine the peak area of each assigned lipid from replicate raw data. The extracted areas of lipid peaks were normalized to the appropriate IS. Multivariate analyses such as principal component analysis (PCA), the sparse projection to latent structure discriminant analysis (sPLS-DA), and analysis of variance (ANOVA) were performed using the MetaboAnalyst website.

Results:

Effects of ALM16 on high glucose-induced cell death and cytotoxicity in HepG2 cells

To determine the protective effects of the ALM16 on high glucose-induced cell death and cytotoxicity, HepG2 cells were pre-treated with the ALM16 for 2 h, and then treated with high glucose (30 mM) for 24 h. The ALM16 (10-200 μg/mL) did not show significant cytotoxic effects. While treatment of the HepG2 cells with high glucose (30 mM) significantly reduced the cell viability to approximately 64.0% compared with the untreated control cells (100%), pre-treatment with the ALM16 (50, 100, and 200 μg/mL) for 2 hours before exposure to high glucose (30 mM) dose-dependently reduced the high glucose-induced cell death to 70.2%, 73.3%, and 78.5%, respectively. In addition, pre-treatment with silymarin (50 μg/mL), as a positive control, reduced the cell death to approximately 83.0%. The effect of the ALM16 on the high glucose-induced hepatotoxicity in HepG2 cells was confirmed by the LDH assay. Whereas an approximately 2.89-fold higher LDH activity was observed from the high glucose-treated cells compared with the untreated control cells (1.00 fold), HepG2 cells pre-treated with the various concentrations of ALM16 were reduced in a dose-dependently manner by 2.78-, 2.40-, and 2.23-fold increases in LDH activity, respectively. The cells pre-treated with silymarin (50 μg/ml) were also reduced LDH activity to 2.53-fold increase.

Lipid profiling of high glucose induced HepG2 cells treated with the mixture of Astragalus membranaceus and Lithospermum erythrorhizon extracts

Next, six samples of HepG2 cells (normal control (Con), Con + glucose 30 μg/mL (Glu30), Glu30 + silymarin 50 μg/mL (Sil50), Glu30 + Alm150, Glu30 + Alm200) were subjected into lipidomic analysis. For the profiling of various lipids from these HepG2 cells, we used a previously constructed method using UPLC-QqQ/MS [13]. Lipid standards were used to optimize the MRM conditions for the analysis of TG, DG, MG, ChE, cholesterol, PC, PE, PS, PG, PI, PA, LPC, LPE, LPS, LPG, LPI, LPA, SM, Cer, dCer, So, Sa, Cer1P, So1P, and Sa1P. In particular, TMSD was used to methylate several highly acidic lipids, such as PS, PI, PA, LPS, LPI, Cer1P, So1P, and Sa1P. By using an octadecylsilyl silica column, various lipid species were separated according to their total carbon chain length (Cn) and total degree of unsaturation (Un). In the MRM data, the RT of each lipid was increased with a higher Cn and a lower Un. In this study, the skyline software was used to assign each lipid peak in the basis of RT and to calculate its peak area in the MRM data. Finally, in the HepG2 cell, a total of 336 lipid species were identified as follows: 23 TGs, 37 DGs, 8 MGs, 16 ChEs, cholesterol, 62 PCs, 28 PEs, 15 LPCs, 5 PGs, 16 PIs, 34 PAs, 17 LPCs, 13 LPEs, 5 LPSs, 7 LPGs, 4 LPIs, 15 LPA, 11 SMs, 11 Cers, 2 dCers, So, Sa, 2 Cer1Ps, So1P, and Sa1P. Furthermore, in the data processing of each lipid class, the calculated peak area of individual lipid species was normalized by the peak area of the IS. And then, the normalized datasets were subjected to statistical analysis.

Statistical analysis of lipid datasets obtained by the profiling of six HepG2 cells

The six datasets of lipids analyzed from Con, Glu30, Sil50, Alm50, Alm100, and Alm200 were subjected into multivariate analyses. Assays were carried out in triplicate (n = 3) for each sample. First, we applied the multiple pattern recognition methods such as PCA and sPLS-DA for visualizing the general clustering trends among the six groups. In the PCA score plot, the six groups were not sufficiently distinguished. For well discriminating the six
groups, we also applied the sPLS-DA that reduce the number of variables in the data to produce robust and easy-to-
interpret model. In the score plot, points of Con were firstly separated with those of Glu30. Furthermore, other four
groups including Sili50, Alm50, Alm100, and Alm200 were scattered between Con and Glu30 in the basis of Component 1. These indicated that cell lipid profiles differ depending on normal control, high glucose induced cells, and high glucose induced cells treated with silymarin or ALM16. In the sPLS-DA score plot, Sili50s were getting closer to Cons. This indicated that the lipid profiles of Sili50 are similar with those of Con comparing to Glu30. Furthermore, the points of Alm200, Alm100, and Alm50 were in order getting closer to those of Con. When the treatment amounts of ALM16 were increased, their points were closer to Cons than others. This indicated that ALM16 treatment is effective to suppress the cellular oxidation and recover the lipid metabolism. In the loading, we also presented the variable importance in projection (VIP) plot of 25 lipids (VIP scores Top 25) that were differently regulated among the six samples of HepG2 cells. Next, ANOVA test was performed to find the differentially regulated lipids (DRLs), which were statistically different lipids among the six groups. As a result, the p-values of 142 lipids were below 0.05 by using the Student’s t-test, and we selected several lipids that have the lowest p-values to find the alteration of their relative levels in the six groups.

Conclusions
In this study, UPLC-QqQ/MS-based lipidomics was used to elucidate the altered lipid metabolism of HepG2 cells affected by the oxidative stress. In the cell experiments, we confirmed that high glucose treatment causes the cellular oxidation. Then, ROS may give effects on the metabolism of various metabolites including lipids. Silymarin is a well-known drug for the anti-oxidation activity. Thus, the treatment of silymarin may suppress the oxidation of high glucose induced cells. Alm treatment also showed the biological activity to suppress the oxidative stress. Thus, the treatment of silymarin or Alm may suppress the severe alteration of lipid metabolism in high glucose induced HepG2 cells. To detail characterize the lipid alterations of HepG2 cells affected by oxidative stress and treatment of silymarin or Alm, six samples of Con, Glu30, Sili50, Alm50, Alm100, and Alm200 were subjected into lipidomic analysis. Furthermore, multivariate analyses such as PCA, sPLS-DA, and ANOVA were applied to characterize the lipid alterations in these six groups. As a result, sPLS-DA well differentiated the lipid profiles of these six groups. In the ANOVA tests, 142 DRLs were identified to find the alteration of their relative levels in the six groups. Finally, we assessed the altered levels of lipids by oxidative stress, and suggested these lipids as the biomarker candidates associated with oxidative stress. Furthermore, we assessed the lipid alteration in the high glucose induced HepG2 cells treated by silymarin or Alm. When silymarin or Alm suppresses the damage of oxidative stress, it can decrease the degree of lipid alteration occurred by oxidative stress. With these results, it was possible to more understand how lipid metabolism of HepG2 cells was affected by oxidative stress. In future aspect, identified DRLs can be used as biomarker candidates to evaluate the oxidation of liver cells.

Novel Aspect:
This study firstly attempted to perform the LC/MS-based lipidomics analysis of high glucose induced HepG2 cells which were damaged by oxidative stress. Various lipids including glycerol lipids (i.e., TG, DG, MG), sterol lipids (i.e., ChE, cholesterol), phospholipids (i.e., PC, PE, PS, PG, PI, PA), lysophospholipids (i.e., LPC, LPE, LPS, LPG, LPI, LPA), and sphingolipids (i.e., SM, Cer, dCer, So, Sa, Cer1P, So1P, Sa1P) were profiled in the HepG2 cells, and their levels were assessed in six samples of Con, Glu30, Sili50, Alm50, Alm100, and Alm200. We also found how the pretreatment with silymarin or Alm before exposure to high glucose dose dependently attenuated the high glucose induced hepatotoxicity and altered lipid metabolism. The DRLs identified by multivariate analyses can be used as biomarker candidates to evaluate the oxidation of liver cells.

References
MALDI-TOF/MS ANALYSES DETERMINED URINARY PHOSPHOLIPIDS AS A POTENTIAL BIOMARKER FOR PROSTATE CANCER

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Keywords: MALDI-TOF/MS; Urine; Prostate cancer; Phosphatidylcholine; Lysophosphatidylcholine.

Introduction:
The variations of phospholipids in plasma or tissues have been reported in several cancers, however they have not been elucidated in prostate cancer (PCa) especially in the non-invasive source of urine (1). Matrix-assisted laser desorption and ionization time-of-flight mass spectrometry (MALDI-TOF/MS) is increasingly used in the lipid research which enables a rapid screening of phospholipids (2).

Methods:
Urinary lipidomic screening was performed by MALDI-TOF/MS (AXIMA Performance, Shimadzu) in the urine samples collected from 20 PCa and 20 benign prostatic hyperplasia (BPH) patients after digital rectal examination (DRE). Lipids were identified by MALDI-TOF/MS2 analyses. The compositions of phosphatidylcholine (PC) and lysophosphatidylcholine (LPC) were compared between the PCa and BPH groups.

Results:
In this study we have focused on the DRE urine. The samples contain many kinds of lipids secreted from prostatic microenvironment, whenable the detection of secreted prostate products as potential sources of PCa specific biomarkers. The lipids were extracted from the DRE urine by the Bligh-Dyer method (3). The urinary phospholipids including PC, LPC, phosphatidylethanolamine (PE) and phosphatidyglycerol (PG) species were detected by MALDI-TOF/MS and identified by MALDI-TOF/MS2 in a reflectron positive mode. Among them, PC and LPC species were relatively abundant and with good reproducibility in the DRE urine samples. The peak intensities of the synthetic PC and LPC molecules spiked in the urine were detected by MALDI-TOF/MS and linearly correlated with their concentrations. The compositions of PC and LPC in the urine samples were significant different between the PCa and BPH groups.

Conclusions:
The urinary phospholipids are detectable by MALDI-TOF/MS and identified by MALDI-TOF/MS2. The relatively quantitative analyses of PC and LPC concentrations in the DRE urine samples were feasible and with good reproducibility. The MALDI-TOF/MS analyses determined the alteration of the compositions of urinary PC and LPC and it may be used a simple and non-invasive biomarker for the PCa.

Novel Aspect:
We revealed the variations of urinary PC and LPC detected by MALDI-TOF/MS in PCa which may offer a simple and non-invasive method for PCa diagnosis.

References:
INVESTIGATION OF ONE NOVEL OXIDIZED 1-PALMITOYL-2-ARACHIDONOYL-SN-GLYCERO-3-PHOSPHOCHOLINE MOLECULE ON HUMAN AORTIC ENDOTHELIAL CELLS BY LC-MS/MS

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Keywords: Phospholipids, oxidation, human aortic endothelial cell, LC-MS/MS.

Introduction:
Phospholipids are the major components of cell membranes. Among them, 1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphocholine (PAPC) is especially important since oxidized PAPC products are closely associated with atherosclerosis. PAPC is composed of a polyunsaturated fatty acyl chain at the Sn2 position, and is highly susceptible to oxidation. Many of the oxidized PAPC products are bioactive.

Methods:
An LC-MS/MS method was developed to analyze one specific ox-PAPC molecule (m/z=846.5) in human aortic endothelial cells [1-5]. The optimal HPLC mobile phase compositions and flow rate were selected. For MS/MS, the optimal sheath gas pressure, capillary temperature, collision energy and injection time were chosen. Endothelial cells were treated with oxidized LDLs, extracted by liquid-phase and solid-phase extractions and then analyzed by LC-MS/MS.

Results:
Elucidation of the chemical structure of m/z =846.5 was based on the MS2 and MS3 spectra [1-5]. Quantitation of m/z =846.5 in HAECs was based on the reconstructed ion chromatogram (RIC) and selected multiple-reaction monitoring (SRM) of LC-MS/MS analysis. An internal standard was used to construct calibration curves for measuring the concentration of m/z =846.5 in endothelial cells under oxidative stress.

Conclusions:
An LC-MS/MS method in conjunction with liquid-phase and solid-phase extractions has been developed to separate and identify a specific ox-PAPC molecule (m/z=846.5) in human aortic endothelial cells. The most possible structure of the ion is PAPC_5-epoxy_8-E/D-IsoP_14-epoxy which is elucidated from its MS2 and MS3 spectra. This new ox-PAPC molecular species has never been reported by other researchers so far.

Novel Aspect:
A novel ox-PAPC molecule (m/z=846.5) in human aortic endothelial cells has been found. Its chemical structure has been identified by LC-MS/MS analysis.

References:
Introduction:
Human plasma contains over 1500 different lipids at the sum composition level.[1] Yet the number of unique lipids present is surely higher, once isomeric contributions from double bond location and fatty acyl regiochemistry are considered. To close this gap, ozone-induced dissociation (OzID) is incorporated into data-independent lipidomics workflows, thus enabling complex extracts to be profiled for unsaturation and regioisomers by construction of 2D maps.

Methods:
Ozone-induced dissociation was implemented on a Thermo Fisher Scientific LTQ Orbitrap Elite high-resolution mass spectrometer, equipped with an ESI source operating in positive ion mode. The instrument is modified such that nitrogen is replaced by ozone (~15% in oxygen) as the HCD collision gas. Lipid standards and complex extracts were introduced into the ESI source by direct infusion (AdvionTriVersaNanomate).

Results:
OzID performance was benchmarked using glycerolipid standards containing unsaturated fatty acyl chains. Isolation of lipid ions in the linear ion-trap, followed by interaction with ozone in the HCD cell yielded diagnostic ions for each double bond position and sn-position in the resulting MS/MS spectrum. The increased ozone concentration in the HCD cell (compared to a single stage ion-trap instrument[2]) significantly improved ozonolysis efficiency. This advance translates into increased lipidome coverage, and improvements in duty cycle for data-independent MS/MS analysis using shotgun workflows. Grouping all precursor ions with a common OzID neutral loss enables straightforward classification of the lipidome by unsaturation position (with respect to the methyl terminus). Glycerolipids exhibiting common HCD/OzID product ions were classified according to the fatty acyl carried at the sn-1 position. 2-dimensional maps provide a straightforward visualization of the data.

Conclusions:
Resolving ambiguity in the molecular structure of lipids is the next frontier in contemporary lipidomics.[3] OzID provides access to this detailed level of molecular characterization. Global profiling of fatty acyl unsaturation within a biological extract provides a snap-shot of enzymatic biosynthesis and could potentially provide indicators of health and disease.

Novel Aspect:
First demonstration of a data-independent strategy combined with ozone-induced dissociation for comprehensive profiling of unsaturation in complex lipid samples.

References
Keywords: Lipidomics, translational research, coronary atherogenesis, asymptomatic patients intermediate Framingham risk score, mass spectrometry.
643 - LIPID PROFILING OF NON-STEROIDAL ANTI-INFLAMMATORY DRUGS (NSAIDs) ON PROSTATE CANCER CELL LINES WITH TOF-SIMS AND DESI-MS

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Keywords (5/5): Prostate Cancer, SIMS, DESI, NSAIDs

Introduction:
Prostate cancer (PCa) is the prime cause of cancer related death in Western men.1 It is not possible to distinguish indolent and aggressive disease, leading to overtreatment and associated complications. NSAIDs can potentially prevent growth of PCa cells, offering an alternative treatment approach.2 The response of NSAIDs on PCa cell lines varying in clinical aggression was studied using MSI.

Methods:
The lipidomic fingerprint was explored post NSAID treatment at a series of concentrations and time points. For DESI (Waters, Xevo-G2-SX), 95% methanol was used at a spatial resolution of 50 µm. For ToF-SIMS (Kore Technology Ltd), the samples were analysed using micro-focussed Au3+ clusters. Due to the hypoxic nature of the prostate, this was repeated under different levels of oxygenation.

Results:
The cell lines used for this study were LNCaP, LNCaP-C42 and LNCaP-C42B, which respectively increase in aggression. Post drug treatment, the proliferation assays demonstrated that trifluorinated ibuprofen (TFIB), ibuprofen (IBP), and orlistat, lead to a decrease in cell growth above 1 mM of IBP concentrations and 10 uM of Orlistat. ToF-SIMS and DESI analysis of the untreated cell line samples highlighted a number of differences in the ratios of lipid classes, including more abundant cholesterol species in the less aggressive LNCaP cells with phospholipids generally being more abundant in the more aggressive cell lines. After drug treatment, multivariate analysis highlighted further differences. Under varying levels of hypoxia, the untreated samples separate with principle component analysis, suggesting future work identifying which more accurately represents the prostate in vitro.

Conclusions:
The administration of NSAIDs leads to a decrease in PCa cell growth irrespective of their phenotype. Untreated PCa cell lines can be differentiated using ToF-SIMS and DESI with multivariate data analysis of the lipid fingerprint. NSAIDs alter these profiles suggesting lipid involvement in the drug pathway. Here we show the potential of MSI technologies to generate therapeutic mechanistic data.

Novel Aspect:
Using imaging MS techniques to examine the lipid profile of PCa cells with differing metastatic properties post drug treatment.
References:

(1) Prostate Cancer (C61): 1993-2014. European Age-Standardised Incidence Rates per 100,000 Populations, Males, UK, 2014.

Introduction:
Marine sponges harbor complex microbiomes and novel bacteria associated with these sponges have been isolated and classified by 16S rDNA sequencing [1]. Sponge-symbiont relationships are not yet well understood. It is clear, however, that symbionts are responsible for a portion of the production of bioactive metabolites previously isolated from sponges. Some membrane lipids also have been shown to play a vital role in bacterial recognition by host cells.

Methods:
An actinobacterium (R45601) was isolated from X.mutaand cultured in ISP2 medium. Cells were lysed and cardiolipins(CLs) were extracted according to the method described by El Hamidi, et al [2] for extraction of LPS. Extracts were dissolved in a 2:1:0.1 solution of chloroform, methanol, and water. Samples were analyzed first by MALDI-TOF using norharmane as matrix. Tandem mass spectra with ion mobility separation were acquired on a Q-IMS-TOF mass spectrometer.

Results:
A comparison of broadband mass spectra of extracted lipids, filtered by drift time in the ion mobility cell and m/z, from a clinical isolate [3] of methicillin-resistant S. aureus (MRSA-M2) and a recently discovered [4], marine sponge-associated actinomycete (R45601) showed that R45601 produces a pool of CLs with greater mean mass and possibly a higher degree of unsaturation than those produced by MRSA-M2. To identify fatty acyl groups incorporated into CLs from each species, MS/MS experiments were performed. The fatty acyl groups from CLs were released in the gas phase upon collisional activation as ionized free fatty acids with a single negative charge. These data show that ions observed in the single stage mass spectra which differ by multiples of 2 Da correspond to CLs with unsaturated fatty acyl groups. CLs from MRSA-M2 contained mostly saturated fatty acyl groups, whereas many CLs from R45601 contained at least one unsaturation. A possible explanation for this difference is that the mechanisms for CL synthesis differ between bacterial phyla.

Conclusions:
Comparisons of molecular classes from different organisms can be useful for generation of testable hypotheses. In this study, there were clear observations of differences in CL structure between representatives from two bacterial phyla. These structural differences may have implications with regard to bacteria-host interactions. CLs may play a role in sponge recognition of microbes. Differences in CL structure will also affect bacterial phenotype and behavior.

Novel Aspect:
Direct comparison of cardiolipins from a terrestrial firmicute and a marine actinomycete.

References
Untargeted lipidomics for the assessment of arsenic exposure in rice (Oryza sativajaponica)

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Keywords: untargeted, lipidomics, LC-MS, arsenic, rice.

Introduction:
There is an increasing interest in how arsenic is assimilated and metabolized in plants-base foodssuch as rice (Oryza sativa japonica), due to the potential health risk that its accumulation might cause through food chain[1]. Lipidomics is a powerful approach to study lipids species present in rice, with the aim of determining the affected metabolic pathways.

Methods:
Two treatments were applied to rice crops: As was applied by irrigation (1 or 1000 μM), and directly to the soil before planting (5 or 50 ppm). LC-MS analysis was conducted using a Waters Acquity UPLC system connected to a Waters TOF mass spectrometer. A Kinetex C8 column (100 x 2.1 mm, 1.7 μm) was used for chromatographic separation. Multivariate tools (PCA, PLS-DA, ASCA) were employed in data analysis.

Results:
In watering treatment, the lower concentration was set at 1 μM, as it is the limit of acceptable As concentration in water by European legislation, whereas the higher concentration was chosen at 1000 μM, because it is expected to induce changes in plants without causing their death [2]. In soil treatment, the lower As limit was set at 5 ppm, as an average concentration of this element in Earth’s crust, and the higher As limit at 50 ppm, as soil is considerate to be contaminated by this element above 10 ppm [1].

Results obtained after chemometric analysis show that there are significant differences between treatments and concentrations, when compared with control samples (without arsenic treatment), especially in the case of the higher concentrations of this element.

The principal lipid families that were affected by As treatments were glycerophospholipids and acylglycerides.

Conclusions:
Only the highest As concentration levels (both in irrigation water or settled in soil) allowed the differentiation between samples. This complies with the European legislation, with permitted levels of the As exposure not showing relevant effects.

As a general lipidomic conclusion, glycerophospholipids and acylglycerides are the main families of lipids that were affected by the arsenic exposure.

Novel Aspect:
A lipidomic approach giving a better understanding of the metabolic pathways affected by arsenic exposure, and able to identify possible biomarkers.

References:
High-Resolution Multiple Reaction Monitoring Method Development for Quantification of Steroidal Hormones in Plasma

Keywords: Lipidomics; MRM-HR; LC-MS/MS; Steroids; Chronic Stress

Introduction:
Advances in MS enabled development of HR-MRM and became the analysis of target analytes more specific in lipidomics approach, contributing in the medical and pharmaceutical fields. Hence, HR-MRM it is a sensitive and very specific virtual modes of acquisition that allows the simultaneous steroidal hormones quantification in low concentrations, such as observed in biological samples.

Methods:
HR-MRM method included cortisol, cortisone, corticosterone, 11-DHC, progesterone and aldosterone. Analytical validation was conducted according to the ANVISA guidelines. Specificity, linearity, intraday and interday precision and accuracy, and the LLOQ were evaluated. Plasma real samples of 129sv male mice exposed to chronic unpredictable stress were used to validate the quantification method.

Results:
The method was conducted according to the ANVISA normative, adopting a coefficient of variation (CV), as well as relative standard deviation (RSD) and relative error (RE) lower than 15% in linearity, intraday and interday precision and accuracy. For cortisol, corticosterone, and their inert metabolites (cortisone and 11-DHC), the LLOQ was 3.9 ng/mL, while those for progesterone and aldosterone was 7.8 and 15.6 ng/mL, respectively. The linearity of the method was between LLOQ-500 ng/mL for each analyte. HR-MRM analysis showed that animals submitted to stressors have 4.5 times more corticosterone in their serum than non-stressed mice and the levels of 11-DHC does not vary significantly in both groups. The development method showed adequate selectivity and sensibility for quantification of target analytes and can be used in several studies according the observed validation parameters.

Conclusions:
The results indicate that the method can be applied for quantification of steroids in several biological samples, such as human or animal plasma with selectivity and confidence. Thereby all studies that involve the balance of the target analytes can be conducted using this method for quantification.

Novel Aspect:
The HR-MRM method allows the monitoring of different steroids from human/animal origin, facilitating its application in several biological situations.

References:
985 - MICRO-UPLC-MS HIGH-THROUGHPUT SCREENING ASSAY FOR SPHINGOLIPIDS PATHWAY ANALYSIS

Kristen Randall (1) - Helen Klodnitsky (1) - Drew Tietz (1) - Clifford Phaneuf (2) - Maureen Olszewski (3) - Elina Makino (3) - Keeley Murphy (4) - Alexei Belenky (1)

Sanofi, Pre-Development Sciences, Waltham (1) - Sanofi, Pre-Development Sciences, Walham (2) - Sanofi, In Vitro Biology, Waltham (3) - Thermo Fisher, Marketing, San Jose (4)

Keywords: microflow liquid chromatography, high-throughput screening, lipidomics

Introduction:
In the presentation we will describe the Micro-UPLC-MS system based on Vanquish Focused+ LC (Thermo) and TSQ Altis Triple Quadrupole Mass spectrometer (Thermo) for lipid analysis.

Methods:
Replacing conventional tubing of the system with micro ID tubing, adding a second pump and optimizing the injection strategy allows us to convert conventional system to micro with parallel chromatography capabilities. Run time using a 5 cm length column was less than one min per injection.

Results:
This system was successfully used for affinity screening of biomarkers from lipid pathways. This strategy is valuable when highly sensitive biomarker quantification in large sample set (library screening) is required.

Conclusions:
In the poster we will demonstrate performance of the micro-LC-MS system using endogenous sphingolipid biomarkers from cell extracts at sub ng/ml (limit of quantitation is 0.05ng/ml) concentration. We have evaluated Acclaim C8 0.3mm ID and BEH C18 1mm ID columns using gradient and isocratic chromatography. C8 was found to be more rugged but C18 enabled us to discriminate isobaric lipids.

Novel Aspect:
We have developed LC-MS based screens using microflow chromatography.
254 - CERAMIDOME PLASTICITY THROUGH ISO-ENERGETIC PRECURSOR AND NEUTRAL LOSS DISCOVERY SCANS.

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Ceramidome plasticity through iso-energetic precursor and neutral loss discovery scans.

Keywords: Ceramide, epidermoside, collision-induced dissociation, screening, triple quadrupole,

Introduction: Mass spectrometric methods of identification and measurement are pivotal to investigate the multifaceted biological role of ceramides and the dose-effect relationship of pharmaceuticals that modulate ceramide flux. Triple-quadrupole tandem MS embodies the highest flexibility to discover, identify and measure expected and unexpected molecular species, and an unprecedented scan mode is presented[1].

Methods: An API3000LTQ LC-MS system is operated under manufacturer’s instructions to perform extensive tandem MS fragmentation studies on standard ceramides with C6-C24 fatty acids, lipid extracts of animal tissues and of nutraceutical seeds and nuts. LC separation and MS operation is optimized for the separation, detection, identification and quantification of expected and unexpected ceramides.

Results: The equation linking instrumental to center-of-mass collision energy (CoM-CE) in collision-induced dissociation is rearranged to calculate laboratory-frame inter-quadrupole potential drop (LF-CE) corresponding to distinctive positions, such as that of the CoM-CE maximum fragment yield, in the breakdown curves for protonated ceramides with molecular masses up to 1,000 Th. The value of the CoM-CE for distinctive fragments is calculated for representative ceramide FA homologs from accurate recordings of the spectra in the 0 to 90 DV interval of LF-CE. A scan function of the q2 collision cell is synchronized with the Q1 scan in the precursor ion mode and with the Q1 and Q3 scans in the Neutral Loss mode. Several lipid extracts containing ceramides are examined by infusion and by LC, and unexpected components, such as Z-ceramides in nut extracts are tentatively identified.

Conclusions Precursor and Neutral Loss scans with iso-energetic CID in the triple quadrupole MS allow to characterize ceramide mixtures that contain unexpected components with a common substructure to known ones. Individual response factors for (semi)-quantification of unexpected components can be calculated from the fractional intensity of the reporter fragment in the spectra of known components.

Novel Aspect: A novel scan function of a triple quadrupole instrument allows to measure tandem mass spectra at a constant center-of-mass collision energy.

References
1062 - SERUM LIPIDOMIC CHANGES IN ZIKA AND DENGUE VIRUS INFECTION REVEALS POTENTIAL TARGETS FOR ASSERTIVE CLINICAL DIAGNOSIS

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Keywords: Zika virus, Dengue virus, lipidomic, serum

Introduction:
The Zika (ZKV) and Dengue (DENV) Virus have been a major public health challenge in the world due to large number of underreporting, inaccurate and false diagnoses. The lipidomic-based method to detect biomarkers of the physiological variations at all stages of the infection and immunological responses. Herein we showed that lipidomics-based mass spectrometry can be applied for molecular diagnostic of Zika and Dengue and co-infection of infected serum [1,2].

Methods:
The serum samples were separated in four groups through ELISA (IgG and IgM) protocol: 30 ZKV, 30 DENV and 30 controls. The method of metabolites extraction from 50 µL serum infected was Bligh-Dyer. The upper phase was collected for lipidomic analysis using an Agilent 1290 Infinit LC-MS system coupled with an Agilent 6550 Q-TOF/MS mass spectrometer equipped with an electrospray source, in positive and negative modes.

Results:
The chromatograms were processed by XCMS software and multivariate analysis was made in the Metaboanalyst 3.0 software. The PLS-DA was applied to classify the serum samples according to the type of virus infection. Based in different class of lipids, the serum infected by ZKV and DENV were clearly distinguishable into two clusters by PSL-DA plot. The majors lipids identified as responsible for the differentiation of the two clusters, by variable importance in projection (VIP plot), were phosphatidylcholine (PC), lysophosphatidylcholine (LysoPC), phosphatidylinositols (PI), glycolipids and glycosphingolipids. The Gangliosides, particularly GM1, GM2, GD1a, GD1b, and GT1 were upregulated only under infection of the ZKV.

Conclusions:
Our study identified altered lipids phosphatidylcholine (PC), lysophosphatidylcholine (LysoPC), phosphatidylinositols (PI), glycolipids and glycosphingolipids of Zika and Dengue infection. The identification of specific biomarkers of the Zika and Dengue virus infections can perform the differential diagnosis of these arboviruses and closer to the actual clinical state of the patient.

Novel Aspect:
The lipids identified allude to possible novel diagnostic targets, pharmacological studies and advance our understanding on the mechanisms of arbovirus pathogenesis.

References:
244 - FISHING FOR LACTONES:
METHOD DEVELOPMENT FOR SELECTIVE IDENTIFICATION OF LACTONE-CONTAINING LIPIDS

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Keywords: LC-MS, lactones, PON1, polyunsaturated fatty acids (PUFA), structure determination

Introduction:
PON1 is an anti-atherogenic HDL-associated lactonase, whose native substrates remain unknown. PON1 knockout mice have low blood pressure and high levels of arachidonic acid (AA) metabolite 5,6-EET [1]. Under physiological conditions, 5,6-EET transforms to the δ-lactone 5,6-δ-DHTL [2], a PON1 substrate [3]. We hypothesize that additional lactone PUFA metabolites are potential PON1 substrates.

Methods:
To identify and characterize these lactones, we react lipid extracts from mouse kidneys with an amine to convert lactones to lactams that can be isolated using solid phase cation exchange. We apply high resolution liquid chromatography-mass spectrometry to determine the molecular formula of the original lactones. Molecular databases are used to find potential structures, and standards will be used to confirm or negate these structures.

Results:
The selective chemical reaction was developed using standards of different lipid functional groups (free fatty acid, ester, and two lactones of different ring size). Conditions were optimized to achieve reaction of the lactones and not of the ester. However, reaction of the fatty acid did occur. LC-MS analysis of similarly reacted lipid extracts from w.t. and PON1KO mice provided over 60 different chemical formulae of potential lactones, most of which were present in all samples (n = 3 pools of 5 kidneys for each strain). Using molecular databases, hundreds of potential lactone structures were identified. We are currently investigating ways to limit the search results to the most biologically relevant and likely structures. Standards of those structures will then be subjected to the same chemical reaction, extraction, and LC-MS method in order to confirm the structures of the unknowns in the biological samples. MS/MS experiments may be applied. It will then be investigated whether the confirmed structures are PON1 substrates.

Conclusions:
Chemical formulae of potential lactones were repeatedly identified in separate mouse kidney samples using LC-MS, while their precise structures are not yet known. Presently, further repetitions are underway to determine the differences between w.t. and PON1KO and pinpoint PON1 substrates. We hope that this research will shed light on the potential role of PON1 in blood pressure regulation.

Novel Aspect:
PON1 has been thoroughly studied in relation to atherosclerosis, yet the substrates of PON1 linking the enzyme to blood pressure regulation remain unknown.

References:

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Challenges of lipid isomers and Isobars in mass spectrometry-based biomarker research
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Keywords: Isobars and isomers, lipidomics, high-resolution accurate-mass (HRAM) spectrometry, multiple-reaction monitoring (MRM)

Introduction
Interfering isobaric & isomeric species still constitute a major issue in the unambiguous attribution of lipid biomarkers to disease. MRM scanning allows to distinguish the main lipid classes, but not always isobaric subclasses. HRAM spectrometry (MS1) allows the distinction of these isobaric species but is not able to distinguish isomers. We are comparing two flow-injection analysis Methods:

Methods
Two commercially available kits using MRM or HRAM screening are being compared. They are used to measure a range of small molecules & lipids and have been validated for human plasma & serum, the AbsoluteIDQ® p180 Kit (1-3) for Waters XevoTMTQ-S, Sciex QTrap®5500 or ThermoScientific TSQ VantageTM, and p400 HR Kit (2017)(ThermoQ ExactiveTM systems) (both Biocrates Life Sciences AG).

Results
Derivatization removes especially most phosphatidylethanolamines, the main interfering species, leading to more specific detection of PCs.
All realistically possible species covered by a result should be listed. The given name of the signal should refer to the most relevant species; lists with isobaric / isomeric species being provided.
In both cases, chromatography will assist the separation of species, but is limited due to high throughput requirements and the need for internal standards for different sections of the chromatogram to correlate with suppression effects.
The reliable quantitation of additional lipid classes can be achieved, e.g. as a service (4), but requires additional effort compared to the single high-throughput methods discussed here.

Conclusions
Screening using HRAM and low-resolution MRM will by their nature give somewhat different results due to different isobaric & isomeric compounds. The AbsoluteIDQ® p180 and p400 HR Kits provide standardized procedures for the fast and efficient detection and annotation of the major lipid classes. Use of HRAM increases coverage through complementary quantitative high-resolution measurements.

Novel Aspect
New guidance on how to interpret the different results obtained by lipid biomarkers screening via MRM versus HRAM.

5. References
407 - APPLYING TRAPPED ION MOBILITY SEPARATION (TIMS) IN COMBINATION WITH PARALLEL ACCUMULATION SERIAL FRAGMENTATION (PASEF) FOR ANALYSIS OF LIPIDOMICS SAMPLES

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Keywords: PASEF; Ion mobility; Lipidomics; timsTOF PRO; Plasma

Introduction:
Untargeted lipidomics workflows are aimed at profiling changes in the lipidome which can lead to the discovery of lipids as potential biomarkers. We present an improved lipidomics identification workflow that is based on the combination of LC-MS and TIMS separation together with a data dependent MS/MS fragmentation (PASEF).

Methods:
LC separation was performed using a Bruker Elute UHPLC system. Data acquisition was performed with separate positive and negative ionization methods on a Bruker timsTOF PRO instrument. The instrument was in TIMS mode while acquiring MS/MS fragmentation (PASEF). The data sets were processed in MetaboScape 4 using 4-dimensional feature finder. Features were annotated using SimLipid software.

Results:
The TIMS technology adds an additional dimension of separation. To evaluate this effect on lipidomics analyses a direct infusion method was applied to critical pairs of isobaric and close isobaric lipids. A pair of isobaric phosphocholines (PC 18:1) differing only in the position of the unsaturation (9Z vs 6Z) could be separated with a mobility peak resolution of 175. A second almost isobaric pair (phosphoethanolamine & phosphocholine) could be fully baseline separated with a mobility resolution of 180.

LC-TIMS-MS measurements in PASEF mode were performed on extracts of commercially available plasma samples from different species (bovine, chicken, pig and human). The 4-dimensional feature finder in MetaboScape was used to extract the features from all analyses. The main precursor mass and the most intense fragment spectrum were exported to SimLipid software for identification.

Conclusions:
First results show 200-400 unique lipid IDs (depending on animal species). Compared to analyses performed without TIMS/PASEF an increase of 30-50% in identifications can be observed. Additional measurements will be presented to further highlight the benefit of PASEF.

Novel Aspect:
Lipidomics workflow combining positive/negative ion data using PASEF mode on an LC-TIMS-QTOF instrument.
Introduction:
The mass spectrometric analysis of lipid mediators and other endogenous compounds remains challenging and rich in pitfalls. Variations between runs and even within runs impairing the validity of the data are hard to identify. We established a systematic approach to ensure the quality of data in the analysis of prostanoids in plasma samples by evaluating control samples using a control chart.

Methods:
A mass spectrometric method for the quantification of PGE2, PGD2, TXB2, PGF2α and 6-keto-PGF1α in human plasma was validated according to EMA and FDA guidelines. After successful validation, a control chart was set up: blank plasma was spiked with prostanoids and aliquots were stored at -80 °C. In every analytical run, one control plasma aliquot was analysed in the beginning and one at the end of the batch, in addition to six freshly prepared quality control samples.

Results:
After analysis of 24 control samples over six weeks, for all analytes alert limits and control limits were defined depending on mean value and corresponding standard deviation. Exemplarily, results for PGE2 were: MV 93.2 pg/mL, SD 8.7 pg/mL, alert limit ±2*SD (75.8 pg/mL or 110.6 pg/mL), control limit ±3*SD (67.1 pg/mL or 119.3 pg/mL). After six more weeks, a tendency of increased concentrations of all prostanoids in the control samples was observed, indicating instability of the analytes in plasma samples at -80 °C after 12 weeks. During validation, stability had been proven for 8 weeks.

Surprisingly, concentrations went back to the initial level after 10 more weeks. Detailed investigation revealed that the slopes of the calibration curves of the runs with increased concentrations of control samples differed from the slopes during the validation. These variations cannot be discovered when using only quality control samples. Thus, the control chart revealed difficulties in the preparation of calibration and quality control standard solutions.

Conclusions:
Due to lack of interlaboratory surveys for lipid mediators and other endogenous compounds, alternative tools for quality assurance are urgently required. Monitoring of control samples using control charts is a useful tool to ensure the constancy of the analytical results. Careful evaluation can reveal systematic errors, problems concerning stability of samples or analytical standards, lack of robustness and other issues relevant to quality.

Novel Aspect:
A systematic approach to ensure quality and validity in the analysis of endogenous compounds.
344 - STUDY OF ACETOGENINS IN A NATURAL EXTRACT USING SFC-HRMS/MS AND POST-COLUMN METAL CATIONISATION

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Keywords: supercritical fluid chromatography, high resolution tandem mass spectrometry, post-column metal cationisation, lipid.

Introduction:
Acetogenins, lipids from the polyketide class and specifically found in plants from the Annonaceae family, are food toxins suspected to be responsible of atypical Parkinsonian syndromes. Acting as an inhibitor of mitochondrial complex I, acetogenins are also proposed as potential anti-cancer molecules. Previous dereplication studies highlighted some limitations of the HPLC-MS approach including long analysis time and low chromatographic resolution.

Methods:
Nowadays, supercritical fluid chromatography (SFC) can be considered as an alternative to LC for the study of hydrophobic compounds. Indeed, physicochemical properties of supercritical CO2 allow excellent solubility of such analytes and give analytical performances above that of LC. However, examples using SFC-HRMS/MS to analyze natural extracts are poorly developed in the literature.

Results:
Due to the rarity of standards, a SFC-HRMS/MS method was directly developed using a crude seed extract of Annona muricata L. with a reduced analysis time by a factor of 4. Nevertheless, the fragmentation of protonated or sodium/potassium cationized species only led to limited structural information. For the first time, post-column lithium cationisation was fully optimized after SFC in order to form ion fragments of structural interest but of very low intensity. Finally, copper salt was employed allowing the detection of intense odd and even ion fragments related to unique radical-driven fragmentation pathways.

Conclusions
The final SFC-HRMS/MS method significantly improved the structural characterization of acetogenins highlighting the power of SFC-HRMS/MS in the field of natural products.

Novel Aspect:
For the first time, SFC-HRMS/MS including post-column cationization was implemented for the structural characterization of natural lipids.

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MODELLING CANCER LIPOGENESIS USING LA-REIMS METABOLIC FLUX ANALYSIS IN BREAST CANCER CELL LINES

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Keywords: [U-13C]-palmitic acid, LA-REIMS, metabolic flux analysis, breast cancer, lipogenesis

Introduction:
Understanding alterations in the lipid metabolism of cancer cells is important due to the role that lipids play in membrane fluidity, energy storage and signalling processes. Using U-13C-labelled palmitic acid, biosynthetically incorporated into fatty acids, provides a further understanding of lipogenesis in in vitro studies. Here, laser assisted rapid evaporative ionisation mass spectrometry (LA-REIMS) was performed to monitor metabolic flux during cell proliferation.

Methods:
SkBr3 cells were grown in DMEM containing 1% fatty acid free-BSA, and treated with [U-13C]-palmitic acid under normoxic conditions for 24h. After centrifugation, cell biomass pellets were analysed directly with LA-REIMS analysis without further sample processing steps. LA-REIMS spectra were obtained in the mass range m/z 50-2500 in positive and negative ion modes and putative identification was performed using the LIPID MAPS database.

Results:
LA-REIMS analysis of cell lines resulted in specific lipidomic profiles that enabled the identification of different cell lines. Common adduct formations such as [M-H]- and [M-H2O]- in negative mode and [M+H−H2O]+, [M+H]+ or [M+Na]+ in positive modes were detected in the metabolic flux experiment for SkBr3 cells. [U-13C]-palmitic acid was detected with m/z 271.2 in negative ion mode in SkBr3 cells. From the data obtained, the labelled lipid composition of SkBr3 cells in negative mode was observed to consist of phosphatidylethanolamines (e.g. PE (P-38:6) at m/z 746.5), sphingomyelins (e.g. SM (34:1) at m/z 701.5), and phosphatidylserines (e.g. PS(36:0) at m/z 790.5). In positive mode, diacylglycerides (e.g. DAG(32:1) at m/z 549.4) and ceramides (e.g. Cer(42:0) at m/z 634.6) were found in the cell line material.

Conclusions:
The use of stable-isotopically labelled palmitic acid in conjunction with LA-REIMS analysis allowed specific biochemical information to be collected. This approach will help to further understand how palmitic acid is being incorporated into the lipid biosynthesis pathway in a rapid and sample preparation free approach. This preliminary data demonstrates the applicability of LA-REIMS for the characterization of metabolic flux experiments using labelled metabolites.

Novel Aspect:
LA-REIMS analysis can be used in metabolic flux analysis experiments in rapidly proliferating cells to model breast cancer lipogenesis – potentially identifying novel diagnostic markers and drug targets.
Introduction:
Theoretically, mammalian cells can contain many thousands of individual lipid molecular species, whilst it is unlikely that all are present in a single cell. Lipidomic experiments have demonstrated the presence of more than a thousand species. The integrated regulation of changes in lipid species that occur can regulate cell functions, including signalling and metabolism, highlights the need for bioinformatics analysis to fully interpret lipidomics data.

Methods:
We have adopted two experimental systems: lipids extracted from colorectal tumour tissue and rhinovirus-infected human bronchial epithelial cells, were identified by LC-MS/MS. Pathway analysis of lipid metabolising enzymes coupled to network optimising Prize-collecting Steiner tree problem methodology was adopted to identify key enzymatic changes which could be considered as therapeutic targets.

Results:
Lipidomic analysis that compared colorectal tumour tissue of defined stage with matched normal tissues showed changes in more than 700 lipid species, this also demonstrated changes in acyl chain saturation and chain length accompanying tumour progression. In the rhinovirus-infected cells, there was a temporal-associated change in a number of lipids including PIP, cardiolipin, PC and DG, there were also clear changes in the acyl chains of neutral and phospholipids. Pathway analysis highlighted key changes in sphingolipid metabolising enzymes in both systems including sphingosine kinase and ceramides synthase activities. Further, adopting the network optimising Prize-collecting Steiner tree problem methodology, we were able to identify fatty acid synthases, acyltransferases and ceramidases as potential therapeutic targets. The importance of a number of these activities in viral infection was confirmed by observing inhibitory effects of selective inhibitors upon infection of human bronchial epithelial cells by rhinoviruses.

Conclusions
The lipidomics-determined changes identify potential novel therapeutic targets, they additionally suggest roles for remodelling of membrane lipid acyl chain changes in membrane fluidity, protein binding or curvature, may have important roles in infection and disease progression.

Novel Aspect:
Lipidomics with appropriate bioinformatics analysis can point to key enzyme activities that are responsible for disease-associated change. This can therefore point to novel therapeutic targets that can be subsequently explored.
Improved Lipid Annotation Utilizing Positive and Negative Ion MS2 / MS3 HCD and CID Spectra

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Introduction:
We present improvements to Thermo Scientific LipidSearch software. New algorithms are introduced to reduce false positives, improve quantitation and to automate searching of LC-MSn data obtained by higher collisional energy (HCD) and linear ion trap collisional induced dissociation (CID) fragmentation Methods: The use of LC-MSn is applied to the more complete characterization of triacylglycerols in total lipid extracts from bovine tissue and insect larvae.

Methods:
Bovine liver and insect larvae total lipid extracts were analyzed by data dependent LC/MS2 and MS3 using a Tribrid Orbitrap instrument. The MSn data were searched against the m/z of selected lipid precursor ions in the LipidSearch database and their predicted product ions and neutral losses. Each lipid adduct ion is ranked by mass tolerance, match to the predicted fragmentation and fraction of total MS-MS intensity.

Results:
The number of lipid species annotated in each experiment were assessed at the sum composition (MS) and isomer (MSn) levels. LC-dd-MS2/MS3 spectra for potential lipid species were annotated separately from positive and negative ion adducts. Each lipid annotation was correlated within a chromatographic time window by merging the positive and negative ion MSn data into a single lipid result. This approach provides lipid annotation that reflects the appropriate level of MS2/MS3 product ions and neutral losses from the entire dataset giving higher confidence in lipid annotations. The merged results were filtered by minimum number of data points, signal-to-noise ratio, adduct ion, match score, ID quality, and coefficient of variation from replicate sample injections. Compared to the results generated only from dd-MS2 HCD results, a combination of HCD and CID LC-MSn gave significantly higher quality lipid identifications. From bovine liver the number of PC and TG results with complete acyl chain information improved by 12% to 23%, respectively.

Conclusions
Lipid annotation is often over-reported because the current software/database approaches match mass spectral evidence to exact lipid structures[1]. The lipidomics community is working towards reporting the correct level of annotation based upon the available mass spectral information. Utilizing a combination of HCD MS2 and intelligent product ion or neutral loss triggered CID MS2/MS3 data improves lipid annotation available within a single LC/MSn experiment.
Novel Aspect:
LipidSearch 4.2 new software for improved lipid annotation and increased confidence using positive/negative ion, HCD/CID LC/MSn data from Orbitrap mass spectrometers

References


For information please contact: scientific@imsc2018.it
Introduction:
Targeted lipidomics (TL) using selected ion monitoring (SRM) enable high sensitive quantitative analysis for certain lipids in biological sample. However, overlapped chromatographic peaks from isomer and fluctuations in retention time of chromatograms cause miss alignment among samples. Here we developed novel lipidomics platform named MetaboAlign which can solve these issues by combining TL with Non-targeted lipidomics on data dependent acquisition (DDA).

Methods:
MetaboAlign, which is consisted of peak detection module, alignment module, and viewer was written in Java. SRM data for rat plasma samples (n10) measured by 4000 Qtrap (Sciex) were converted to MzML by ProteoWizard. DDA data were measured by QExactive HF (Thermo Fisher Scientific), which were used for lipid identification by LipidSearch4.2TM (Mitsui Knowledge Industry). Both results were imported to MetaboAlign, quantitative profiles of lipids were calculated.

Results:
MetaboAlign performs quantitative analysis including peak detection, alignment, identification and calculation of concentration automatically. RT correction algorithm enables large number of peaks in each sample to align with high accuracy. Combination with non-targeted lipidomics (NTL) using high resolution mass (HRMS) can also assign lipid to each peak in distinction from its isomer. We applied this software to rat liver sample (n10). As a result, 861 kinds of lipid species were identified by NTL, 496 of which were acquired as lipid profiles by TL.

Conclusions
Novel lipidomics platform combined TL with NTL was developed. Rapid and accurate quantitative analysis of large sample sets was successfully achieved using the software.

Novel Aspect:
Comprehensive and effective platform for large scale quantitative analysis using combination of targeted and non-targeted lipidomics is presented.

For information please contact: scientific@imsc2018.it
Keywords: Desorption electrospray ionization mass spectrometry, medulloblastoma, lipid profiling, rapid pathology, tissue smears

Introduction: Medulloblastoma (MB) is a heterogeneous pediatric brain tumour comprised of 4 molecular subgroups that appear morphologically identical, under the microscope for conventional intraoperative, rapid pathologic assessment [1-3]. While each MB subgroup presents its own survival rate, unique response to chemotherapy and the necessary aggressiveness of resection, they remain indistinguishable intraoperatively.

Methods: Desorption electrospray ionization mass spectrometry is an effective technique for tumour type identification based on profiling of tissue lipid and small metabolites [4,5]. Here we subjected the rapidly prepared smears of murine models of medulloblastoma tumours to DESI-MS analysis to harness both the specificity and the sensitivity of this technique in determining MB subgroup affiliation in less than 1 min of sample preparation and analysis time.

Results: Mouse cell-line derived xenograft tumours of MB Group 3 and SHH subgroups were subjected to preparation of smear samples (30 seconds) and 100 seconds of DESI-MS data collection for the preparation of multivariate statistical model that can distinguish between the two subgroups using. The model was comprised of 400 data points (200 per subgroup) comprised of 5 independent cell lines and 10 independent tumours, and showed clear separation of data points belonging to each subgroup. The model was validated using 20% leave out test. The predictive ability of the model was then assessed using blind tests. Smear spreads from two independent tumours belonging to each MB subgroup were prepared in 30 seconds and subjected to additional 10 seconds of DESI-MS data collection and real time analysis against the multivariate model using binned spectral data. A success rate of greater than 95% was achieved using the combined platform of rapid DESI-MS analysis of smears and online multivariate recognition and model comparison platforms.

Conclusions: Rapid determination of medulloblastoma subgroups based on DESI-MS lipid profiles in less than 1 min of smear sample preparation and analysis time is shown to be possible. This observation allows prognostic information regarding surgical margin extent tailored to the specific requirement of each MB subgroup to be fully utilized within the time constraints of surgery, further highlights specificity of lipid profiling with DESI-MS.

Novel Aspect: Identification, within 1 minute, of morphologically identical subgroups of same tumour type with DESI-MS analysis of tissue smears based on lipid profiling is possible.

References:


A.10 LIFE SCIENCES - GLYCOMICS, SACCHARIDES AND GLYCOCONJUGATES

1324 - TOP DOWN PROTEOMICS OF HUMAN ALPHA-ACID-GLYCOPEPTIDE:
FAST AND UNAMBIGUOUS GLYCAN ANALYSIS

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Keywords: Top-Down Proteomics, Glycan Analysis, negative ion mode, Alpha-Acid-Glycoprotein, ESI-MS

Introduction:
Glycosylation is an important posttranslational modification as it modulates biological functions of proteins. An altered glycosylation is found in a variety of diseases such as cancer, neuronal diseases and inflammation. Therefore, monitoring changes in glycosylation leads to a better understanding on how glycosylation influences the development and progression of these diseases and can be applied for biomarker development.

Methods:
We present a top down approach for qualitative and quantitative analysis of highly glycosylated human alpha-acid-glycoprotein (AGP). After purification of AGP from human plasma, subsequent ESI-q/TOF-MS measurement in negative ion mode revealed the broad mass distribution caused by glycosylation. To support data interpretation, AGP was also analyzed after desialylation. In-house developed bioinformatics tools were used for data interpretation.

Results:
Analyzing AGP as native glycoprotein and after treatment with neuraminidase reduced sample preparation and led to a fast and robust method for glycan analysis. We were not only able to determine the proteins glycosylation pattern but also to identify various genetic variants for AGP-1 and AGP-2. The variants did not show significant differences in their glycosylation pattern. The fully sialylated glycoprotein showed an almost complete termination with sialic acids of all antennae, carrying on average 16 to 17 sialic acid residues. To demonstrate the possible use of this method for biomarker development, we analyzed one sample from a patient diagnosed with liver cirrhosis and one diagnosed with hepatocellular carcinoma. In both cases, drastically increased fucosylation over all glycosylation sites was observed.

Conclusions:
Our method presents a way of detecting different genetic variants and determine the glycosylation pattern specific for each variant at the same time. Bioinformatics tools analyzed the complex heterogeneity in glycosylation unambiguously. Changes in glycosylation as e.g. increased fucosylation due to cancer diseases can be detected and quantified easily.

Novel Aspect:
Ionizing highly sialylated glycoproteins in negative ion mode enhances signal intensity and can be applied for quantification of glycans in use as clinical biomarker.
Deciphering the sequence of carbohydrates is a major challenge in glycosciences today. Whereas spectroscopic approaches (NMR, Raman, ...) and Mass Spectrometry (MS) offer valuable information on carbohydrate structure, they also suffer from severe technical limitations: on one hand, spectroscopy provides refined structural detail but requires a relatively large amount of purified sample, on the other hand MS applies to complex samples in small amounts, but does not disambiguate all carbohydrate isomerisms.

In this context, we have developed a unique hybrid Glycoanalytical approach, which adds a spectroscopic dimension to MS analysis. Based on this technology, it is possible to measure simultaneously the MS spectrum and the spectroscopic fingerprint of a carbohydrate. This enables the disambiguation of all kinds of carbohydrate isomerisms (monosaccharide content, regiochemistry and stereochemistry of the glycosidic bond, and positional isomers of functional modifications) without the need for chemical modification, labeling, or time-consuming purification.

While this new carbohydrate metric mitigates the lack of structural specificity of MS without the limitation of traditional spectroscopy, the identification of a carbohydrate however still relies on the availability of referenced standards. In order to enable true “de novo” analysis, we have established a top-down sequencing strategy based on the demonstration of a memory of the carbohydrate sequence within MS fragmentation products. [1] The performance of de novo carbohydrate sequencing will be illustrated on various classes of carbohydrates.

REDUCTIVE ALKALINE RELEASE OF N-GLYCANS GENERATES A VARIETY OF UNEXPECTED, USEFUL PRODUCTS

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Keywords: N-glycans, alkaline release, PGC-LC-ESI-MS

Introduction:
Release of O-glycans by reductive β-elimination has become routine in many glyco-analytical laboratories and concomitant release of N-glycans has repeatedly been observed. Revisiting this somewhat forgotten mode of N-glycan release revealed that all kinds of N-glycans including oligomannosidic and complex-type N-glycans from plants with 3-linked fucose and from mammals with or without 6-linked fucose and with sialic acid could be recovered.

Methods:
Typical conditions for the alkaline borohydride treatment of glycopeptides were varied in case of temperature and concentration of chemicals. Following PGC SPE cleanup, yield and chemical nature of the products were investigated using MALDI-TOF MS and PGC-LC-ESI-MS. Reducing glycans were labelled with 2-aminobenzamide, reducing glycosylamine and 1-amino-1-deoxy-glycitol with InstantPC™ dye. The fluorescent products were separated by HILIC-FLD-HPLC.

Results:
Even after 16 h incubation in 1 M sodium borohydride, a large part of the glycans occurred in reducing form. Moreover, about one third emerged in the form of the stable amino-functionalized 1-amino-1-deoxy-glycitol. Relative yields of the products reducing glycosylamine, reducing N-glycan, 1-amino-1-deoxy-glycitol or glycitol could be controlled by the release conditions.

Conclusions:
Chemical release of N-glycans constitutes a cost-saving alternative to enzymatic hydrolysis for the preparation of precursors for the production of reference compounds for various formats of N-glycan analysis. Moreover, it allows to obtain a stable amino-functionalized glycan derivative, which can be employed to construct glycan arrays or affinity matrices.

Novel Aspect:
While the principle observation of the release of N-glycans by alkali under reducing conditions was made decades ago, the very nature of the generated products has not been recognized so far.

References
R. Figl, F. Altmann, Proteomics 2018, 0, 1700330.

For information please contact: scientific@imsc2018.it
Analysis of mouse brain N-glycans by (PGC-)LC-ESI-MS using isotopically labeled standards

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Introduction
N-glycosylation is the most complex and heterogeneous post translational modification. It is important for protein folding, stability and biological interactions of many proteins. The large number of possible isomers yielding from one glycan composition necessitates powerful separation techniques (porous graphitic carbon LC) and the use of defined standards combined with MS/MS.

Methods
Proteins of mouse brain tissue was purified, treated with PNGase F to release the N-linked glycans and reduced with sodium borohydride. The glycans were purified and directly subjected to a PGC-LC-ESI-MS system (QTOF instrument). Prior to the measurement defined isotopically labeled N-glycan standards were added to identify and quantify the isomers occurring. The incorporation of different numbers of heavy isotopes at varying positions (13C6- and 13C1galactose, reduction with sodium borodeuteride or deutero acetyl groups ofGlcNAc) allowed the assembly of internal multiplex standards mixtures.

Results
The major glycan was found to be a bisecting, fucosylatedGnGn (GnGnF (bi) -proglycan nomenclature, www.proglycan.com). All main isoforms could be identified and relatively quantified. The ability of PGC to discriminate about 30 different isobaric mono-fucosylated N-glycans was shown (H5N4F1). Absolute quantification could be furthermore achieved by the quantitation of one C13 labelled standard by amino sugar analysis. This one internal standard allowed for the calculation of the other glycoforms by the use of instrument dependent correction factors (calculated ratios known from an equimolar standard mix [1]).

Conclusions
A powerful method for the analysis of N-glycans in complex tissue samples is presented. The use of different labeled standards allowed identification and quantification of glycan isoforms in a single MS-run.

ASSIGNMENT OF LINKAGE, ANOMERIC CONFIGURATION, AND BRANCH LOCATION OF OLIGOSACCHARIDES VIA A LOGICALLY DERIVED MSN APPROACH

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Keywords: carbohydrate, branched and linear oligosaccharides, structural determination

Introduction:
Carbohydrates (or glycan) play influential roles in cellular recognition processes and many other biological processes. However, the structural determination of oligosaccharides remains an obstacle and constitutes a bottleneck in carbohydrate research. An approach for de novo structural determination of linear and branched oligosaccharides based on logically derived procedure of tandem mass spectrometry was demonstrate.

Methods:
A commercial linear ion trap mass spectrometry (LTQ XL, Thermo Scientific) coupled with electrospray ionization was employed in this study. The selection of structural decisive fragment ions for the subsequent CID was guided using a procedure that we built from the understanding of the saccharide dissociation mechanism. The complete structural identification is obtained by combining the structural information from each step of CID.

Results:
A specially prepared disaccharide CID spectrum database consisted of 16 mannobioses was build. Low-energy collision-induced dissociation (CID) of sodium ion adducts was used to facilitate the cleavage of desired chemical bonds during dissociation. The linkages, anomeric configurations, and branch locations of oligosaccharides are determined by comparing the CID spectra of oligosaccharide with the fragmentation patterns based on dissociation mechanism and our specially prepared disaccharide CID spectrum database. The applications of this method to structural determination of mannose-oligosaccharides were demonstrated. The structure of a given trisaccharides from 88 isomers and tetrasaccharide from 928 isomers can be determined by only 3-5 properly selected CID mass spectra according to the logical procedure we derived. The results show that this method provides a rapid structural identification procedure for mannose oligosaccharides and can be extended to glucose and galactose oligosaccharides as well.

Conclusions:
We demonstrated a simple method that an oligosaccharide can be identified from hundreds isomers by only using 3-5 properly selected CID spectra. The required CID spectra for structural identification can be determined from the schemes we derived according to the dissociation mechanisms. The entire procedure for these four CID spectrum measurements only take few minutes, demonstrating this approach is a simple and rapid method for the structural determination of oligosaccharides.

Novel Aspect:
The approach can be built in a computer controlled mass spectrometer and ultimately developed into a automatic procedure for structural identification of oligosaccharides.

References

FRAGMENTATION OF OLIGOSACCHARIDES AND SUPPRESSION OF METAL-SALT INDUCED ADDUCTS USING ELECTROSPRAY IONIZATION

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Keywords: Mass spectrometry, FT-ICR, Oligosaccharides, CID, alkaline adduction

Introduction

It is known that alkaline adduct-ions influence CID and PD induced fragmentation mechanisms of oligosaccharides [1,2]. For proteins the use of ammoniumacetate to counteract cation induced signal suppression in electrospray ionization is published [3]. To increase the intensity of the non-adducted molecular ion the application of ammoniumacetate to oligosaccharide containing samples is of interest.

Methods

The experiments were performed on an APEX Qe FT-ICR mass spectrometer, with a 9.4 T superconducting magnet (Bruker Daltonik, Bremen, Germany). Maltopentaose (MP) was dissolved in MeOH/H2O and ionized using an Apollo III ESI-source. To analyse the suppression of alkali-adducts NH4Ac was added to the samples. Fragmentation of the molecular cations were achieved by SORI-CID with argon as collision gas.

Results

Several spectra of maltopentaose with different concentrations of NH4Ac could be recorded. The S/N-ratio of the non-adducted molecular ion could be increased by nearly sixty times compared to the measurement without the use of ammoniumacetate. However, no complete suppression of the adducted molecules could be achieved. The use of NH4Ac leads to an increased fragmentation - similar to CID experiments. Nevertheless, it allowed the comparison of SORI-CID indicated fragmentation behaviour of the different species. The spectra of the protonated species show no cross-ring cleavage, but loss of H2O and glucose. Compared to that the CID spectra of the Na-adducts show cross-ring cleavages, also in combination with loss of glucose. In this regard the study of the effect on chromophore labelled maltopentaose and subsequent CID/PD fragmentation would be part of interest and is indeed part of ongoing research.

Conclusions

Using ammonium acetate to reduce alkaline adduct ions of oligosaccharides was successfully applied to the example of MP, whereby MS2 experiments of the [M+H]+species could be performed. Doing this, no cross-ring cleavages are observable. Due to the symmetry of MP, an assignment of the fragments to the reducing or non-reducing end is not possible. Because of the precipitation of acetates and ammonium salts, this method requires a higher cleaning effort.

Novel Aspect

The use of ammonium acetate for the suppression of alkaline adduct-ions to improve the S/N-ratio of the [M+H]+species measuring oligosaccharides.

References

MALDI-MS ANALYSIS OF SUCROSE USING A CHARCOAL MATRIX

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Keywords: sucrose, charcoal, MALDI, glycosidic bond, cationization agent

Introduction:
Matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) analysis of low molar mass compounds using a conventional matrix, such as 2,5-dihydroxybenzoic acid (DHB) or α-cyano-4-hydroxycinnamic acid (CHCA) is not commonly performed due to matrix-related peaks [1, 2]. For the effective MALDI-MS analysis of sucrose whose average molar mass is only 342.3 Da, a charcoal matrix with different cationization agents was utilized.

Methods:
Different cationization agents such as Li+, Na+, K+, Rb+, Ag+, and Cs+ were used for the MALDI-MS analysis of sucrose with a charcoal matrix. The matrix (1 μL) was placed on a MALDI plate and dried at room temperature. Then, 1 μL of 100 mM sucrose stock solution was deposited on top of the matrix. To compare the effectiveness of cationization agents, each additive cation (1 μL) was loaded on top of the dried spots. Mass spectra were obtained in the positive ion reflection mode.

Results:
A higher cation-adducted sucrose peak with significantly reduced interference was observed with a charcoal matrix rather than DHB or CHCA. However, the charcoal matrix caused glycosidic bond cleavage, resulting in sucrose fragment peaks. These could not be removed, even when we reduced the laser intensity. The degree of sucrose fragmentation was inversely related to the size of the cation additive. More sucrose fragmentation occurred with small cationization agents (Li+, Na+, or Ag+), while little fragmentation occurred with relatively large cationization agents (K+, Rb+, or Cs+).

Conclusions
Charcoal has a higher energy transfer efficiency than DHB or CHCA. This may explain the increase in sucrose peaks and fragmented peaks observed when sucrose was analyzed with a charcoal matrix. The degree of sucrose fragmentation was inversely related to the size of the cation additive. The highest degree of sucrose fragmentation was observed when the cation additive was Li+, followed by Ag+, Na+, and K+. No fragmentation was observed with additives Rb+ and Cs+.

Novel Aspect:
Charcoal matrix was utilized for the analysis of sucrose in MALDI-MS with different cationization agents such as Li+, Na+, K+, Rb+, Ag+, and Cs+.

References
COMPETITIVE UNIVERSAL PROXY RECEPTOR ASSAY (CUPRA) FOR QUANTITATIVE HIGH-THROUGHPUT GLYCAN LIBRARY SCREENING

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Keywords: Glycan recognition; library screening; affinity; specificity; ESI-MS

Introduction:
The absence of quantitative high-throughput (Q-HT) glycan screening assays is a significant impediment in glycomics. Glycan microarrays, wherein oligosaccharides (OS) are immobilized on glass surfaces, are the dominant HT screening technology. However, glycan microarray screening has many well-documented limitations. Here, we introduce a Q-HT electrospray ionization mass spectrometry (ESI-MS)-based method for OS library screening.

Methods:
The Competitive Universal Proxy Receptor Assay (CUPRA) assay is based on competitive protein binding and employs a universal proxy protein (UniPproxy), which binds all components of a library of modified OS (OSmod) through a high affinity tag introduced to the OS. Changes in abundances of the (UniPproxy+OSmod) complexes in solution upon introduction of a glycan-binding protein (GBP) allows for the simultaneous identification and quantification of OSmod ligands.

Results:
Implementation of CUPRA was demonstrated using a library of 70 OSmod, produced from OS with structures found in humans, and GBPs with known OS binding specificities. Application of CUPRA to cholera toxin B subunit homopentamer (CTB5) correctly identified the OSmod containing the Gal\(^1\)-3GalNAc\(^1\)-4(Neu5Ac\(^2\)-3)Gal\(^1\)-4Glc (GM1) and Fuc\(^1\)-2Gal\(^1\)-3GalNAc\(^1\)-4(Neu5Ac\(^2\)-3)Gal\(^1\)-4Glc (Fuc-GM1) structures as the only high affinity ligands in the library. Moreover, the measured affinities agree, within a factor of 2, with the affinities measured for the corresponding oligosaccharides. Application to Maackiaamurensis agglutinin (MAA), which is a Neu5Ac\(^2\)-3Gal\(^1\)-4GlcNAc/Glc specific GBP, identified Neu5Ac\(^2\)-3Gal\(^1\)-4Glc (3SL) and Gal\(^1\)-4(Neu5Ac\(^2\)-3)Gal\(^1\)-4Glc (GM2) as ligands. A single ligand, containing the Neu5Ac\(^2\)-6Gal\(^1\)-4Glc structure was found for Sambucus nigra lectin (SNA). This result is consistent with the reported specificity of the GBP for Neu5Ac\(^2\)-6Gal structures.

Conclusions:
Together, these results demonstrate that CUPRA allows for the simultaneous screening and quantification of glycan ligands with affinities \(\geq 10^3 \text{ M}^{-1}\). Future efforts will focus on expanding the size and diversity of the OSmod library, with the goal of producing a library of \(\sim 200\) components derived from purified OS. Natural libraries of OSmod will also be produced from OS extracted from human milk and N-glycans released from isolated glycoproteins and tissue. The OSmod libraries will then be used for the discovery of biologically-relevant glycan interactions involving human, bacterial and viral GBPs.

Novel Aspect: Development of the first high-throughput and quantitative glycan screening assay
MULTIPLEXED ISOPOE LABELED STANDARDS FOR ISOMERIC ANALYSIS OF N-GLYCANS

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Introduction
Porous graphitic carbon (PGC) columns have proven extraordinarily useful for the LC-ESI-MS analysis of N- and O-glycans. Their shape selectivity has been demonstrated for different sialic acid and galactose linkages. The ability of PGC to discriminate about 35 different isobaric mono-fucosylated N-glycans will be shown. Application to mouse brain N-glycans revealed them to contain hybrid type glycans with fucose at the core and Le x (but not Le a or blood group H) determinants.

Methods
The notoriously poor reproducibility of retention times on PGC prompted us to generate internal standards with different mass increments due to thoughtful incorporation of one or two molecules of either 13C6- or 13C1-galactose, of BH4 or BD4 reduction and finally de-N-acetylation and re-N-acetylation using stable-isotope labeled acetic anhydride. The obtained multiplexed reference glycans can easily be told apart by their specific mass increment.

Results
Since expression of retention times as glucose units is not useful for LC on PGC, we propose to scale any chromatogram to a reference run and express the retention in the form of virtual minutes (vimin). Absolute quantification was furthermore achieved by the quantitation of one 13C6 labelled standard by amino sugar analysis. This one internal standard allowed for the calculation of the other glycoforms by the use of instrument dependent correction factors (calculated ratios known from an equimolar standard mix [1]).

Conclusions
A strategy is proposed by which a set of multiplexed standards can serve to assign the structure of any N-glycan structure already by retention time, thus providing a new level of confidence in the analysis of structural isomers that supports and expands the conclusions drawn from MS/MS spectra.

References
**Development of a New Method for the Identification of Follicle Stimulating Hormone (FSH) Glycoforms Involved Human Fertility.**

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Keywords: FSH (Follicle Stimulating Hormone), human fertility, glycosylation, mass spectrometry

**Introduction:**
Follicle-stimulating hormone (FSH) plays a key role in the proliferation and development of ovarian granulosa cells in women and spermatogenesis in men [1-3]. The glycosylation of FSH is extremely important since it has a significant influence on its biological activity. The quantitative determination of FSH at the various stages of the menstrual cycle is fundamental, for this reason, it is important to develop new methodologies that can lead to the simple and sensitive determination of FSH.

**Methods:**
During preliminary experiments, standard FSH was digested with trypsin and the resulting peptide mixtures directly analyzed by LC-MS/MS methodologies. Specific peptides were selected on the basis of their molecular ion and their most intense fragments and used as targets to set up the Multiple Reaction Monitoring (MRM) method. The set of peptides, defined by their specific MRM transitions, can only be associated to the FSH protein leading to unambiguous identification and quantitation of FSH even within a complex biological matrix like serum with unmatched specificity and sensitivity.

**Results:**
Tandem mass spectrometry analysis allows us to characterize the glycosylation profile of several FSH molecules including the pituitary form in young and postmenopausal women, and some existing commercial forms. The definition of the glycoformswas carried out using cutting-edge techniques such as ETD fragmentation using a high resolution Orbitrap mass spectrometer. An immunoaffinity chromatography protocol was performed on a serum sample to isolate selectively FSH protein and to identify it. Finally, an innovative methodology for its identification and quantification was set up by MRM mass spectrometry. For the optimization of the MRM method, more unique peptides were selected and monitored in order to uniquely identify FSH in complex matrices. The building up of a calibration curve of standard peptides has allowed to quantify FSH in human serum. The research activities are performed in collaboration with the MERCK Group, leader in FSH production.

**Conclusions:**
This study is addressed at defining the relationship between the glycosylation pattern of FSH and its biological activity and defining similarities and differences between the various forms and the standard molecule. Because of the extreme importance in determining the exact amount of FSH during the various phase of the menstrual cycle as well as in therapy, the development of a sensitive and robust method for the identification and the quantification of specific FSH glycoforms by advanced mass spectrometry methodologies was of great importance.

**Novel Aspect:**
The quantitative determination of FSH is typically measured with the immunological assays [4] that suffer from numerous limitations correlated with the presence of false positives. These limitations can be overcome by innovative mass spectrometry techniques in Multiple Reaction Monitoring mode.

**References:**
N-glycan analysis of various Chlorella strains by MALDI-TOF and LC-ESI-MS

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Keywords: Chlorella, MALDI-TOF, LC-ESI-MS, N-glycosylation

Introduction:
Microalgae of the genus Chlorella are candidates for the production of polyunsaturated fatty acids, various dietary supplements and they are also debated as novel expression systems for recombinant proteins. Our work shows that different Chlorella species display widely varying N-glycan profiles with unique masses not known from embryophytes (land plants).

Materials and Methods:
Total N-glycan spectra of a panel of Chlorella vulgaris and Chlorella sorokiniana strains as well as of a broad selection of commercial Chlorella tablets - many of them labeled with the outdated taxonomical name Chlorella pyrenoidosa - were investigated by MALDI-TOF-MS and LC-ESI-MS.

Results:
Most samples typed as C. vulgaris contained oligomannosidic glycans with several O-methyl groups, while the C. sorokiniana culture strains exhibited two differing profiles with pentose containing glycans. The commercial samples -almost irrespective of their label- yet again occurred as four distinct structural groups. One resembled C. vulgaris, two showed rather complex patterns of methylated glycans, and one contained just one very dominant structure that turned out to contain three arabinoses, one of them methylated and a galactofuranose linked to the second N-acetylglucosamine residue.

Conclusions:
Chlorella N-glycans differ greatly from plant and vertebrate N-glycans by the lack of fucosylation, the presence of arabinose and galactose residues and the prominent O-methylation of sugars. These properties foresee a large variety of unknown glycosyltransferases and potentially immunogenic glycan structures. The occurrence of groups with clearly distinct N-glycan patterns suggests that N-glycan profiles could constitute a valuable tool for taxonomic classification as well as for determination of the origin of Chlorella food supplements.
Introduction:
Saponaria officinalis is a common perennial plant from the Caryophyllaceae family. It is well-known for its detergent property and has been used as a soap in ancient times, later also as an expectorant in bronchitis and in rheumatic disorders. Decoctions and infusions of this plant can be used as an emulsifier for food and cosmetic products (masks, toothpastes, shampoos). However, the fact that its properties depend on the organ and time of harvest is often overlooked.

Methods:
Saponins were tracked by LC-MS system using semi-targeted metabolomic approach. Changes in the metabolome compositions were registered by following 400 transitions in negative ion mode by 6460 ESI MS/MS system coupled to RPLC. For each parent ion (selected from the plant's analysis in the scanning mode, range 350 – 1700 m/z) at least two product ions were selected to track changes of metabolomics profiles for different organs and harvest time.

Results:
Different triterpenoid saponins, saponariosides A-M named and classified by Kazuo Koike and Zhonghua Jia [1], were detected in extracts from the whole plants of Saponaria officinalis. Throughout the ESI-MS profiling of RPLC eluate, it was recognized that lower molecular weight saponins of S. officinalis were present in the very polar fractions eluted faster. Very high diversity of the structures was observed as different sugars were found to be attached to aglycone, such as pentose, hexose, and deoxy-sugars. This was indicated as the difference between m/z values for signals such as 132, 162 and 146 for deoxyhexosyl moieties. Cluster analysis of the collected peak areas for individual saponins were visualized as a dendrogram and heat map in respect to time of harvest. We found that flowering and formation of seeds influences significantly the relative amounts of different saponariosides. The observed changes in composition show good correlation with parameters illustrating the ability of extracts to form stable and dense foam.

Conclusions
The HPLC–MS/MS analysis allowed us to detect and identify saponins, and perform their semi-quantification. Developed LC-MS/MS platform allowed to track changes in saponin’s composition for different organs obtained for S. officinalis harvested in the period from June to October.

Novel Aspect:
For the first time, the stage of the development and organ-specificity of the crop were included in studies of S. officinalis.

References

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HILIC MS-HCD/FT-IT AND HYBRID SEARCH ENHANCE THE STRUCTURE ELUCIDATION OF OLIGOSACCHARIDES IN NIST HUMAN MILK REFERENCE MATERIAL

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HILIC MS-HCD/FT-IT and Hybrid Search enhance the structure elucidation of oligosaccharides in NIST human milk reference material

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Keywords: Human Milk Oligosaccharides, Fourier Transform-ITMS, HILIC-MS-HCD, Isomers, MS library

Introduction:
Elucidation of complex mixture of underivatized isomeric glycans is challenging when commercial standards are not available. The NIST SRM* 1953 consist of human milk oligosaccharides (HMO) from 100 healthy breastfeeding mothers in the US was used. However, the information on these functional molecules in the reference material is limited. This study focuses on the development of MS database of annotated spectra of oligosaccharides in human milk.

Methods:
The human milk SRM 1953 underwent sample preparation [1]. The purified oligosaccharides were analyzed on Hydrophilic Liquid Interaction Chromatography-electrospray ionizationin Fusion Lumos Orbitrap-based MS using HCD&Fourier transform ion trap (FT-IT)fragmentation techniques. The spectra are extracted from LC-MS raw data and processed using in-house algorithm then analyzed using different tools including NIST Tandem mass spectral library and hybrid search.

Results:
The high mass accuracy both for precursor and product ions from FT-IT and HCD data was sufficient to enable the assignment of HMOs including novel oligosaccharide structures with different adduct types in negative/positive detection mode. Core group (Gal-Glc) with different terminal sugars, Fuc-Gal-Glc eluted prior to Sia-Gal-Glc based on the HILIC chromatogram. The elution profile of oligosaccharides (>4 sugar units) with 3-4 isomers, such as fucocolacto-N-octaoses and sialylated fucocolacto-N-hexaoses displayed different retention times on HILIC that enhances oligosaccharide identification. A clear distinction in the HILIC elution pattern of isomeric oligosaccharides related to size and polarity also confirmed the identifications. Moreover, the hybrid search can reliably find modified oligosaccharides in comparison to non-reduced analogs, and vice versa, enabling an increase in match factors.

Conclusions
The HILIC MS/MS and NIST hybrid search facilitates the structural assignment of 70 complex oligosaccharides of 470 spectra including newly identified glycan isomeric structures. The MS library of HMOs demonstrated to aid in identifying glycans in other biological samples, which represents a new variety of mass spectral library – a concept that can be extended to other varieties of biological fluids such as detection of glycans and non-digestible artificial sugars in human & bovine milk samples.

Novel Aspect:
A comprehensive structural and tandem MS library of annotated spectra of oligosaccharides derived from the NIST human milk reference material: a robust method of detecting other compounds in milk products.

Reference
* SRM (Standard Reference Material) 1953 (human milk); SRM 1849 Infant formula
Introduction:
Glycosylation changes of serum glycoproteins associated with liver disease progression were described previously. Most of the studies were profiling detached glycans from crude human sera or isolated proteins from patient sera. Recent studies described more detailed site specific glycosylation analysis of isolated glycoproteins or site specific analysis of particular glycan modifications such as core-fucosylation after glycosidase treatment. Our study is focused on the site specific analysis of incompletely processed glycan structures as oligomannose and hybrid glycans in liver tissue associated with liver disease progression (cirrhosis, HCC). Lectin affinity enrichment combined with HR mass spectrometry analysis allowed us to identify large set of high mannose, hybrid and paucimannoseglycopeptides in complex liver tissue glycoproteome.

Methods:
Human Liver tissue biopsies divided to tumor and adjacent portions were analyzed as whole tissue lysate and lectin enriched glycopeptides. Tryptic digestion including reduction and alkylation steps was followed by ConA lectin enrichment of oligomannose and hybrid glycopeptides. Whole liver proteome and enriched glycopeptides were analyzed by HR LC-MS/MS using Orbitrap Fusion Lumos mass spectrometer working in DDA mode coupled with 3000RSLC nano-chromatography (Dionex). Peptides and glycopeptides were separated on the 50cm C18 capillary column using 90 min acetonitrile gradient. Data were processed using Proteome discoverer (Thermo), Byonic and GPS software. All glycopeptide spectra were confirmed manually.

Results:
Complex site specific glycosylation analysis as one of the most difficult analytical approach was never completely solved because of glycopeptide mass spectrometric fragmentation similarity. Using combination of selective structure enrichment, HR mass spectrometric analysis, chromatography reproducibility we were able to identify large set of high mannose and hybrid glycopeptides (2429) in human liver tissue. Byonic, GPS software and retention behavior prediction help us to identify additional high mannose and hybrid glycopeptides which were masked in crude liver tissue analysis by oligomannosylated peptides from abundant complex type glycopeptides. Large portion of identified ConA enriched fraction were trimmed paucimannoseglycostructures (734) related to increased lysosomal activity. Some of the paucimannoseglycostructures of lysosomal glyco-proteins for example prosapposin, TPP, Cathepsins or LAMP are elevated in liver HCC.

Conclusions
We have identified and quantified 2429 high mannose and hybrid glycopeptides in 6 human liver biopsies samples divided to tumor and adjacent tissue. We have detected elevation of proteins and glycoforms in tumor related to lysosomal activity.

Novel Aspect:
High mannose and hybrid type glycoproteomics related to liver disease.
Keywords: structural analysis, IRMPD spectroscopy, hyphenated MS-based approach, carbohydrate sequencing

Introduction:
Sequencing techniques have been established for proteins and DNA and have revolutionized modern biology but routine techniques do not exist for glycans. In particular, top down analysis of glycans by mass spectrometry is often ambiguous due to the presence of various isomerisms. This lack of structural tools is identified as a crucial bottleneck, limiting the full development of glycosciences.

Methods:
Our instrument combines Mass Spectrometry and Vibrational Spectroscopy (InfraRed Multiple Photon Dissociation) in the 3 µm spectral range. It offers the structural resolution of spectroscopic techniques for mass-selected ions and operates at typical MS conditions, i.e. it requires significantly less sample than traditional spectroscopy.

Results:
First, we have shown that the IR signature in the 3 µm range obtained with our instrument is a powerful metric to resolve simultaneously all isomerisms: the nature of the monosaccharide content, the regiochemistry of the glycosidic bond, the anomer stereochemistry (α or β), the structure of the monomeric ring (pyranose or furanose), and the position of modifications such as sulfation or acetylation. Then the conservation of the molecular structure of a precursor ion within MS fragments has been revealed on disaccharides, opening the way to top-down, de novo MS analysis.[1] Following this demonstration, we have established a set of carbohydrate sequencing rules using a combination of Mass Spectrometry and IRMPD Spectroscopy. Finally, we have applied our approach to the determination of the sequence of various classes of saccharides.[2]

Conclusions
Without chemical derivatization of the glycan and with a minimal database of monosaccharide standards, the monosaccharide content and the stereochemistry of any oligosaccharide can be retrieved using our hybrid analytical approach based on MS-sequencing strategies and the structural resolution of carbohydrate isomerisms by IRMPD spectroscopy.

Novel Aspect:
Our approach is to combine the best of two worlds: Mass Spectrometry and Spectroscopy, for the identification of glycan isomers.

References
Keywords: Behcet disease, N-Glycan, Isomer, Quantitation, MRM-MS

Introduction:
Glycosylation is widely recognized as the powerful resource of biomarkers for cancers and infectious diseases. Behcet disease (BD), a typical immune disease, is a vasculitis disorder characterized by oral sores and arthritis. Until now several diagnostic criteria of BD have been proposed however, there is no reliable diagnosis/treatment method. Here, we developed glycomic-based novel approach for BD monitoring using PGC-MRM MS.

Methods:
Sera of Behcet patients (n=47) and health controls (n=47) were obtained from Samsung Medical Center in Korea. Each sample was enzymatically treated with PNGase F to release N-glycans. Native N-glycans were enriched by PGC-SPE prior to MS analysis. Targeted acidic glycans were chromatographically separated to obtain isomer-specific information and each glycan was relatively quantified by PGC-MRM MS. Total eleven N-glycan isomers were monitored.

Results:
Glycans released from human serum were initially profiled by nanoLC/MS and about 100 glycan compositions were identified. Of them, sialylated glycans which are highly present in immunoglobulin-related proteins and in human serum were further selected for quantitative analysis. Total eleven N-glycan isomers consisting of three major compositions were successfully separated on a PGC column and quantitated using MRM-MS. In particular, four glycans including mono/di-sialylated bi-antennary N-glycans (Hex5 HexNAc4 NeuAc1-2) were highly expressed in BD patients compared with control group. These glycan isomers showed high diagnostic efficacy having AUC of ROC curve over 0.98. As well as disease-specific glycans, we found the glycan expression level of female control groups was ten times higher than that of male.

Conclusions:
While global profiling of serum N-glycome has already revealed potential biomarkers for various diseases, glycan quantitation is analytically challenging due to the complexity caused by glycan isomers with a wide dynamic range of intensity. In this study we developed new technology to quantify isomer-specific glycans using PGC based MRM-MS for Behcet disease monitoring.

Novel Aspect:
The first study of isomer-specific glycan quantitation using MRM MS for Behcet Disease Monitoring

References
Introduction
Cancer research is one of the most studied areas of science and prostate cancer (PCa) is one of the most common
types of cancer among men. Tissue samples, especially biopsies are often used in mass spectrometry based
biomarker research and have a great potential in understanding biochemical mechanisms underlying diseases such
as cancer. Tissue microarrays (TMA) consist of several biopsies (generally 1.5 mm in diameter) of different patients
fixed on a microscope slide.

Methods
Glycosaminoglycan (GAG) chains were digested into smaller fragments (GAG disaccharides) on the tissue surface
with bacterial lyase enzymes prior to the nanoLC-MS analysis. The development and optimization of a
chromatographic and MS method for the investigation of the GAG disaccharides was performed involving the use of
novel self-packed HILIC-WAX capillary columns. For the statistical evaluation of datasets T-test and principal
component analysis were used.

Results
The aim of our work was to develop and apply advanced nanoLC-MS techniques to reliably identify GAGs from the
surfaces of PCa TMAs.
As for the separation method, effects of the ionic strength, the pH and the eluent strength were investigated and an
isocratic separation method was found to be suitable for separating the GAG disaccharides. The limit of detection
was ca. 1 fmol for each disaccharide and the limit of quantitation was between 10-50 fmol.
The nanoLC system was on-line coupled to mass spectrometer operated in negative mode and several TMA cores of
different pathological grades of PCa biopsies were analyzed.
Statistically significant changes in heparan-sulphate composition were observed. The ratio of the different GAG
disaccharides was found to be a potentially useful indicator of prostate cancer progression. A good correlation
occurs between the cancer grade and sulphation patterns of the heparan-sulphate chains; the ratio of doubly and
triply sulfated disaccharides increases with cancer progression.

Conclusions
GAGs play critical roles in cancer progression and our new isocratic HILIC-WAX-nanoHPLC-MS method provides
extremely high sensitivity and reproducibility thus enabling the determination of the sulfation pattern from single
TMA cores (<10 µg tissue sample). The ratio of the different GAG disaccharides correlates with PCa progression; the
amount of doubly and triply sulfated disaccharides shows an increase with cancer grade.

Novel Aspect
Our novel HILIC-WAX nanoHPLC-MS method enables determination of the GAG content of single PCa TMAs and sulfation pattern of these TMA cores may be a useful indicator of PCa grade.

Acknowledgement
Gábor Tóth appreciates the financial support of the Mass Spectrometry Division of the Italian Chemical Society providing the IMSC 2018 participation fellowship.
Thin-layer chromatography coupled with nano-matrix based MALDI-TOF mass spectrometry for structural elucidation of glycans

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Keywords (5 words): Carbohydrate, Thin-layer chromatography, nanoparticle, MALDI-MS

Introduction:

Our recent study demonstrated that 2,5-dihydroxybenzoic acid conjugated Fe3O4 nano-matrix (DHB@MNPs) was powerful for simultaneous ionization and fragmentation of oligosaccharides by MALDI MS [1]. However, analysis of more complex mixtures of carbohydrate requires further separation. In this study, we aimed to directly couple thin layer chromatography (TLC) separation of carbohydrate with DHB@MNPs based MALDI MS detection as a novel method of analysis.

Methods:

Our new approach incorporates several stages of development. First, saccharides mixture separation was carried out on C18 modified reverse phase (RP) TLC plate. Second, we used ionic liquids as nanoparticle dispersion media. Third, dispersed DHB@MNPs was then deposited on TLC surface by spin coating technique. Fourth, the separated carbohydrates spots were directly scanned by MALDI TOF MS. Finally, the method was applied to human milk oligosaccharide analysis.

Results:

Compared to normal phase (NP) TLC, carbohydrate samples were found to be better separated on RP-TLC and yields higher fragment ion intensity which may be due to weak retention of polar carbohydrate on non-polar RP-TLC. Spot homogeneity of spin coated and ionic liquid dispersed DHB@MNPs were evaluated by acquiring spectrum from different distance from the origin. Coefficient of variation (CV) of molecular and fragment ions intensity of each separated spot fall in acceptable range (CV < 15%). Besides the molecular ions of D-Glucose and β-Lactose, several fragment ions of Lewis Y were able to be detected by direct MALDI MS scanning which enabled unambiguous structural elucidation with single MS. The isomeric pairs of Lewis B and Y were also discriminated by DHB@MNPs-induced unique fragment ions [Z1α (372.3) and Y1α (390.4) unique for Lewis B; 2,4A3/Y2α (391.5), 0,3A3α (421.1) and Z2α (534.5) unique for Lewis Y]. Using our optimized method more than 32 neutral human milk oligosaccharides were identified from a donor at after one month lactation time.

Conclusions:(400 characters)

With our integrated platform we were able to demonstrate separation and detection of a model saccharide mixtures. DHB@MNPs matrix was dispersed well by ionic liquid and uniformly deposited on TLC surface by spin coater. Automatic scanning allowing more accurate, faster and reproducible data acquisition. Overall, our approach
provides alternative efficient carbohydrate analysis without the need of the common prior chemical derivatization and tandem MS.

Novel Aspect:

RP-TLC separation and spin coating of ionic liquid dispersed DHB@MNPsfollowed by MALDI MS scanning as a facile method for structural elucidation of carbohydrate mixture.

References

APPLICATION OF A NOVEL FUNCTIONALIZED RESIN WITH PHENYLBORONIC ACID FOR CAPTURING OF GLYCATED AND GLYCOSYLATED PEPTIDES AND THEIR MS IDENTIFICATION.

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Keywords: glycopeptides, glycosylation, capturing, PTMs, LC-MS

Introduction:
Proteins post-translational modifications, eg. glycation and glycosylation are investigated as specific biomarkers for diagnosis. Limitations of standard bottom up approach are connected with peptides low relative abundance, low ionization efficiency and sample complexity. The further isolation and concentration methods are required, which can be achieved by phenylboronic acid affinity materials.

Methods:
The ChemMatrix® Rink Resin functionalization with 4-carboxyphenylboronic acid moiety was performed according to the solid phase peptide synthesis procedure. The glycoconjugates were captured by incubation with the functionalized resin. Capturing and liberation progress were monitored both by the mass spectrometric methods: LC-MS, FT-ICR-MS, MS/MS (all with ESI ion source) and UV spectroscopy.

Results:
Solid supports were efficiently functionalized with various spacers and 4-carboxyphenylboronic acid. The linker was characterized by LC-MS and UV and the substitution level of the resin was determined. The resin is highly stable to various reaction conditions as confirmed by LC-MS results. Synthetic glycoconjugates were synthesized using sugar-modified building blocks. The labeling of glycopeptides by dabcyl moiety allowed the monitoring of capturing by UV. The glycated peptides were captured efficiently by phenylboronic acid solid support (70%). The capturing progress depends on glycoconjugates concentrations as monitored by UV. The captured products were identified by LC-MS, FT-ICR-MS and MS/MS.

Conclusions:
We developed and tested the new method of glycopeptides enrichment. The affinity of glycopeptide to resin functionalized with 4-carboxyphenylboronic acid depends on the structure of saccharide moiety. LC-MS allows identification and quantification of captured of specific glycoconjugates. This method has potential applications in biomarkers identification in medical diagnosis.

Novel Aspect:
The application of ChemMatrix® resin allows convenient and fast preparation of supports with various structures, properties and specificity for glycoconjugates capturing.

References:
Noortje de Haan (1) - Karli Reiding (1) - Stefanie Holst (1) - Bram Heijs (1) - David Falck (1) - Guinevere Kammeijer (1) - Viktoria Dotz (1) - Manfred Wuhrer (1)

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Keywords: sialic acid, ethyl esterification, dimethylamidation, lactonization, isomer differentiation

Introduction:
Glycosylation is a highly complex post-translational modification that plays a key role in the organization of multicellular life. Many human proteins are glycosylated, and changes in their glycosylation occur with many disease, a phenomenon which is still poorly understood. We here present mass spectrometric methods for analyzing glycosylation at higher throughput with differentiation of biologically important sialic acid linkage isomers.

Methods:
Glycans were enzymatically released from proteins and subjected to sialic acid derivatization in a linkage-specific manner introducing a mass difference for linkage differentiation. The approach was hyphenated with MALDI-MS [1], and imaging MS [2]. Furthermore, the method was combined with capillary electrophoresis(CE) – sheathlessnanoESI-MS after reducing-end labeling with a positively-charged tag, achieving glycan analysis at ultrahigh sensitivity.

Results:
High-throughput glycan analysis after linkage-specific sialic acid derivatization was applied to human serum and plasma samples of thousands of individuals to define disease-associated glycosylation signatures of major human diseases including inflammatory bowel disease, colorectal cancer, rheumatoid arthritis, and diabetes type 2. In addition, glycosylation signatures of age, sex, pregnancy, obesity and inflammation were determined. The derivatization method was transferred to tissue sections in order to allow glycan mass spectrometry imaging with a focus on sialic acid linkage differentiation providing valuable insights into the glycosylation heterogeneity of various types of tumors. The method was further expanded to a CE-MS variant allowed the analysis of the human plasma N-glycome with unprecedented analytical depth which proved particularly valuable for analyzing scarce biological samples that are available in very restricted amounts.

Conclusions:
Glycan isomers differing in sialic acid linkages can be readily differentiated using the tailored derivatization toolbox in combination with MS detection. The method revealed glycosylation signatures of major human diseases and provided insights into tumor heterogeneity with respect to glycosylation.

Novel Aspect:
A high-specificity derivatization step in the glycan MS workflow allows the rapid differentiation of sialic acid linkage isomers providing novel biomedical insights.

References:
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Keywords: chip nanoESI MS; ion mobility separation mass spectrometry; human adult hippocampus; gangliosides; collision-induced dissociation.

Introduction:
The central nervous system possesses the highest content of gangliosides (GGs) [1]. Considered as biomarkers of brain development, aging, and certain diseases, the gangliosides expressed in an adult hippocampus (AH) specimen, the first brain region affected in neurodegeneration [2], were here systematically investigated in a combined approach based on chip nanoelectrospray ionization (ESI) QTOF MS and ESI ion mobility separation (IMS) MS.

Methods:
GG solution of 5 pmol/µL concentration in methanol was investigated in negative ion mode using an orthogonal hybrid QTOF MS coupled to a fully automated chip-based nanoESI robot and in parallel by IMS using a Synapt G2s. A 2 kV ESI voltage was used for both experiments, while the sample flow rates were 50nL/min for QTOF and 2µL/min for IMS. For the IMS experiments, IMS wave velocity was set at 650 m/s, IMS wave height at 40 V. CID MSn were performed at energies within 10-50 eV.

Results:
The generated data clearly showed similarities of ganglioside pattern. However, significantly lower extent of in-source-induced fragmentation of GGs using chip-based ESI versus the conventional ESI-based ion source was observed. This attribute represented the major advantage considering the content in polysialylated species and complex structures modified by fucosylation or acetylation, respectively, which are prone to in-source detachment. On the other hand, ESI IMS MS separated the components based on their differential mobility through a buffer gas, isolating the GGs based on the carbohydrate chain length and the degree of sialylation. Altogether, the optimized conditions for both experiments led to the identification of over 100 distinct ganglioside structures in human adult hippocampus, some of them modified by fucosylation, acetylation and N-acetyl galactosamine attachment. Furthermore, by applying CID MSn, novel GG species, not identified before in AH, were structurally investigated in details.

Conclusions:
In this work, we report on a systematic and comparative assay based on two high-performance MS platforms for the determination of GG structural diversity in a native mixture purified from normal adult human hippocampus. The results demonstrate that the combination of chip-based nanoESI QTOF MS and ESI IMS MS and MSn data is proficient to unequivocally detect and characterize complex glycolipids with potential biomarker role and discover new structures.

Novel Aspect:
The two-step approach based on chip- nanoESI QTOF MS and ESI IMS MS provided novel insights into the pattern and structure of GGs in adult hippocampus.

References
A.11 LIFE SCIENCES - MS TOOLS IN DOPING & TOXICOLOGY

1121 - COUPLING MASS SPECTROMETRY AND CHEMOMETRICS FOR STRUCTURAL CHARACTERIZATION OF ANDROGENIC ANABOLIC STEROIDS

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Keywords: Anabolic Androgenic Steroids, Doping Control, Mass Spectrometry, Multivariate Analysis, Predictive Models

Introduction
The Androgenic Anabolic Steroids (AAS) are one of the main and most used classes of doping substances, included in the S1.I section of the prohibited list of the World Anti-Doping Agency (WADA)[1]. An increasingly widespread phenomenon is that of designer steroids, molecules designed and synthesized by modifying the structure of known AASs, in the aim of maintaining the same biopharmacological activity but resulting undetectable at the anti-doping test.

Methods
Predictive models were constructed based on the MS characterization of 133 AAS compounds, to identify new steroids and obtain information on their molecular structure. Starting from Principal Components Analysis different chemometric methods were applied, such as classification and clustering methods, outlining a spectral and structural characterization for each subclass of AAS.

Results
Mass spectrometric data obtained by GC-qTOF experiments performed on the TMS-derivatives allowed to group the AAS in 5 subclasses. PCA showed the high similarity between the class of 4-ene-3-keto steroids and the steroids substituted on the A ring.

A discriminant classification step by PLS-DA was performed to build a predictive model to separate the subclasses and to identify the most significant variables, like the unsaturation in position 17 on D ring, characteristic of the 3-hydroxy steroids category.

The use of a modeling classification method, which operates by building individual models for the different categories, allowed to define the characteristics of uniqueness of the investigated steroid subclasses. This approach resulted particularly effective to identify the salient features of the class of 5-ene steroids.

Conclusions
All the information has been summarized by identifying class specific common fragments and spectral trends.
The results of this study suggest that it is possible to trace, as quickly as possible and with the highest degree of certainty, any novel doping substances – and especially “designer AAS” – illicitly synthesized in such a way as to be invisible to the current tests.

The newly developed methods to reduce the time for the identification of unknown substances by fitting/unfitting them in one of the specific subclasses.

Novel Aspect
The predictive nature of the chemometric models was based on the characterization of 133 AAS, and considering the contribution of more than 30 variables.

References
1119 - DEVELOPMENT AND VALIDATION OF MULTI-TARGET ANALYTICAL PROCEDURES FOR THE IDENTIFICATION OF POTENTIAL DOPING AGENTS IN ‘OVER THE COUNTER’ PRODUCTS

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Development and validation of multi-target analytical procedures for the identification of potential doping agents in ‘over the counter’ products

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Keywords: Multi-target procedures, Doping analysis, Nutritional supplements, Designer drugs

Introduction
The regular use of nutritional supplements by athletes is a very common practice [1]. Contamination and/or adulteration of these products has been claimed to be the cause of numerous adverse analytical findings [2]. Different research groups confirmed the unlabeled presence of anabolics and stimulants in various products [3-4]. For the above reasons it becomes necessary to develop methods for the detection of the widest variety of substances included in the WADA list [5].

Methods
Both liquid and gas chromatography coupled to mass spectrometry were utilized. Different pre-treatment techniques including liquid-liquid extraction and solid phase extraction were evaluated. Validation was evaluated through specificity, sensitivity, repeatability, and recovery.

Results
Two different analytical protocols were developed: the former was specifically designed for the analysis of anabolic substances extracted from nutritional supplements by liquid–liquid extraction with n-pentane, and the latter for the remaining compounds included in the Prohibited List by solid phase extraction with weak cationic exchange columns. The procedures were validated and presented high specificity (no interferences at the retention time of all analytes and no carryover), sensitivity (all compounds could be detected at or below 20 ng/g), repeatability (variability of the relative retention times lower than 0.1% and of relative abundances of selected ion transitions lower than 15% for all the analytes considered in the study) and good recoveries (over 60% for all analytes). The efficacy of the multi-analyte procedures here developed was tested by analysing different products. Results revealed the presence of testosterone in a dietary supplement, while higenamine, a β2-agonist, was found in an herbal massage cream.

Conclusions
Two different multi-target analytical methods (the former based on GC-MS and the latter on LC-MS) were designed, developed, validated and successfully applied. Both procedures allow to detect the presence of doping compounds in different products, confirming that both the newly proposed protocols match the requirements of routine analytical methods for the detection of trace amounts of prohibited substances in products available “over the counter”.

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Novel Aspect
Two complementary newly developed methods allow the detection of banned substances in both nutritional supplements and in “over the counter pharmaceutical” formulations.

References
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Keywords: Anabolic androgenic steroids, Doping control, Isotopic Ratio Mass Spectrometry, Programmed Temperature Vaporizer Injector

Introduction
The confirmation of the synthetic origin of endogenous anabolic androgenic steroids is performed in all doping laboratories accredited by WADA through a GC-c-IRMS analysis, due to the different carbon isotope abundance, 13C/12C (δ‰), between endogenous steroids and synthetic ones. The high sensitivity requirements of current method and the limited volume of urine disposable for the analysis, led us to improve the injection conditions using the PTV technique [1].

Methods
Blank urine samples were spiked with boldenone and 19-norandrosterone standards and cleaned up by two sequential HPLC steps. The purified samples were injected in a GC-c-IRMS instrument, replacing the splitless injection mode with the use of a programmed temperature vaporizing injection (PTV). The initial sample volumes of urine was fixed at 6 mL, whereas different final injection volumes (2 to 10 µL) were evaluated.

Results
At present, the anti-doping laboratories must activate the confirmation analysis for boldenone and 19-norandrosterone by GC-c-IRMS whenever their concentration is greater than the limits defined in reference Technical Documents (TD). In details the “TD2016IRMS” [2] and the “TD2017NA” [3] impose the GC-c-IRMS confirmation for samples with a concentration in the range 5-30 ng/mL for boldenone and 2-15 ng/mL for 19-norandrosterone. In the ordinary method, 21 mL of urine are used, leading to a final injected volume, in splitless mode, of 2-3 µL [4][5]. These conditions are necessary to obtain adequate signals within the linear range of the instrument (400 to 7000 mV) considering the low LOD of boldenone and 19-norandrosterone (4 and 2 ng/mL, respectively). We investigated the possibility to increase the injection volumes, reducing progressively the initial urine volumes. We noticed that the compounds response signals improved proportionally to the larger sample volumes injected, without any significant increase of the background noise.
Conclusions
The results proved that the possibility to adjust the injection volumes, choosing injection volumes larger than those traditionally used in the splitless mode, allows to reduce and standardize the initial urine sample volumes, making faster and less laborious the preliminary sample preparation steps. The reduced urine volume also ensures a reduced risk of column overloading and a decrease the matrix effect in the instrumental analysis.

Novel Aspect
The PTV technique could be extended to detect the exogenous origin of other compounds with a very low concentration.

References
WADA Technical Document – TD2016IRMS Detection of Synthetic Forms of Endogenous Anabolic Androgenic Steroids by GC/C/IRMS.
936 - IS GC-MS ANALYSIS SUFFICIENT FOR IDENTIFICATION OF ILLICIT DRUGS FROM FESTIVE GATHERINGS?

Sophie Magréault (1) - Christel Grondin (2) - Catherine Lamoureux (3) - Emmanuel Bourgogne (4)


Keywords:
GC-MS; clinical toxicology; illicit drug; Festive drugs

Introduction:
Poisoning by recreational use of psychoactive substances is increasingly common in Europe and is responsible for death. Intoxicology, identification needs different extractions procedures and derivatization before GC-MS analysis. Developing systematic strategies for screening drug compounds in tablets, powders, herbs or liquids remains a challenge. Through different French festive events, samples were collected with the main objective to discuss advantages and limitations of such systematic analytical procedures using conventional GC-MS.

Methods:
For solids, analytes are dissolved in EtOH. For liquids, analytes are dissolved in water and MeOH, pH was measured and acidic and basic liquid-liquid extractions were performed. After evaporation, samples were reconstituted in ethanol before injection onto a GC-MS operated in scan mode. Derivatization (acetylation, silylation, propylchloroformate) are performed and each spectra compared to different commercial (MPW, SWGDrugs, NIST) or in-house libraries.

Results:
Between July and October 2016, 81 recreational substances were collected during 10 interventions in festive gatherings. Using a systematic workflow, drugs were found in every sample, whatever the matrix (solid, liquid, resin, oil) except in blotters where LSD was expected. Drugs found were mainly “classic drugs” such as cocaine, amphetamines, or cannabis. Different derivatization is mandatory for complete identification of drugs enabling increasing sensitivity for low concentration diluents, cutting agents, adulterants and other psychoactive substances. Propylchloroformatederivatization allows covalently binding on Nitrogen heteroatom, whereas acetylation or silylation bind on oxygen heteroatom. As an example, in “Cocaine” samples, levamisole, phenacetin, caffeine, diltiazem, hydroxyzine were detected. Three new psychoactive substances (NPS) were also found: ethylphenidate, dibutylone (bk-DMBDB) and thiopropamine (tieno-amphetamine) highlighting the need for up-to-date and different mass spectra libraries.

Conclusions
The applied decision tree allowed finding all except one drugs in the samples, whatever the matrices. Drawbacks are the hard laboratory work, choosing the dilution identifying major analytes but also low concentrated cutting agents; different derivatization for exhaustive identification. Up-to-date different mass spectra libraries are also mandatory for NPS drug discovery. Without the latter, skilled technicians are required. GC-MS is not devoid of limitation. A designer drug (1p-LSD) present in blotting paper could not be identified and accurate mass analysis solved this issue. Ambient pressure ionization coupled to accurate mass is definitely the present/future for direct sample analysis. Nevertheless, conventional and systematic approaches efficacy should not be underestimated.

Novel Aspect:
Application of a systematic decisional tree for identification of illicit drugs in solid/liquid/oil/plants samples by GC-MS in clinical toxicology
Introduction:
The aim of the present study was to investigate the phase I and phase II metabolism of 2-methiopropamine, a structural analogue of methamphetamine[1-3], after acute administration in mice, in order to select the most appropriate marker(s) of intake, also defining the excretion windows in urine. In vitro metabolism studies were carried out in order to enzymatically synthesize the metabolites of MPA and to set up the most appropriate sample pre-treatment procedures for LC-QqQ analysis.

Methods:
A dose of 10 mg/kg was selected for the in vivo metabolism studies of MPA and urine samples were collected every 3 hours in the range of 0-9 hours after the injection. The in vitro studies were carried out with HLM (human liver microsomes). Samples from both studies were analysed, after extraction with tert-butyl-methyl ether at pH 9, using an Agilent 1200 HPLC equipped with a SUPELCO C18 column coupled to an API4000 triple quadrupole (Sciex) with ESI source, operating in positive-ion mode.

Results:
To reproduce the in vivo metabolism, the in vitro metabolism protocol was optimized considering different conditions: substrate concentration 20 µM, proteins concentration 0.5 mg/mL, phosphate buffer 0.1 M at pH 7.4, and an incubation time of 4 h at 37°C. Three main metabolites were identified: Nor-MPA, Hydroxy-MPA, and Nor-hydroxy-MPA. To optimize sample pre-treatment different extraction solvents (tert-butylmethyl ether, diethyl ether and ethyl acetate) and pH values (7, 9 and 12) were evaluated. The best recoveries (higher than 70%) for the principal metabolites were obtained at pH 9. In vivo metabolism studies showed the formation of three principal phase I metabolites identified as: Nor-MPA, Hydroxy-MPA, and Oxo-MPA. 2-MPA and its metabolites show a maximum of excretion in the first 3 h from administration to mice showing an increasing conversion into phase II metabolites both glucorono and sulfo-conjugates for MPA, Nor-MPA, Hydroxy-MPA; Nor-Hydroxy-MPA was detected only as a phase II metabolite.

Conclusions:
The excretion profile of 2-MPA after acute administration was evaluated identifying its principal metabolites and their excretion windows in mice. MPA and Nor-MPA are the best markers of intake in the first 0-24 hours after the administration of MPA. The present method was qualitative validated for MPA and Nor-MPA according to WADA technical document[4-5] defining: recovery, LOD, selectivity and repeatability.
Novel Aspect:

There are few studies about MPA metabolism [3]. This is the first study identifying MPA excretion profile including phase I and phase II, also selecting the best markers of intake.

References

Keywords: barbiturates, validation, UPLC-MS/MS, forensic toxicology

Introduction:
Barbiturates are a group of medicinal substances with a wide spectrum of central nervous system inhibitory effects, from mild sedation to total anesthesia [1]. They have a similar chemical structure to each other. Here, we present rapid, accurate and sensitive UPLC-MS/MS methods for the simultaneous quantitation of five barbiturates (butobarbital, butalbital, pentobarbital, amobarbital and secobarbital) in human urine.

Methods:
Compounds were extracted from human urine samples using the LLE. The sample analysis was performed using Thermo Ultimate 3000 UPLC coupled with TSQ Vantage triple quadrupole mass spectrometer. Chromatographic separation was performed on an Acquity UPLC BEH C18 (2.1 mm × 100 mm, 1.7 μm) column coupled with Acquity UPLC BEH C18 (2.1 mm × 50 mm, 1.7 μm) guard-column. The 0.1 % formic acid in water and acetonitrile were used as A and B mobile phase, respectively.

Results:
The main difficulty behind this study is to develop a method, allowing to distinguish structural isomers pentobarbital and amobarbital. The appropriate compounds separation was achieved by linear gradient from 27 % to 95 % for 8.5 min in 0.35 ml/min at 60°C. The method was fully validated for human urine in the range of 50 – 1000 ng/ml. Correlation coefficients (r2) for five various barbiturates were > 0.99. Recovery, repeatability, and within-laboratory reproducibility lie in the range of 89 – 97%, 3 – 5%, and 7 – 8%, respectively.

Conclusions:
A rapid and robust UPLC-MS/MS method has been developed and validated for the simultaneous determination of butobarbital, butalbital, pentobarbital, amobarbital and secobarbital in human urine. We proposed the LC conditions for the pentobarbital and amobarbital separation which allows to differ these compounds. The method are suitable for quantitation of barbiturates not only in toxicology, but in pharmacokinetic studies and clinical trails.

Novel Aspect:
High chromatographic separation power for the specificity of five various barbiturates by UPLC-MS/MS in human urine was achieved in the field of forensic toxicology.

References
A Systems Biology Approach for Discovering Biomarkers Indicative of Exposure to Carfentanil

Elizabeth S Dhummakupt, Bao Q Tran, Daniel O Carmany, Gabrielle Boyd, Phillip M Mach, Michael Feasel, Jennifer Sekowski, Trevor Glaros

Keywords: opioids, metabolomics, proteomics, biomarkers

Introduction: There are very few validated assays that are field-capable of rapidly detecting carfentanil, an ultra-potent, synthetic opioid related to fentanyl. Carfentanil has been responsible for numerous reports of mass localized overdoses in the United States. In order to improve the ability to detect this compound, a systems biology approach was utilized to discover host-based markers that are amplified upon carfentanil exposure.

Methods:
New Zealand white rabbits were exposed to a 0.1 ECT50 concentration of carfentanil through nose-only inhalation. Blood was drawn immediately before and after exposure, then at time points out to thirteen days post-exposure. Blood plasma was then analyzed utilizing proteomics and untargeted metabolomics pipelines. For proteomics, a multidimensional fractionation method was developed for deeper coverage, and isobaric tandem mass tags (TMT) were utilized for quantitative assessments.

Results:
For the proteomics, each animal served as its own control, due to the high inter-animal variability. By utilizing this method and stringent data processing filters, over 700 proteins were selected for statistical analysis. Of those, over 40 proteins were identified to have a two-fold ratio change in at least one time point, and 19 proteins resulted in significant p-values from ANOVA analysis. The metabolomics analysis identified over 100 features that had both a two-fold ratio change and a significant p-value from ANOVA. The PLS-DA showed significant separation when comparing pre- and post-exposure to the six-hour time point. Separation continued to become more pronounced as time progressed in both PLS-DA and PCA; however, after the first day the separation appeared to be maximized out to the latest time point. Identified proteins and metabolites suggest carfentanil exposure causes increased reactive oxygen species, possible vascular damage from coagulation and clotting, and possible myocardial infarction.

Conclusions
Proteomics and metabolomics pipelines were leveraged for a long time course study of low-dose exposure to carfentanil. A set of 19 proteins and over 100 metabolite features were identified as significant via fold-change and ANOVA. Both sets of data indicated significant changes could be seen six to 24 hours after exposure. Remarkably, a handful of metabolic perturbations were observed in samples taken immediately post exposure.

Novel Aspect:
First utilization of proteomic and metabolomic pipelines for time course analysis following carfentanil exposure.

Funding for this project is provided by the Defense Threat Reduction Agency – Joint Science and Technology Office for Chemical and Biological Defense.
Hepatotoxicity of prescription and over-the-counter medication is resulting in major drug-induced adverse effects. Acetaminophen (APAP) and clozapine (CLZ) are known to form reactive metabolites, which can covalently bind to cysteine residues in hepatic proteins. We have investigated the protein binding of these two compounds to different glutathione S-transferase (GST) isoforms.

Methods
Our developed method uses in vitro activation of APAP and CLZ to NAPQI and CLox, respectively, with either liver microsomes or CYP3A4 Supersomes, while adding purified GSTs to the incubation. Further sample preparation employed tryptic or peptic digestion, coupled to LC-MS/MS analysis. Results between SPE, immunoprecipitation and offline LC fractionation have been compared. We have developed two LC-MS/MS based strategies for screening for modified peptides.

Results
Method optimization was carefully performed on different cytosolic Homo sapiens GSTs. LC-MS/MS of information dependent acquisition (IDA) files were searched with UniProtKB/Swiss-Prot protein database. The search algorithm was altered to find NAPQI- or CLox-cysteine adducts. Multiple reaction monitoring (MRM) was also used as a targeted screening technique. Optimal conditions for incubations were determined (3 h at 37 °C), followed by either tryptic or peptic digestion at 37 °C for 4 h or 1 h, respectively.

The first LC-MS/MS screening technique used a traditional shotgun proteomics workflow (IDA) on a QqTOF with subsequent database searching. Then, a targeted MRM method for all cysteine-containing peptides of the individual GST isoforms was tested. The GSTM2 and P1 were found to be a target of NAPQI in vitro, with more sites identified using the MRM based screening method. Cys174 in M2 and Cys48 in P1 were found using IDA and MRM; while additional sites Cys115 in M2, and Cys15 and Cys170 in P1 were confirmed by MRM only.

Conclusions
Currently, CLox-protein binding, as well as, IP purification are being optimized to provide highly purified NAPQI- and CLox-peptide extracts for fast LC-MS/MS analyses of complex samples, including exposed liver microsomes, S9 fractions and homogenates. Comparison of the performance of IDA and MRM analyses showed that MRM represents a complementary technique to untargeted IDA, which can be easily applied in future drug-protein binding investigations.

Novel Aspect
High sensitivity screening and identification of drug-related protein binding to glutathione S-transferases using LC-HRMS/MS and LC-MRM
Introduction:
The gold standard method for analyzing androgenic anabolic steroids (AAS) in equine urine, in the context of anti-
doping control, is GC-MS/MS after deconjugation and derivatization steps. LC-HRMS can offer an alternative option
for the direct analysis of AAS under their intact conjugated forms in horse urine. The aim of this study is to develop
an analytical strategy allowing the extraction of conjugated AAS and their analysis by LC-HRMS using the Q-Exactive
HF-X.

Methods:
AAS are mainly under their sulfo or glucuruno-conjugated forms in equine urine. Therefore an adapted analytical
strategy was settled for their detection. The sample preparation was optimized assessing different SPE cartridges
and conditions, which have been compared in terms of extraction recovery. The chromatographic separation and the
mass spectrometry parameters were also optimized for conjugated steroids detection with higher sensitivity and
selectivity.

Results:
SPE cartridges with C18 or strong anion exchange (SAX) stationary solid phases were evaluated during the study.
Extraction of conjugated AAS from horse urine were performed on C18 cartridges using percentages of methanol in
wash steps ranging between 5 and 40%. Higher percentages of methanol resulted in cleaner extracts and lower
matrix noise impact. However, increasing this percentage induced a loss of some glucuronide and di-glucuronide
steroids. Unlike the C18 cartridges, the anion exchanger cartridges resulted in a higher extraction recovery of
glucuronide steroids and lower extraction yields for sulfo-conjugated forms. The selected chromatographic condition
using C18 column showed well-resolved chromatographic peaks and a good separation profile. Finally, the MS
parameters were optimized using the ESI negative mode to reduce background noise and enhance conjugated
steroids ionization. A scan mode combining Full MS and PRM was settled and AGC parameters optimized to enhance
sensitivity and selectivity.

Conclusions
The developed analytical strategy was based, first, on a steroid extraction performed using a C18 SPE cartridge
characterized by an efficient extraction of conjugated steroids from horse urine together with an optimized washing
step reducing matrix effect during the LC-MS analysis. Then, the optimization of chromatographic and mass
spectrometry parameters permitted to obtain satisfying separation and detection of sulfo and glucuruno-conjugated
forms.

Novel Aspect:
Unlike gold standard GC-MS/MS strategy, LC-HRMS allows conjugated AAS analysis in equine urine under their intact form insuring a gain in time and avoiding potential artifacts.

References
**Introduction:**
The use of androgenic anabolic steroids (AAS) as growth promoters for equine performance enhancement is forbidden according to article 6 of the International Federation of Horseracing Authorities (IFHA) [1]. The latter bans the use of substances capable of giving horses an advantage, contrary to the horse’s inherent merit. Moreover, the use of these substances is supposed to result in biological and behavioral modifications, considered dangerous for the animal.

**Methods:**
The gold standard method for analyzing AAS in equine urine is GC-MS/MS following hydrolysis and extraction steps. The duration of the analytical run is circa 30 minutes per sample which consists an issue for high throughput routine analysis. In order to reduce the analysis runtime and enhance performances, a Fast GC-MS/MS method has been developed using a shorter column of 20 m instead of 30 m with tighter i.d and thinner film in comparison to GC columns.

**Results:**
Two FastGC columns, Drug-1 (10 mx 0.18 mm x 0.18µm) and ZB-5MS-PLUS (20m x 0.18 mm x 0.18µm), were evaluated and compared to the currently used GC column DB-5MS-UI (30m x 0.25 mm x 0.25µm). The chromatographic profile of the 47 steroids of the study, when performing the separation on the 10 m Drug-1 column, exhibited phenomena of peak tailing and carry-over. These phenomena were caused by the matricial charge density versus the low quantity of stationary phase. The use of a FastGC column ZB-5MS-PLUS (20m x 0.18 mm x 0.18µm) with an optimized gradient permitted to separate the different steroids of the study and resulted in a well-resolved chromatographic profile. The FastGC-MS/MS strategy using the 20 m Fast-GC column was performant in terms of repeatability, sensitivity and selectivity following the analysis of blank and spiked urine samples and reference standards of a 47 steroids pool mixture. The developed strategy led to a considerable gain in analysis time and hence, presents a significant interest for high throughput routine analysis.

**Conclusions**
The developed Fast GC-MS/MS method using the 20 m ZB-5MS-PLUS column allowed to obtain well-resolved chromatographic peaks for the different steroids of the study. The performance of the Fast-GC-MS/MS strategy were satisfactory namely in terms of sensitivity and robustness. In comparison to the currently used GC-MS/MS method, a gain in analysis runtime of 8 minutes per sample and 5 hours per instrument per day was obtained.

**Novel Aspect:**
Fast-GC-MS/MS strategy results in a significant gain in analysis time and insures good performances which is crucial for high throughput routine analysis of AAS in equine urine.

**References**
Introduction:
Although studies have reported the toxicological effects and underlying mechanisms of toxicity of silver nanoparticles (AgNP) in a variety of organisms, the interactions of AgNP with environmental contaminants are poorly understood. We used biochemical assays and mass spectrometry-based proteomics to assess the molecular effects induced by a co-exposure of HepG2 cells to AgNP and cadmium ions.

Methods:
Human HepG2 hepatocellular carcinoma cells (Sigma-Aldrich) were cultured as a monolayer in high-glucose Dulbecco’s modified Eagle’s medium (DMEM) and subjected to spherical AgNP (10 nm), cadmium, or a combination of the two for 4-h and 24-h’s for comparison. Cell viability, ATP/ADP ratio assays were performed in parallel to quantitative proteomics (Q Exactive HF) using isotopic labeling.

Results:
Cell viability and energy homeostasis were slightly affected after 4-h exposure to AgNP, Cd2+, or a combination of the two; these endpoints were substantially altered after a 24-h co-exposure to AgNP and Cd2+, while exposure to one of the two contaminants led to minor changes. Proteomics analysis followed the same trend: while a 4-h exposure induced minor protein deregulation, a 24-h exposure to a combination of AgNP and Cd2+ deregulated 43% of the proteome. The toxicity induced by a combined exposure to AgNP and Cd2+ involved (1) inactivation of Nrf2, resulting in downregulation of antioxidant defense and proteasome-related proteins, (2) metabolic adaptation and ADP/ATP imbalance, and (3) increased protein synthesis intended to reestablish homeostasis. The adaptation strategy was not sufficient to restore ADP/ATP homeostasis and to avoid significant cell death.

Conclusions:
Co-exposure for 24 h to AgNP and Cd2+ induced more toxic responses than individual exposures. The toxicity induced by AgNP+Cd2+ was the upregulation of oxidative phosphorylation and lipid metabolism, suggesting a metabolic adaptation, as HepG2 cells usually produce ATP mainly by glycolysis, and alternative nutrients, such as lipids, may have been recruited to reestablish energy homeostasis.

Novel Aspect:
Our results provide information on the toxicological interaction of AgNP and Cd2+ ions in HepG2 cells, and insights into possible toxicity mechanisms.

References

1412 - EVOLUTION AND APPLICATION OF MASS SPECTROMETRY –BASED ANALYTICAL METHODS IN ANTI-DOPING ANALYSES

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Keywords: anti-doping, WADA accredited laboratory, prohibited substances, sport, mass spectrometry

Introduction:
Accredited anti-doping laboratories operate within strict regulations of WADA, such as the International Standard for Laboratories (ISL) and technical documents, which aim at harmonizing performance and reporting of routine analyses. Due to the sensitivity and selectivity required from confirmation analyses, mass spectrometry (MS) coupled to either gas (GC) or liquid chromatography (LC) is the analytical technique of choice for identification of the analytes.

Methods:
Whereas the first routine applications were based on the GC/MS techniques, implementation of LC/MS-methodologies during the last 15-30 years dramatically extended the menu of prohibited substances amenable for MS-identification. Both platforms are, however, required for adequate coverage of analyses, which have mainly qualitative but also some quantitative targets, and benefit greatly of technical advances of sensitivity, resolution and data processing.

Results:
In the evolution of the GC/MS-techniques, the cornerstones include the implementation of enhanced trimethylsilyl (TMS) reagent for keto-group, which had a significant impact on the robust analysis of not only exogenous, but also endogenous anabolic androgenic steroids (AAS) with keto-structure. Despite of roots originating to 1980’s, this technique is still actively in use in steroid profiling for athlete biological passport (ABP) purposes. Regarding the endogenous AAS, such as testosterone, another major advance has been the development of an analytical approach where GC separation, combustion to CO2, and isotope ratio mass spectrometry (GC/C/IRMS) was combined for the determination of doping use, i.e. exogenous origin of AAS. Along the LC/MS-based methods, especially those with soft ionization methods such as electrospray, anti-doping analyses have become more sophisticated for prohibited substances with low volatility (corticosteroids), instable derivatives (diuretics), and higher molecular mass (conjugates, peptides).

Conclusions:
The spectrum of target compounds in anti-doping analyses is wide, dynamic and strongly dependent on the pharmaceutical drug development. In order to keep up with the analytical capabilities for emerging performance enhancing substances, the laboratories should maintain the flexibility of analytical pipelines but also follow the development of mass spectrometers and their performance to update the processes for improved sensitivity, selectivity and robustness.

Novel Aspect:
Samples can be stored up to ten years. With re-analyses, potential of non-restrictive data acquisition and digital matrix are proposed to increase deterrence effect of doping control.
Aplication of dispersive liquid-liquid microextraction (DLLME) for identification of synthetic cannabinoids in plasma by LC-MS/MS

Keywords: Synthetic cannabinoids, DLLME, LC-MS/MS, New Psychoactive Substances

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Introduction: (Limit of 400 characters without spaces)
The detection of synthetic cannabinoids (SCs) in biological fluids is challenging and requires sensitive analytical methods. The dispersive liquid-liquid microextraction (DLLME) is a sample preparation technique based on the partition of analytes using small volumes of solvent mixture (dispersive and extractor). The aim of this work is to optimize a DLLME extraction to analyze SCs in plasma by LC-MS/MS.

Methods:
Sample (400 µL) was fortified with 40 µL of SCs mixture, vortexed and 1200 µL of dispersive solvent was added and centrifuged at 5500 rpm/5 min. Water (1 mL) and sodium tetraboratesaturated solution (100 µL) was added to 500 µL of supernatant and 100 µL of the extraction solvent was rapidly added and the mixture was centrifuged at 5500 rpm/5 min. An aliquot (180 µL) was transferred to a vial, evaporated, reconstituted in 100 µL of methanol and injected (2 µL) into LC-MS/MS.

Results: (Limit 900 characters without spaces)
The dispersive and extraction solvents were chosen by design of experiment (D-optimal), based on their solubility and extraction capacity, in order to ensure the formation of the cloudy (dispersive effect). The dispersive solvent had to be miscible with both aqueous and organic phases. The design of experiment pointed out that the best solvents for extraction were acetonitrile as dispersive solvent, and chloroform as extractor solvent. This combination of solvents allowed the efficient extraction of SCs as low as 0.1 ng/mL using small plasma volume (0.4 mL). DLLME still needs to be improved and further experiments will be carried out, to evaluate the optimum condition of sample volume, pH, salting-out process and centrifugation conditions.

Conclusions
The extraction method initially optimized and applied for the extraction of SCs was adequate to recover the analytes in plasma samples at low concentrations (0.1 ng/mL), concentration expected to be found in real cases. We’re improving our method, performing other studies to improve even more the extraction steps, and thus the method detectability, to finally validate and apply in real samples.

Novel Aspect:
DLLME is a microextraction technique, which requires low volume of solvents, and was successfully applied to SCs analysis, extremely toxic emergent drugs.
Development and Validation of an LC-MS/MS Method for the Determination of Maternal and Fetal Tissue Distribution of Permethrin and Its Metabolites in Pregnant Rats

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Keywords: UHPLC-MS/MS
Permethrin
Toxicokinetic
Gestation
Rat

Introduction:
Animal studies suggest that gestational exposure to permethrin can cause neurodevelopmental toxicity [1]. Permethrin can cross the placental barrier [2]. However, toxicokinetic data are not available to quantify this transfer. The aim of our study was to develop an UHPLC-MS/MS method to quantify permethrin isomers and their metabolites in blood, urines, feces and several tissues of interest: brain, liver, kidney, mammary gland, fat and placenta in pregnant rats.

Methods:
The extraction was performed by liquid/liquid extraction. Chromatographic conditions for cis and trans-permethrin and their metabolites (cis and trans-DCCA and 3-PBA) were optimized using different analytical columns, mobile phases and way of elution.
The final method was validated for recovery, specificity, linearity, precision and accuracy before its application to quantify samples of a toxicokinetic study in pregnant rats exposed daily to permethrin.

Results:
Chromatographic separation was accomplished on a HSS T3 column with a gradient elution system. Two different mobile phases were used. For permethrin, the mobile phase consisted of ammonium acetate buffer and acetonitrile. For metabolites, acetonitrile and water containing 0.1% of formic acid were selected.
Relative recoveries were found to be in the range of 66 to 117% for permethrin and 43% to 140% for metabolites. No interference from other components in the matrix were observed at the retention times of the compounds.
Matrix-matched calibration curves had satisfactory linearity up to 2000 ng/mL. The LOQ ranged from 10 to 200 ng/mL for the compounds depending on the matrix. The intra-and inter-batch precision and accuracy were better than 15%.
The validated method was applied to analyze samples collected in the toxicokinetic study. Cis-permethrin, trans-DCCA and 3-PBA were detected in all matrices, including fetal matrices. Trans-permethrin and cis-DCCA were detected in all matrices except placenta and liver respectively.

Conclusions
We developed a rapid analytical method with limited sample pre-treatment sufficient to allow the simultaneous quantification of permethrin and their metabolites in biological matrices in pregnant rats and fetuses.
The method showed good performances in terms of accuracy and precision. The LOQ were better compared to a previously published method targeting the same compounds in a large panel of biological matrices in rats [3].

Novel Aspect:
This method is the first method used to successfully quantify permethrin isomers and their metabolites in a various panel of biological matrices in pregnant rats and fetuses.

References
938 - DETECTION OF CHEMICAL WARFARE AGENTS-RELATED PHENYLARSENIC COMPOUNDS IN MARINE BIOTA AND IDENTIFICATION OF THEIR METABOLITES IN IN VITRO STUDIES BY ORBITRAP HRMS

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Keywords: Chemical warfare agent, arsenic, fish, metabolism, high resolution mass spectrometry

Introduction: Phenylarsenic compounds, Clark I, Clark II and Adamsite, were designed as riot control agents during World War I[1]. Nowadays, these chemicals are classified as chemical warfare agents (CWAs) by Chemical Weapons Convention. These agents were produced in a large scale during World War I and II. After World War II, their disposal began by sea-dumping. Hundreds of thousands of tons of chemical ammunitions (mainly sulfur mustard and phenylarsenic compounds) were dumped in the Baltic Sea and Skagerrak area between Norway and Denmark [2]. Several investigations have shown that sediment samples collected near these dumping areas are contaminated with CWAs as a result of leaking containers [3]. These leaking toxic chemicals are posing a threat to marine environment. In aqueous environment phenylarsenic compounds degrade forming hydrolysis and oxidation products. Previously traces of oxidation form of Clark I and/or II have been detected from lobster and flatfish samples collected from dumpsite area near Swedish coastline[4]. In order to study uptake of arsenic containing CWAs in fish in larger scale, fish samples were collected from different dumpsites and analysed. One goal of the ongoing EU-project DAIMON (Decision Aid for Marine Munitions) (2016-2019) is assessment of munitions’ impact on marine biota. One aim is to develop specific and sensitive methods to assess the fate and impact of toxic CWAs on marine biota [5].Quantitative analysis of these chemicals and their degradation products are needed to prove presence of these products in aquatic biota in order to support risk assessment for possible accumulation in food chain. There are hardly any studies about transference of arsenic based CWAs into aquatic biota. Due to the lack of information how these agents behave in living organism it’s impossible to predict interactions or transformations of these chemicals. In order to study transformations of arsenic containing CWAs in fish we performed metabolism studies with fish liver homogenates. Information achieved from metabolism studies is essential when developing novel analysis methods for arsenic containing CWAs from fish samples.

Methods: Fish samples obtained from three different dumpsite areas were analysed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) in the selective reaction monitoring (SRM) mode. Qualitative identification criteria based on EU guidelines were used to reliable identification of detected CWA-related compounds. In order to study transformations of phenylarsenic compounds in fish in vitro we incubated five CWA-related arsenic compounds in cod liver homogenates (S9 fraction). Protein concentration of liver homogenates was determined by using the Lowry method. Samples from metabolism studies were analysed by liquid chromatography-Orbitrap high resolution mass spectrometry.

Results: Fish samples collected from three different dumpsites contained CWA-related arsenic compounds at parts per billion (ppb, ng/g) concentration levels. These arsenic containing chemicals were analysed as their pentavalent forms by LC-MS/MS method. Metabolism studies with cod liver homogenates proved that phenylarsenic compounds as trivalent intact form as well as oxidized pentavalent form formed glutathione (GSH) conjugates. Structures of these metabolites were determined by high resolution mass spectrometry. Also reference chemicals of detected glutathione metabolites were synthesized and their structures were confirmed additionally by nuclear resonance spectroscopy. MS spectra of detected metabolites from in vitro metabolites studies were found to match spectra of synthesized reference chemicals.
Conclusions: There are hardly any studies about transference of arsenic based CWAs into aquatic biota. Previously these arsenic-containing chemicals have been detected from marine biota samples as their pentavalent oxidation state. Metabolism studies with fish liver enzymes proved that these oxidized arsenic compounds were reduced by glutathione and formed glutathione conjugates. There are no data available about toxicities of these trivalent phenylarsenic-glutathione metabolites neither how these metabolites behave in fish. It’s possible that these glutathione metabolites are excreted into bile or urine. Based on this, more studies are needed to evaluate total arsenic-related CWAs concentrations in aquatic biota.

Novel Aspect: CWA-related arsenic compounds have been detected in different marine species for the first time. In order to get more specific information about transformations of these arsenic compounds in fish, metabolism studies with fish liver homogenates were applied. Phenylarsenic compounds formed glutathione conjugates which structures were confirmed by Orbitrap high resolution mass spectrometry. None of these metabolites have ever been detected from marine biota samples and this is the first time when metabolism of phenylarsenic compounds have been studied with fish liver homogenates in vitro.

References

PROTON TRANSFER REACTION – MASS SPECTROMETRY AND ITS APPLICATIONS TO HOMELAND SECURITY: DETECTION OF ILLICIT DRUGS

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Keywords:
Proton transfer reaction mass spectrometry, illicit drugs, thermal desorption, homeland security

Introduction:
Proton Transfer Reaction Mass Spectrometry (PTR-MS) is an analytical technique for the detection of trace chemical compounds in complex environments through soft chemical ionization useful in many fields including health sciences, environmental sciences and homeland security. PTR-MS is based on the volatility of the targeted chemicals and its ability to work without initial separation or preconcentration makes it suitable for on-line, real time measurements.

Methods:
PTR-MS is a suitable analytical technique to monitor volatile organic compounds (VOCs) in the air. However, compounds of interest in the field of homeland security can have low pressure, which makes their detection challenging. To amend this, a Thermal Desorption Unit (TDU) in conjunction with a PTR-MS instrument can be used. It has recently been applied to the detection of explosives [1] and here we apply it to the detection of illicit drugs.

Results:
In this study we demonstrate the application of a TDU for the on-line detection of trace quantities of the most widely used societal drugs, the reactions of which with H3O+ at just one reduced electric field, 120 Td, have already been reported [2].

The results shown were achieved by measuring some hundreds of nanograms deposited in a teflon swab which was subsequently inserted into the TDU for the desorption of the sample into the PTR-MS instrument. The two modes of the instrument, namely DC and RF modes, were used. In DC mode, a uniform electric field draws the ions downstream in the reactor, while in RF mode a radio frequency field is superimposed to the uniform electric field to deliver extra collisional energy to the molecules.

Conclusions:
This study shows that the TDU is a suitable tool to use in conjunction with PTR-MS for the detection of illicit drugs. The relative distribution of product ions in the DC mode agrees with the literature [2], but the results from the RF mode is controversial. For heroin, morphine and codeine both modes present similarities, showing more fragmentation of the parent ion in the RF mode, whereas for cocaine and MDMA they totally differ.

Novel Aspect:
The novelty of these results lies in the use of the TDU and the RF mode in a PTR-MS instrument for the detection of illicit drugs.

References:
ASSESSMENT OF CHEMICAL TRACE ELEMENTS IN COSMETICS' EYESHADOWS

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Keywords: trace elemental analysis, eye shadows, cosmetics, ICP-MS, CV-AAS

Introduction:
Among color cosmetics eyeshadows deserve special attention since they are applied in the area where facial skin is thin. Many studies suggest that cosmetics belonging to the eye makeup segment contain high amount of toxic metals. The size of particles used in their production also plays a key factor in safety issues. All may contribute to the increase of allergic reactions risk or development of other side effects appearing with their prolonged application [1-2].

Methods:
ICP Mass Spectrometry with Time-of-Flight Analyzer (Ag, Ba, Bi, Cd, Pb, Sr and Tl) and Cold Vapor Atomic Absorption Spectrometry (Hg) techniques were applied for inorganic chemical elements determination in eyeshadows products. In total 94 samples (in 12 colors) obtained from the local market and originating from 8 most commonly used brands were purchased for the analysis. The samples were coded depending on their price, color, type, origin and producer.

Results:
They are no current data concerning the potential assessment of the risk connected with application of eyeshadows. The safety of their use is dependent upon e.g. the type of products (source and form of the components, size of the particles), time and frequency of the application, etc. In this study eyeshadows samples were grouped by similarity to the concentrations of the chosen chemical elements obtained by ICP-MS and CVAAS techniques. The price of products, their color, brand, origin, destiny (for kids and adults), type (mat and pearl) were used as criteria of their classification. Multivariate methods as PCA and CA were applied to observe the relationships and interactions of several analyzed elements and assess influence of chosen factors. It was shown that the average level of Cd in one sample was few times above the permissible limit (0.5 mg/kg), while the concentration of Pb and Hg in two samples should not be ignored since they slightly exceeded the polish regulations regarding cosmetics products (10 mg/kg and 1 mg/kg, respectively).

Conclusions:
Summarizing the influence of chosen factors it can be concluded that factors having the least impact on the concentration of studied elements are color and type of eyeshadows. Factors affecting the majority of concentrations of the determined elements are the brand of the eyeshadow (Sr, Ag, Cd, Pb, Bi, Hg), country of origin (Sr, Ba, Pb, Bi, Hg) and shadow price (Sr, Ba, Pb, Bi, Hg). For the following elements: Sr, Pb, Bi, Hg the influence of the same factors was observed.

Novel Aspect:
The examination of the content of chosen trace metals in the locally sourced eyeshadows is quite relevant since there are no updated reports regarding homegrown products safety.

References
A generic extraction method combined with LDTD-MS/MS analysis was developed for fast turnaround screening of drugs in saliva. This new method could give police officers rapid and accurate answer in less than 10 minutes allowing on-site screening on a police roadblock.

Methods:
Drugs are spiked at 50%, 100% and 150% of the decision point in saliva. 50 μL is mixed with a solution composed of 100 μL of internal standard and 75 μL of buffer. The mixture is vortexed 20 seconds and centrifuged 30 seconds. 8 μL of the upper layer are deposited in LazWell-HDE plates. The plates are dried for 3 minutes. Seven drugs are analyzed in positive APCI mode and one in negative APCI mode in a single run. LDTD’s laser power was ramped from 0 to 55% of maximum power in 6 seconds.

Results:
The decision points in saliva are 15 ng/mL for THC, 50 ng/mL for Amphetamine/Methamphetamine/MDMA and 10 ng/mL for Cocaine/ Benzoylecgonine/Morphine/6-AM. Using the SWGTOX validation guidelines, a blank preparation of saliva is spiked with 8 previously enumerated drugs at concentration representing 50%, 100% and 150% of the cut-off. Validation of this new screening approach, the CV at each concentration must not exceed 20% and the average concentration ± 2 times the standard deviation must not overlap with other concentration at the decision point. Triplicate extractions are performed for each concentration over the course of 5 days, for validation. Additional blank of saliva, one with Internal standards (IS) and the others without are extracted and analyzed. The THC is analyzed in negative mode and the others in positive. No overlapping at the decision points is observed and a %CV lower than 15% is obtained. Series of tests were timed from the saliva collection to the output result giving and average of 9.3 minutes for the complete workflow.

Conclusions:
Simple extraction method combined with LDTD-MS/MS analysis allow specific measurement of narcotic in saliva. The generic method enables accurate on-site analysis of saliva collected from an individual potentially driving under the influence of drugs with expected feedback results obtained under 10 minutes by the police officer.

Novel Aspect:
In situ High Throughput targeted screening of narcotic in saliva using LDTD-MS/MS

References
Introduction
The steroidal Athlete Biological Passport aims at revealing use of testosterone. It is implemented by GC-MS quantification of selected endogenous androgens in urine. Sensitivity of this approach suffers from confounding factors e.g. enzyme polymorphism, bacterial contamination and ethanol consumption [1]. Among the improvement strategies, quantification of steroid hormones in serum has shown promising results and a complement to the urinary module [2, 3].

Methods
A steroidomic approach was used to highlight new serum biomarkers of testosterone doping. The developed analytical workflow consisted of a SPE clean-up of steroids and their phase II metabolites, followed by UHPLC-HRMS analysis. Full Scan MS and data dependent MS/MS spectra were acquired in both positive and negative ionization modes. Samples from testosterone administration study (n=380) were analyzed in 11 analytical batches.

Results
Positive and negative ion mode data from UHPLC-HRMS analyses were treated separately: firstly, data processing including noise filtering, chromatographic alignment and peak picking was performed by means of dedicated software and resulted in datasets with approximately 40000 features for each ionization mode. Then, in order to reduce data dimensionality, ion annotation was carried out assigning confidence level 1 (confirmed with authentic standard) and 2 (consistent with predicted properties) employing a newly created in-house steroid database (350 steroids) together with retention time prediction model [4]. Intensities of annotated features (64 and 174 in positive and negative mode, respectively) were corrected with LOESS regression in order to minimize the batch effects and finally Analysis of variance Multiblock Orthogonal Partial Least Squares (AMOPLS) was performed to highlight new promising blood biomarkers of testosterone intake, similarly to what was previously done on urine matrix [5].

Conclusions
The study revealed a set of biomarkers for both oral and transdermal testosterone abuse, including hydroxylated metabolites of androgens, as well as their glucuronidated phase II metabolites. In comparison with urinary steroid profiling, these targets could increase sensitivity and extend the current detection window. In the near future, targeted quantitative UHPLC-MS/MS method will be developed for serum to validate the results of this study.

Novel Aspect
This work represents a first application of steroidomics on serum samples for anti-doping purposes, demonstrating the usefulness of holistic approaches in such a context.
References

In-vitro/vivo study of Andarine metabolism, a selective androgen receptor modulator (SARM), in the context of horse doping control

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Keywords: Doping, horse, metabolism, SARM, in-vivo/vitro

Introduction:
Andarine is a selective androgen receptor modulator classified as non-steroid anabolic agent. Its use is forbidden by the International Federation of Horseracing Authorities at any time in horse career. This molecule has the ability to stimulate androgen receptors in muscles and bones and thus to enhance athletic performances. To control the fraudulent administration of andarine, a horse metabolism study has been performed to identify the best target metabolites.

Methods:
This metabolism study of andarine was carried out first by in-vitro approach using microsomal fractions of equine hepatocytes (S9) and then in-vivo on one horse via a subcutaneous administration route. Both in-vitro reaction products and blood/urine samples, collected up to 4 days, were analyzed by LC-HRMS2 and LC-SRM to characterize phase I and phase II andarine metabolites.

Results:
The in-vitro results obtained using S9 equine hepatocyte fractions showed an important phase I metabolism of andarine. Indeed, six phase I andarine products were identified i.e. three metabolites with a functional loss (methyl, acetyl or phenyl), one with the nitro group reduction and one which was identified as cleavage product of andarine (4-nitro-3-trifluoromethyl-phenylamine) with its hydroxylated form. The post administration study has shown the presence of the native molecule in both urine and plasma. In urine, the results were consistent with those obtained in-vitro: the metabolite of andarine 4-nitro-3-trifluoromethyl-phenylamine and its hydroxylated form were also identified. These two metabolites were much longer detected in urine than the native molecule and presented high degree of phase II conjugation with both sulfate and glucuronic acid. Limit of detections (LOD) of andarine were 1 pg/mL in plasma and 5 pg/mL in urine. Concerning the two urine metabolites their LOD was around 50 pg/mL in andarine equivalent.

Conclusions:
Andarine is prohibited at any time. Therefore, it was important to study its metabolism in horse to identify the best target metabolites to detect its misused. The in-vitro results demonstrated that andarine was strongly metabolized. These results have been confirmed by the in-vivo experiments. This study demonstrated that the control of andarine must be performed in urine on its two major metabolites which were much longer detected than the native molecule.

Novel Aspect:
Combination of in-vitro and in-vivo approaches to study by LC-HRMS2 horse metabolism and kinetic of elimination in plasma and urine of andarine in the context of horse doping.

References:
A FIT-FOR-PURPOSE UHPLC-MS/MS METHOD FOR ROUTINE SCREENING OF SARM RESIDUES IN BOVINE AND EQUINE SERUM

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Keywords: SARMs, UHPLC-MS/MS, serum, doping control, food safety

Introduction:
Selective Androgen Receptor Modulators (SARMs) are an attractive alternative to anabolic-androgenic steroids despite the fact they are not yet approved as pharmaceutical drugs [1]. Consequently, there is much evidence of their availability through black and grey market sources. In this study, a semi-quantitative UHPLC-MS/MS method was developed to monitor the abuse of 15 compounds belonging to eight different SARM families, in bovine and equine animals, respectively.

Methods:
Serum samples underwent protein crash (0.5 mM NH4OH in MeCN), induced phase separation (NaCl) and defatting (n-hexane) before concentration under nitrogen. Extracted SARM residues were subsequently analysed by UHPLC-MS/MS operating in ESI+/ESI-. Chromatographic separation was performed using a Luna Omega Polar C18 column at 45 °C, and a binary gradient elution of 14 minutes employing H2O and MeOH, both containing 0.1% (v/v) CH3COOH as mobile phases at a flow rate of 0.4 mL/min.

Results:
Validation of the semi-quantitative assay was carried out according to the Community Reference Laboratories Residues (CRLs) 20/1/2010 guidelines [2]. The following performance studies were carried out during the validation process: specificity, selectivity, detection capability (CCβ), absolute recovery as well as applicability, ruggedness and matrix effects. The detection capability was calculated by assessing threshold value (T) and cut-off factor (Fm) and CCβ values were determined at the following concentrations: 0.5 ng mL⁻¹ (CDS-025139), 1 ng mL⁻¹ (AC-252636, PF-06260414), 2 ng mL⁻¹ (bicalutamide, GLPG-0492, LGD-2226, S-1, S-6, S-22, S-23) and 5 ng mL⁻¹ (BMS-564929, LGD-4033, RAD140, S-4, S-9), respectively. Moreover, ongoing work aims to identify in vivo metabolites of selected SARMs compounds following animal administration studies, which could serve as additional marker residues to be included within the current test method.

Conclusions:
SARMs are widely recognised as emerging drugs of abuse in animal sport, and also as potent candidates for misuse in food producing animals. The developed method is currently being employed as a rapid, simple, environmental friendly and cost-effective tool for the screening of serum samples to evaluate the abuse of SARM drugs in stock farming and to monitor for doping practices in racing horses, ensuring customer safety and fair play in animal sport competitions.

Novel Aspect:
The developed high-throughput assay allows, through a relatively short run and a small test sample volume (400 µL) of serum, the analysis of a total of 50 samples in a single day.

References

Acknowledgements:
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Analysis of removal of pesticides and drugs in waste water by plants using Flow Injection Analysis (FIA) Magnetic Resonance Mass Spectrometry (MRMS)

Keywords: Pesticides, Flow Injection Analysis, Drug detection, Screening

Introduction:
Treatment of wastewater is important to remove pollutants, drugs and other potentially environmentally toxic molecules before release into natural compartments. Constructed wetlands in France are used to treat water from small villages. Here we present the profiling of plants to understand if pesticides and drugs can be removed from waste water through an accumulation in plants or degradation by environmental factors.

Methods:
Two poplars (Populus nigra) were planted - one close to the border of a pond and the other away from the pond (control). Mature leaves of these two plants were collected and extracted. Each sample was analyzed in 8 technical and 3 measurement replicates in FIA-ESI using positive and negative ion mode. A solariX 2xR (Bruker Daltonik GmbH, Bremen) MRMS system was used for these studies. Analysis of data was performed with MetaboScape 3.0 (Bruker Daltonik GmbH, Bremen).

Results:
The data of the ESI(+) and ESI(-) were combined for feature analysis. More than 3400 features have been found for the plant extract samples. Roughly 90% of the detected features could be assigned with a molecular formula using a mass tolerance of only 0.5 ppm. More than 360 compounds have been annotated in the samples using a food data base with nearly 16000 entries using the same mass tolerance. Features responsible to differentiate treated and non-treated plant sample were detected by T-test and Principle Component Analysis (PCA). These compounds were analyzed in detail as possible drugs and pesticides. Based on these results 6 pesticide and 4 drug candidates were found by screening versus pesticide and drug data bases.

Conclusions:
Pesticides and their metabolites can be detected by FIA-MRMS in plant samples. Statistical analysis such as T-test and PCA can be used to find compounds responsible to distinguish treated and non-treated plants based on FIA-MRMS data. This method can be used to understand if pesticides and drugs can be removed from waste water.

Novel Aspect:
FIA-MRMS can be used to study the effect of removal of drugs and pesticides in waste water.
49 - IMPROVEMENT FOR HIGH SENSITIVITY OF THE DRUG SCREENING BY THERMAL DESORPTION AND PYROLYSIS COMBINED WITH DART-MS (TDP/DART-MS)

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Keywords: drug screening, urine, TDP/DART-MS

Introduction: (396 /400 characters)
In recent years, new drugs appear one after another, including new psychoactive substance that threaten society. Comprehensive analytical method for drug screening is greatly desired, since it is difficult to examine individually the analytical conditions that are appropriate for each one. Thus, we are developing a comprehensive drug screening method for urine or blood sample without pretreatment by thermal desorption and pyrolysis-DART-MS (TDP/DART-MS).

Methods: (373 /400 characters)
Standard drug mixture solution and urine added drugs (i.e., blank urine with several kinds of drug mixture added) were used as samples. TDP/DART-MS were composed by the combination TripleTOF 5600+ (Sciex) Q-TOF mass spectrometer equipped DART ion source (IonSense) and ionRocket TDP device (BioChromato). Mass spectra were measured as samples were heated from room temperature to 300 °C at a rate of 60 °C/min, the measurement time was 5min.

Results: (764 /900 characters)
At first, analysis of standard drug mixture by TDP/DART-MS, each drug was separated and detected through adding gradient heating, and thermal desorption profiles were highly reproducible for individual drug. Additionally, the detected ions were correctly identified according to their measured accurate mass and MS/MS spectra. So, it was confirmed that TDP/DART-MS can be a suitable analytical method for our investigation. Next, we examined the minimum pretreatment such as deproteinization by ACN, since for analysis of urine added drugs without any pretreatment, it was indicated that we had to improve the detection sensitivity. Comparing non-pretreated and deproteinization pretreated, the detection intensity of each drug in urine sample pretreated deproteinization were increased considerably. Moreover, the calibration curves using urine were linear in that ranging 0.01–1 μg/ml.

Conclusions: (362 /400 characters)
In summary, TDP/DART-MS can identify drugs in urine, directly and rapidly. In addition, since the calibration curves were linear in that ranging 0.01–1 μg/ml. Although our issue is to improve the detection intensity of drugs, TDP/DART-MS will be useful for rapidly quantitative analysis of drugs in urine in the future, and this analysis method will be a useful method in the field of required rapidity such as hospital and police.

Novel Aspect: (89 /150 characters)
TDP/DART-MS is useful analysis method for rapid identification and screening of drugs in urine samples.
DETECTION OF IGF-1 IN SERUM AS A BIOMARKER OF GROWTH HORMONE ABUSE BY ATHLETES

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Keywords: IGF-1, trypsin digestion, LC-MS/MS, doping-control

Introduction
The measurement of concentrations of insulin-like growth factor-1 (IGF-1) and N-terminal pro-peptide of type III collagen in serum was suggested for detection of growth hormone abuse by athletes [1]. The method for IGF-1 concentration determination by the quantification of two “signature peptides” (T1, T2) by UHPLC-MS/MS after trypsinolysis was proposed [2]. Here, we describe our results obtained after optimization and validation of the above method.

Methods
T1 and T2 were obtained after IGF-1 binding protein complex dissociation followed by protein precipitation by acetonitrile and trypsinolysis. Calibrations curves were made using rat serum fortified with human IGF-1. Also we used calibrators that were stored at -80°C. T1 and T2 peptides were analyzed by Thermo Ultimate3000 UHPLC with TSQ Vantage triple quadrupole mass spectrometer. The structures of peptides were confirmed by HRMS on Q Exactive mass spectrometer.

Results
The range of bottlenecks was revealed during method implementation. Calibration solutions that allow the estimation of the inter-day precision show remarkable changes in the standard concentration over time. The critical step in sample preparation was DTT reduction. Incubation for more than 90 min at 60°C led to T1 signal disappearance. Thus, we suppose that milder DTT reduction conditions are apparently more preferable for working with long peptides. For UHPLC-MS/MS method, the use of the gentle gradient (5-45%, 20 min, 0.22 mL/min) at 40°C and high dwell time (200 ms) were necessary for achieving a good response at low levels. The method was fully validated in the range of 50 – 600 ng/mL. The correlation coefficients (r²) for T1 and T2 were 0.996±0.004/0.996±0.003, respectively. Interestingly, the correlation coefficients (r²) for T1 and T2 in the range of 50 – 1000 ng/mL were 0.984±0.003/0.984±0.003, respectively. Therefore, the calibration point at 1000 ng/mL of IGF-1 was recognized as outlier and removed.

Conclusions
The method was fully validated and implemented for anti-doping purposes. Determination of T1 peptide was the critical point of the method, probably, due to its longer amino acid sequence as compared to T2. It was determined that high temperatures should be avoided during the reduction step. For LC conditions, the gentle gradient, low flow rate and column temperature should be used to facilitate separation T1. High dwell time is crucial for method sensitivity.

Novel Aspect
The incubation conditions in the presence of DTT, as well as UHPLC-MS/MS parameters have a significant effect on the T1 peptide sensitivity.

References
A.12 LIFE SCIENCES - CLINICAL CHEMISTRY

976 - IMPACT OF CYP2D6 GENETIC POLYMORPHISM ON THE PHARMACOKINETICS OF TRAMADOL AND ITS THREE MAIN METABOLITES IN KOREANS

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Keywords: Tramadol, CYP2D6, LC-MS/MS

Introduction:
Tramadol is a centrally acting analgesic used in the treatment of moderate to severe pain. Tramadol is mainly metabolized by the CYP2D6, CYP2B6 and CYP3A4 to O-desmethyltramadol (ODMT), N-desmethyltramadol (NDMT) and N,O-didesmethyltramadol (NODMT) [1]. We investigated the effects of CYP2D6 genetic polymorphism on the pharmacokinetics of tramadol and its three main metabolites.

Methods:
A multiple oral dose of tramadol was administered to twenty-two subjects with CYP2D6EM(n=14) and CYP2D6IM(n=8). Blood samples were collected up to 72 hr after drug intake, and plasma concentrations of tramadol and its metabolites (ODMT, NDMT and NODMT) were determined by validated HPLC-MS/MS method.

Results:
AUC of tramadol (p<0.001), NDMT (p<0.05) and NODMT (p<0.001) in CYP2D6IM group was significantly higher than those in CYP2D6EM group. The AUC ratios of ODMT to tramadol in the CYP2D6IM group was decreased to 44.9% of that in the CYP2D6EM group (p<0.001). The AUC ratios of NDMT to tramadol in the CYP2D6IM group was increased to 148.8% of that in the CYP2D6EM group (N.S.).

Conclusions:
The CYP2D6 genotype was found to play a significant role in the metabolic conversion of tramadol into ODMT and, as a compensatory consequence, impacted the conversion of tramadol into NDMT.

Novel Aspect:
CYP2D6*10 allele is found to affect the pharmacokinetics of tramadol and its phase I metabolites in Koreans.

References

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A combined micro-extraction by packed sorbent-UHPLC-ESI-MS/MS procedure for the analysis of prostanooids and isoprostanoids in dried blood spots of preterm newborns with patent ductus arteriosus

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Keywords: tandem mass spectrometry, micro-extraction by packed sorbent, dried blood spots, isoprostanoids, preterm newborns

Introduction:
An innovative UHPLC-ESI-MS/MS method for the analysis of prostaglandin-E2, 8-iso-prostaglandin-F2α and 8-iso-prostaglandin-E2 in dried blood spots is proposed. The non-invasive and painless monitoring of these inflammation and oxidative stress mediators in preterm newborns suffering from Patent Ductus Arteriosus could assess their potential role as predictors of response to therapy [1].

Methods:
Analytes were extracted from dried blood spots (20 µL x 3) with 70:30 v/v methanol:water mixture. The extracts were purified by an automated micro-extraction by packed sorbent (MEPS) before injection in the UHPLC-MS/MS instrument. Analytes were separated by a reversed-phase Polaris-C18 column and a gradient elution with aqueous formic acid (0.1%) and 50:50 v/v methanol:acetonitrile mixture. Triple quadrupole mass spectrometer operated in ESI(-) and MRM mode.

Results:
The fast and easy sample clean-up by MEPS guaranteed a high reproducibility (RSD < 5%) and extraction yield (>70%) in less than ten minutes, and limited the required volume of both solvents (30-100 µL) and sample (100 µL), with respect to conventional solid phase extraction [2]. The MRM acquisition ensured high sensitivity and selectivity and the presence of the analytes in the samples was confirmed by a q/Q ratio within ±20% of the value measured in reference standards. Limits of detection below 20 pg/mL, linearity (R2>0.99) over three orders of magnitude and satisfactory overall intra- and inter-day precisions (RSD <10%) were obtained. The deuterated internal standard was successfully laid on the filter paper instead of the common addition to the extraction solvent [2], in order to control both storage and extraction steps [3]. Finally, particular attention was paid to stability issues.

Conclusions:
To the best of our knowledge, this is the first analytical method combining a fast and reproducible MEPS procedure and a highly sensitive and selective UHPLC-ESI-MS/MS analysis for the determination of prostaglandin-E2, 8-iso-prostaglandin-F2α and 8-iso-prostaglandin-E2 in dried blood spots. The method is suitable for monitoring preterm newborns with Patent Ductus Arteriosus.

Novel Aspect:
The very reliable combination of tandem mass spectrometry and fast, semi-automated, re-usable MEPS for prostanooids and isoprostanoids analysis in dried blood spots.

References:
Introduction
In premenopausal women the role of estrogens is not thoroughly understood due to the large variations in estrogenic concentration throughout the menstrual cycle [1-6]. Lifestyle factors, such as smoking, physical activity and alcohol consumption may affect menstrual cycle outcomes [7]. Vice versa, the variations could influence fertility [8], the sexual behavior [9] and some diseases, such as schizophrenia [10], stress [11], insomnia [12] and anxiety [13].

Methods
A Gas Chromatography coupled to Mass Spectrometry (GC-MS) method capable of detecting 15 estrogens (17α-estradiol, 17β-estradiol, estrone, estriol and their principal metabolites) in urine was developed following the experimental design approach and fully-validated in accordance with the 17025:2005 principles. Multivariate data analysis was performed applying PARAFAC, Principal Component Analysis (PCA) and Linear Discriminant Analysis (LDA) to the data.

Results
The following validation parameters were successfully investigated: linearity range, selectivity, specificity, LOD, LOQ, intra-assay precision and accuracy, repeatability, matrix effect, extraction recovery and carry-over. Urine samples derived from 9 childbearing female volunteers (age between 25-35) who collected their urine every day for 28 days, that is a complete menstrual cycle. The data were evaluated by PARAFAC and the natural fluctuation of the concentration of each estrogen throughout the 28-day menstrual cycle was extract. Then, PCA was carried out on the data and the Scores Plot showed the occurrence of three different clusters corresponding to the three phases of the menstrual cycle: follicular phase, ovulation and luteal phase. Plotting PC1 and PC2 as a function of the menstrual cycle day in two separate graphs, the transitions between the phases turned visible by identifying the starting point of both the ovulation and the luteal phase. Lastly, a preliminary LDA model was built.

Conclusions
The application of a GC-MS method coupled to multivariate statistical techniques demonstrated great potentialities in the study of the natural rhythmic fluctuations of estrogens throughout the menstrual cycle. Therefore, the next step will be the application of this innovative approach to examine in depth the relationship between the endogenous estrogens and the increased risk of female reproductive cancers in postmenopausal women.

Novel Aspect
For the first time, not only the variation of the parent estrogens was studied, but also of their principal metabolites.

References
Bergemann N., Parzer P., Runnebaum B., Resch F., Mundt C., Psychological Medicine, 37, 1427–1436 (2007).
1354 - ANALYSIS OF SWEETENERS IN URINE USING NOVEL STATIONARY PHASE AND SELECTIVE LC-MS/MS METHOD

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Keywords: sweeteners, urine, biomarkers, HILIC, UPLC-MS/MS

Introduction:
There are only a limited number of published methods for monitoring of sweeteners intake. Most of these are focused on determination of small biomarker groups [1] and investigation their role in human metabolism. Moreover, these methods were either published deep in the past[2,3]or use chemical derivatisation[4,5]. In our study we aim to develop a novel and simple LC-MS/MS method for measurement of several intake biomarkers for different groups of sweeteners.

Methods:
Analysis of the sweeteners was performed using an ACQUITY UPLC System (Waters, Elstree, United Kingdom) coupled to a Xevo TQ-XS Triple Quadrupole mass spectrometer (Waters, Elstree, United Kingdom). For optimal separation of structurally related compounds different stationary phases, including C18, Amide, HILIC and Biphenyl, were tested. Moreover, in order to achieve greater ionization efficiency for small molecules novel UniSpray ion source was tested.

Results:
Final separation was achieved using a HILIC stationary phase with gradient of buffer and acetonitrile. The mobile phase gradient separated artificial sweeteners from sugar alcohols and natural sugars over a total 7.0 minute run time. For accurate detection of analytes ionization in negative mode and specific MRM transitions were used.

Conclusions:
The novel UPLC-MS/MS method with HILIC separation of structurally related sweeteners in human urine was developed in this study. This method was sensitive enough to comprehensively detect the broad spectrum of artificial and natural sweeteners without applying complicated derivatisation steps.

Novel Aspect:
This unique method enables rapid analysis of several different groups of sweeteners in human urine in one run. It may serve to identify biomarkers in nutritional research.

References:

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Introduction:
Myriocin (Myr) is a suicide inactivator of ceramide synthesis with a complex lipid multifunctional structure. Its biological activity is exerted at very low doses, and thus highly performing quantitative method are needed [1]. The pharmacological development of Myr to modulate ceramide levels also requires currently unavailable ADME information in healthy and pathological animal models.

Methods:
A 3200 qTRAP system was operated under manufacturer’s instructions to perform extensive tandem MS studies. LC and MS were optimized for the separation, detection, identification and quantification of Myr in aqueous humour, vitreous humor and retina of rabbit after different treatments. MRM scan mode was used as protocol, to tackle the ambiguities of some samples, EPI scan, MS3 and the use of HR-MS on a 6600 qTOF was also crucial.

Results:
Tissue levels of Myr are related to the dose, pharmaceutical formulation, administration route, and administration schedule. Four type of eye drop formulations were evaluated in rabbits: Myr in buffer solution, Myr in two different types of liposomes (neutral and cationic), Myr encapsulated in a novel type of solid nanolipid particles. In particular, solid nanolipid particles was found to be extremely efficacious for the delivery of Myr in the different areas of the eye. The slow release formulation produced a Tmax between 180-240 minutes, with a maximum concentration respectively of 10 µg/mL in vitreous and 1.7 µg/g in retina. Biological activity of Myr was always confirmed in rabbit’s retina by a significant decrease in ceramides levels, compared to the physiological level established from untreated animals. Some untreated animals demonstrated the presence of a peak with spectroscopic and chromatographic characteristics of Myr. EPI scan, MS3, and the use of a HR-MS as protocol confirmed an upstream contamination.

Conclusions
A new pharmaceutical delivery system, such as solid lipid nanoparticles, allows Myr to accumulate into the retina by a non-invasive eye-drops administration, and to modulates sphingolipid levels. These results would be promising to study its efficacy in mouse pathological model of retinitis pigmentosa. MRM measurement needs alternative and/or complementary techniques to confirm eventual false-positives samples.

Novel Aspect:
The use of HR-MS as protocol should be quite advantageous for predicting new metabolites and for removing unwanted matrix interferences.

References
Pharmacokinetics and bioavailability of different acetylsalicylic acid formulations assessed by Liquid Chromatography-Tandem Mass Spectrometry in healthy subjects

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Pharmacokinetics and bioavailability of different acetylsalicylic acid formulations assessed by Liquid Chromatography-Tandem Mass Spectrometry in healthy subjects

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Keywords: pharmacokinetics, LC-MS/MS, aspirin, enteric-coated formulation

Introduction

Low-dose acetylsalicylic acid (ASA, 100 mg/die) is used in thromboprophylaxis. Enteric-coated formulation (EC-ASA) is commonly used for its lower risk of side effects. Some patients on EC-ASA do not respond appropriately and recent studies showed that poor responsiveness is more frequent with EC-ASA [1]. Aim of this study was to validate a method useful to study the pharmacokinetics (PK) of ASA in healthy subjects treated with two different aspirin formulations.

Methods

Liquid chromatography tandem mass spectrometry (LC-MS/MS) technique was used for determination of ASA and salicylic acid (SA) in human plasma, using the respective deuterated isotopomers as internal standards (ASA-d4, SA-d4). The method was validated according to FDA guidelines and was applied to evaluate the PK of ASA and SA at different times (30 min-24 h) in healthy subjects (n=11) treated for 1 week with 100 mg of EC-ASA or plain Aspirin (plain-ASA).

Results

Analytes and internal standards were extracted from human plasma by protein. The compounds were separated on a reverse-phase column with an isocratic mobile phase. Compounds parameter were optimized to have the higher signal to noise ratio in negative multiple reaction mode. Ion transition recorded were 179→137 for ASA, 183→141 for ASA-d4, 137→93 for SA and 137→141 for SA-d4. The calibration curves were linear (r²≥0.99) over the concentration range of 20-2000 ng/mL for ASA and 20-8000 ng/mL for SA. LOQ was 20 ng/mL for both. Plasma AUC after EC-ASA administration was 702(604-881) ng h/mL (median, 25%-75% CI). tmax occurred between 2-8 hours after intake. Two subjects did not show ASA absorption within the observation period confirmed by SA trend. PK of plain-ASA showed AUC of 823 (635-1013) ng h/mL and all subjects absorbed the drug with tmax between 0,5-1 h.

Conclusions

The developed method allowed to study the analytical fate of aspirin “in vivo” by comparing two different ASA formulations. Coated formulation showed a variable behavior and in 2 subjects absorption seemed impaired. On the contrary using plain-ASA variation is reduced and all subjects absorbed efficiently the drug. Causes of inadequate response to ASA may be related to different gastro-intestinal availability of coated formulation.

Novel Aspect:

Availability of a validated method is pivotal to study ASA absorption and metabolism in different cohorts of patients known to have poor pharmacodynamics responses to the drug.

References

Comparison of ESI, APCI and APPI ionization techniques for testosterone (T), dihydrotestosterone (DHT) and estradiol (E2) LCMS/MS assay

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Keywords: Comparing ionization techniques for steroids

Title:
Comparison of ESI, APCI and APPI ionization techniques for testosterone (T), dihydrotestosterone (DHT) and estradiol (E2) LCMS/MS assay.

Introduction:
High sensitivity measurement at low concentrations of non-derivatized testosterone (T), dihydrotestosterone (DHT) and estradiol (E2) requires optimizing ionization technique but few matrix-specific comparisons are available. We compared electrospray ionization (ESI), atmospheric pressure chemical ionization (APCI) and atmospheric pressure photoionization (APPI) for sensitivity and matrix interference in neat solutions and human serum.

Methods:
LC instrumentation and conditions
A Shimadzu Prominence HPLC system was used and consisted of a DGU-20A5 degasser, three LC-20 AD pumps, a SIL-20AT HC autosampler and a CTO-20A column oven (Shimadzu Scientific Instruments, Kyoto, Japan). The column was an Agilent Poroshell 120 SB-C18 (2.1 × 50 mm; 2.7 μm) with 0.3 μm in-line filter (Agilent Technologies). For the determination of LLOQs with standard solutions, column temperature was 40 °C, injection volume 20 μl, and mobile phase of 70% methanol/water, containing 0.1% (v/v) of formic acid for positive ESI and 2.5 mM ammonium hydroxide for negative ESI. APCI and APPI were used without mobile phase additions. For serum and tissue samples, a following gradient was employed (A: water, B: methanol): 0–1 min: 20% B, 1–8 min: 20 > 70% B, 8–10 min: 70% B, 10–12 min: 100% B, 12–16 min: 20% B. Flow rate was 0.3 ml/min, injection volume 40 μl and same mobile phase additions were used as for the isocratic separations. A flow-line selection valve was used to divert the eluent to waste during 0–7 min and 11–16 min. For all experiments with APPI, toluene was used as a dopant solvent, delivered with an LC-20 AD pump with a flow rate 10% of that of the mobile phase.

MS instrumentation and conditions
An API 5000 triple quadrupole MS was used with Turbo V (ESI, APCI) and PhotoSpray (APPI) ion sources (AB Sciex, Toronto, Canada). The APPI lamp was a DC-driven 10.0 eV krypton discharge lamp model PKS100 from Heraeus (Hanau, Germany). Nitrogen was employed as ion source, curtain and collision gas (Peak NM20ZL, Peak Scientific, Inchinnan, UK). For the precursor ion selection and optimiza-
tion of the MS parameters, 5 μM solution of E2 in methanol was infused post-column into the mobile phase (70% methanol/water). The E2 infusion flow rate was kept at 5% of the mobile phase flow. Precursor and product ion selection was based on ion intensity. After establishing the MRM conditions, further optimization of the ion source parameters and the mobile phase flow was made with on-column injections, which enabled signal-to-noise (S/N) calculations. MS parameters not related to ionization were optimized and kept identical for each MRM transition. Both quadrupoles were set at unit resolution, cycle time was 250 ms and no signal thresholding was applied. Data acquisition and proces sing were performed with AB Sciex Analyst 1.5.2 software. The MRM transitions and more detailed instrument settings are described in Table 1.

We used a Shimadzu Nexera UHPLC and SCIEX API 5000 triple quad with Phenomenex Kinetex XB C18 (50mm x 2.1mm x 1.7μm) column at 40°C, injection volume 50 μl, flow rate was 0.5 ml/min, mobile phase of methanol-water. For ESI we used modifiers of 0.1% formic acid for positive and 2.5 mM ammonium hydroxide for negative polarity. APCI and APPI were used without mobile phase additions but toluene as a dopant for APPI.

Results:

Direct infusions of 5μg/ml of T, DHT and E2 carried out to optimize the best ionization and MRM transitions in both positive and negative polarity for E2, whereas only positive mode for T and DHT with ESI, APCI and APPI. With ESI, peak responses increased with 0.1 % formic acid in positive mode and 2.5mM ammonium hydroxide in negative mode for E2 as well for DHT in positive ESI. APCI and APPI both resulted in water loss for main precursor ion for DHT and E2 with the highest intensity, although no modifiers were required for high signal intensity, toluene improved ionization with APPI. T had similar responses for all measurement. Regardless of ionization, methanol in the mobile phase enhanced ionization more than acetonitrile. After establishing the optimized MS/MS conditions serum extracts and neat samples were analyzed, to define LLOQs with a precision <20%, and accuracy of 80–120% and matrix effect. APPI had strongest S/N ratio and better LLOQs for all analytes compared to APCI and ESI.

Conclusions

Ionization technique and ion polarity both contribute to sensitivity and susceptibility to matrix interference. With serum extracts, ESI suffered from severe ion suppression compared to APPI and APCI. We conclude that irrespective of ionization, negative polarity was best for E2 whereas, for T and DHT positive polarity is best. APPI has the highest potential to produce high S/N ratio with least ion suppression.

To maximize the LC–MS assay sensitivity for E2 analysis, various approaches have been described. Sample preparation and LC separation of steroids in biofluids such as serum or plasma are reasonably well established, but the most efficient ionization technique for E2 is still to be determined. E2 can be ionized with all the common commercially available ionizationAPPI is much less prone to ion suppression than either APCI or ESI. Evidence indicates that ESI is especially susceptible to ion suppression effects. The high linear dynamic range and the applicability to a broad range of polarity makes APCI a good choice for steroids, especially when the APPI source is not available.

Novel Aspect:

This study comparing ESI, APCI and APPI for the analysis of T, DHT and E2 in serum provides insight on achieving high sensitivity and choice for optimal ionization to avoid matrix interference.

Accurate measurement of estradiol (E2) is important in clinical diagnostics and research. High sensitivity methods are critical for
specimens with E2 concentrations at low picomolar levels, such as serum of men, postmenopausal women and children. Achieving the required assay performance with LC–MS is challenging due to the non-polar structure and low proton affinity of E2.

Previous studies suggest that ionization has a major role for the performance of E2 measurement, but comparisons of different ionization techniques for the analysis of clinical samples are not available. In this study, female serum and endometrium tissue samples were used to compare electrospray ionization (ESI), atmospheric pressure chemical ionization (APCI) and atmospheric pressure photoionization (APPI) in both polarities. APPI was found to have the most potential for E2 analysis, with a quantification limit of 1 fmol on-column. APCI and ESI could be employed in negative polarity, although being slightly less sensitive than APPI. In the presence of biological background, ESI was found to be highly susceptible to ion suppression, while APCI and APPI were largely unaffected by the sample matrix. Irrespective of the ionization technique, background interferences were observed when using the multiple reaction monitoring transitions commonly employed for E2 (m/z 271 > 159; m/z 255 > 145). These unidentified interferences were most severe in serum samples, varied in intensity between ionization techniques and required efficient chromatographic separation in order to achieve specificity for E2.

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Highly Selective and Fast Identification of Brucella by Immunoaffinity-MALDI-TOF Mass Spectrometry

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Keywords: MALDI-TOF; Mass spectrometry; Brucella; Immunoaffinity

Introduction: The genus Brucella contains highly infectious species classified as biological threat agents [1]. Brucellosis is endemic in parts of Iran, and the annual incidence of the human and animal brucellosis has been reported [2]. The timely identification of the microorganism is necessary not only to treat patients effectively but also to establish outbreak management, source tracing, and threat analyses. Matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOFMS) is a rapid method used to analyze biological differences in microorganisms [3]. In this work, we have developed a highly selective method to quickly and accurately identify bacteria in milk/blood samples combining MALDI-TOFMS and immunoaffinity enrichment/separation.

Methods: Synthesized monodispersed magnetic particles are activated and modified with protein A for oriented immobilization of monoclonal antibodies. 5 mL of human whole blood is spiked with B. melitensis cells. The spiked blood is injected into a blood culture bottle and cultured at 35°C in the presence of 10% CO2. Antibodies-modified magnetic beads are used for extraction of B. melitensis from artificially inoculated milk and human blood samples. After inactivation of enriched bacteria, the ethanol/formic acid extraction procedure is applied and analyzed by MALDI-TOF MS.

Results: MALDI-TOF MS results for the identification of Brucella strains have been investigated after enrichment by the immunoaffinity technique based on immobilization of antibody on the magnetic beads. Initial spectra from B. melitensis biovar 16M have been obtained for pattern matching-based bacteria identification and subsequently the experimental spectrum from a blood/milk sample has been compared with the reference one. Because of the method’s high sensitivity, the blood culture time required for diagnosis can be greatly reduced. As a proof, whole blood/milk spiked with a low initial concentration (102 or 103 cells per mL) of bacteria is cultured, then the antibody coated beads are added to these prepared samples after different blood culturing times. Finally, the bacteria attached on the surface of beads have been eluted and analyzed by the MALDI-TOFMS. Therefore, entire diagnosis process (treatment and mass-spec analysis of Brucellae from blood/milk media) can be accurately accomplished less than 48 hours by using this newly developed method.

Conclusions: We have developed an immunoaffinity-MALDI-TOF MS method possessing a simple pretreatment and high sensitivity for rapid detection of Brucella spp. in humans and animal clinical specimens. This method indeed facilitates early accurate diagnosis of the disease which is required for further well-timed actions including effective anti-bacterial therapy or implementing control measures.

Novel Aspect: Immunoaffinity-MALDI-TOFMS method has been developed for the sensitive and rapid identification of Brucella strains.

References
Introduction:
Targeted protein determination using liquid chromatography tandem mass spectrometry (LC-MS/MS) offers a more reliable alternative to traditional immunometric assays. The interest in applying LC-MS/MS in protein biomarker analysis is hence increasing. For low volume samples (<50 µL) a highly specific sample preparation step normally is necessary to detect low abundance biomarkers (low pM-level).

Methods:
Two different approaches are discussed: 1) NanoLC-MS/MS analysis of human chorionic gonadotropin (hCG) from dried matrix samples (blood, serum, plasma and urine) by affinity sample clean-up of whole protein followed by tryptic digestion. 2) NanoLC-MS/MS analysis of progastrin releasing peptide (ProGRP) from 50 µL of serum using affinity extraction of proteolytic epitope peptide after digestion.

Results:
Ovarian and testicular cancer marker hCG was detected from four different biological matrices (whole blood, serum, plasma and urine) from 15 µL of dried matrix spots at its upper reference level (low pM-level). The detection limits (S/N=3) ranged from 14.5 pM (whole blood) to 30.5 pM (urine). The method was briefly evaluated for dried serum samples providing satisfactory results (RSD(%)=13-29% and accuracy (97-106%)) [1].

The small-cell lung cancer marker ProGRP was also determined at its upper reference level (low pM-level). Detection limits (S/N=3) of 3.9 pM from 50 µL of serum was observed when using monoclonal antibodies developed against intact proteins to capture a proteotypic epitope peptide. Compared to intact protein extraction, this concept, which we call epitope fishing, provided cleaner extracts.

Conclusions:
NanoLC and state-of-the-art triple quadrupole MS, and affinity clean-up is a powerful combination for determination of low abundance protein biomarkers available in low volumes. Both affinity clean-up of whole protein (hCG in 15 µL of dried matrix spot) and of proteotypic epitope peptide (ProGRP in 50 µL serum) have been successfully enabled at low pM-level.

Novel Aspect:
Determination of low abundance protein biomarkers is enabled from small volumes (< 50 µL) by affinity clean-up and state-of-the-art nanoLC-MS/MS.

References:
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Keywords: aspergillosis; non-invasive; diagnosis; siderophore; imaging

Introduction: Aspergillus fumigatus is a ubiquitous saprophytic airborne fungus responsible for more than one million deaths every year. Its siderophores represent important virulence factors contributing to microbiome-metabolome dialog in a host. From diagnostic point of view, the application of Aspergillus secondary metabolites is promising due to non-invasiveness and high speed, sensitivity and potential for standardization.

Methods
Using a model of experimental aspergillosis in immunocompromised Lewis rats [1], the fungal siderophores ferricrocin (FC) and triacetylfusarinine C (TAFC) were monitored in rat urine before and after lung inoculation with A. fumigatus conidia. Molecular biomarkers were separated in high/low dose infection models by high performance liquid chromatography (HPLC) and detected by mass spectrometry (MS). Rat results were compared with those in a set of 11 human clinical samples.

Results
In high dose rat model, the initial FC signal reflecting the aspergillosis appeared as early as four hours post-infection. Results for seven biological replicates showed increasing exponential metabolite profiles in three-day frame of the experiment. TAFC was shown to be less populated biomarker produced by A. fumigatus 1059 CCF strain exhibiting identical kinetic profile to that of FC. Among nine biological replicates in the low dose model, three animals did not develop any infection up to experiment termination (day 10). One animal experienced an exponential increase of metabolites and died on the day six post-infection. All remaining five animals revealed constant or random FC levels and exhibited little or no symptoms. µPET/CT imaging, although it was not as an early tool, further determined the infection location in vivo and allowed to visually follow the infection progression in time. In a set of 11 human ICU patients with probable aspergillosis, siderophore-based approach was more sensitive than standard galactomannan (GM) testing.

Conclusions
Siderophore detection in urine (rat or human) by mass spectrometry represents an early, innovative and non-invasive tool for diagnosing aspergillosis. Further studies are needed to clarify the effect of antifungal prophylaxis to Aspergillus secondary metabolite production in human host. The general application of microbial siderophores in human diagnostics can soon represent the same clinical breakthrough like ribosomal protein typing 10 years ago.

Novel Aspect
Siderophore detection in urine (rat or human) by mass spectrometry represents an early, innovative and non-invasive tool for diagnosing aspergillosis. Further studies are needed to clarify the effect of antifungal prophylaxis to Aspergillus secondary metabolite production in human host. The general application of microbial siderophores in human diagnostics can soon represent the same clinical breakthrough like ribosomal protein typing 10 years ago.

References
1377 - WHICH LC-MS/MS PLATFORM IS MOST APPROPRIATE FOR THE QUANTITATIVE ANALYSIS OF STEROIDS IN URINE, SERUM AND ORAL FLUID FOR CLINICAL RESEARCH

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Keywords: steroids, serum, triple quadrupole, liquid-liquid extraction

Introduction:
Steroid hormones control many physiological aspects in our body. A robust, sensitive, accurate and specific LC/MS/MS analytical method has been developed for the quantitation of 19 steroids in serum. A simple liquid-liquid extraction sample preparation allows achieving the required sensitivity. This research study was carried out to evaluate which LC-MS/MS instrument is best suited to characterize and quantitate the steroids in serum.

Methods:
Thermo Scientific™ TSQ Fortis™, Thermo Scientific™ TSQ Quantis™, and Thermo Scientific™ TSQ Altis™ tandem mass spectrometers and a Thermo Scientific™ Vanquish™ HPLC system were utilized. A 200 µL serum sample was used and a Thermo Scientific™ Accucore™ C18 column achieved baseline chromatographic separation. Quantitative analysis was performed using SRMscan in positive and negative mode. Accuracy of the analytical method was verified using reference samples.

Results:
Good linearity and reproducibility were obtained with the concentration range from 10pg/ml to 1000 ng/ml for the steroids with a coefficient of determination >0.995 for all mass spectrometer platforms. The lower limit of detection (LLOD) and lower limit of quantitation (LLOQ) were determined. Excellent reproducibility was observed for all platforms, being the %CV for each calibration point always < 10% for all steroids. The inter-assay precision was determined by extracting and quantifying three replicates of in-house control material resulting in %CV for the steroids of < 10% deviation from the targeted mean. The inter-assay precision was determined over three consecutive days and was found to have a %CV < 10% for each steroid within its respective linear range for the three levels of pooled serum sample control material respectively. The accuracy was determined by the analysis of in-house control material as the percentage deviation from the targeted mean. The results were < 10% for all levels in each matrix.

Conclusions:
A sensitive, simple, specific and accurate liquid chromatography QQQ mass spectrometry analytical method was developed and verified for the measurement of 19 steroids in serum and applied to any Thermo Scientific™ tandem mass spectrometer platform. The sample preparation technique is quick and easily applicable for high throughput analysis. The reported method achieves research laboratory required analytical performances for steroids analysis in serum.

Novel Aspect:
Utilization of various triple quadrupole mass spectrometers to determine the most appropriate platform for steroid analysis in serum.
Keywords: exhaled breath condensate (EBC) samples, amino acids, underivatization step, type 1 diabetes, LC-MS/MS,

Introduction
Insulin deficiency in type 1 diabetes leads to metabolic changes. Despite technological progress, early detection of the disease is problematic and checking sugar level is burden for 66% of patients. The use of exhaled breath condensates as a non-invasive sampling method might help patients. Liquid chromatography technique coupled with mass spectrometry may help in the detection of differences in amino acid profiles in patients with type 1 diabetes and healthy ones.

Methods
A procedure for preparing EBC samples with underivatized amino acids to chromatography analysis is based on vacuum evaporation. For the determination of low molecular weight amino acids, liquid chromatography coupled to mass spectrometry (LC-MS/MS) with a hydrophilic interaction chromatography (HILIC) column was used. All amino acid profiles were determined in exhaled breath condensates samples.

Results
A sensitive and specific method was used for the simultaneous determination of the entire L-amino acid profile in exhaled breath condensate samples, a matrix taken in a completely non-invasive way, in pediatric patients with type 1 diabetes, and the results were compared with the control group. The use of vacuum evaporation as a sample preparation step has reduced the consumption of solvents, which is consistent with the principles of green chemistry. Thanks to the use of the HILIC column, it was possible to identify 23 amino acids without the necessity to derivatize them. The condensates of exhaled breath of pediatric patients with diabetes in whom the disease has been diagnosed have been analyzed. Therefore it is a stabilized research group, so no statistically significant differences were observed in the amino acid profiles of exhaled breath condensates in children with type 1 diabetes compared to healthy control group.

Conclusions
The results indicate that it seems to be advisable to use the amino acid analysis in EBC, which is a method that does not burden the patient doing multiple blood glucose measurements and treated with insulin. Due to the chronic nature of diabetes, chronic complications are important for the patients' survival time and the quality of life. The EBC can be used as a new method allowing for their early detection and can positively affect the effects of treatment of type 1 diabetes.

Novel Aspect:
A new research method based on LC-MS/MS has been developed, allowing for non-invasive diagnostics of metabolic changes occurring in type 1 diabetes.

References
Introduction:
Exhaled breath condensate (EBC) is a promising specimen for use in diagnostics and therapy monitoring. It consists of condensed water vapor, water soluble volatile organic compounds (VOCs), as well as non-volatile hydrophilic and hydrophobic components [1]. It can be easily and non-invasively obtained, presenting an advantage over sputum, which is a more commonly used sample for respiratory disease assays, but is hard to obtain from children and weak patients.

Methods:
In this study, we explore the effects of meal and sputum induction (SI) on the EBC profile. Samples were collected from 10 healthy volunteers, divided into 2 batches. For each batch, samples were collected for 3 days at different timepoints, before and after SI and meals. Double filtration sterilization was done in consideration of plans for infectious disease biomarkers study. VOCs were analyzed using GC-MS and the non-volatile components using LC-MS.

Results:
Preliminary analysis of GC-MS and LC-MS results using principal components analysis (PCA) showed inter-batch effects for the GC-MS profiles across three days of sampling indicating possible differences in the VOC composition in the sampling environment of the two batches. It is also observed that for Batch 1, there is some separate clustering of samples before and after SI. This could be indicative of a change in the EBC VOC profile after SI. Though there were still some inter-day batch effects observed, samples before a low-fat breakfast clustered closely while the samples for each batch collected 30 minutes and 2 hours after the meal clustered together. Samples before and after a high-fat breakfast were found to cluster together. PCA of LC-MS data showed distinct clustering of samples before and after SI while samples before and after meals were mostly overlapped.

Conclusions:
Results revealed that there can be changes in the VOCs and non-volatile components of exhaled breath after sputum induction. The VOCs profile can also be affected by the meal taken before sampling while the profile of non-volatile components does not significantly vary whether sampling is done before or after meal. This indicates that EBC collection should not be performed after sputum induction of patients and at least 2 hours after a meal in breath biomarkers studies.

Novel Aspect:
This study demonstrates multi-platform profiling of EBC components using GC-MS and LC-MS to establish the effects of meals and sputum induction in EBC collection.

References:
DEVELOPMENT AND VALIDATION OF A HIGH SENSITIVITY ASSAY OF ESTROGENS IN HUMAN PLASMA BY UHPLC-MS/MS WITHOUT DERIVATIZATION.

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Keywords: Estrogens, plasma, high-sensitivity, UHPLC-MS/MS.

Introduction:
Estrogens play important roles in many biological functions as primary female sex hormones. In males, estrogens are present at lower concentrations but are also involved in several mechanisms. Modification of circulating estrogen levels can be associated with both positive and negative health effects.

In the last decade, LC/MS/MS has proven its capabilities in terms of sensitivity and specificity to become the gold standard methodology in steroid analysis. However, measuring estrogens at very low level remained challenging. Many published methods involve complex sample preparation, large sample volumes or derivatization. These processes are not compatible with a routine laboratory workflow. Here we present a UHPLC-MS/MS method to measure estrone (E1), estradiol (E2) and estriol (E3) together in the low pg/mL range.

Methods:
300 µL of plasma were spiked with 30 µL the ISTD solution (deuterated E1, E2 and E3) and then loaded on a SLE+400 96-well-plate (Biotage, Sweden). After 5 min incubation, target compounds were eluted with 2x 500 µL of Ethylacetate/Hexane 75/25 v/v. After evaporation and reconstitution, extracts were analyzed using a Nexera X2 coupled to a LCMS-8060 tandem mass spectrometer (Shimadzu Corp., Japan). Using a gradient of water and methanol with NH4F, all compounds were separated on a Raptor Biphenyl column (Restek, USA) with a cycle time of 3.5 min. Data acquisition used MRM mode with the electrospray ion source operating in negative mode.

Results:
To fully optimize the detection of estrone (E1), estradiol (E2) and estriol (E3) in plasma, the method considered the impact of alternative sample preparation protocols in addition to enhancing ion sampling efficiency and MS/MS ion signals.

Several sample preparation procedures were evaluated including protein precipitation (PPT), solid phase extraction (SPE), liquid-liquid extraction (LLE) and supported liquid extraction (SLE). In this work, SLE resulted in the highest extraction recovery with a minimal matrix interference. SLE was also considered to be a simple technique to use routinely with a relatively fast sample cycle time and created further opportunities in automation. Extraction recovery was between 96 to 97% for E1, 88 to 102% for E2 and 94 to 108% for E3. Matrix effect ranged from 0.90 to 1.00 for E1, 1.19 to 1.29 for E2 and 0.91 to 1.11 for E3.

As SLE selectively extracted E1, E2 and E3 whilst minimizing possible co-extraction of isobaric matrix interferences the peak area variance for the internal standards (ISTD) in patient samples and QC was less than 16% (%RSD was 16%, 9% and 11% for D4-E1, D3-E2 and D3-E3 respectively, n=27).

As a result, we obtained limits of quantification of 0.5 pg/mL (E1), 1 pg/mL (E2) and 5 pg/mL (E3). To our knowledge, such limits of quantification have never been reported with a simple sample preparation and no derivatization. The linear calibration range was then 0.5-500 pg/mL, 1-500 pg/mL and 5-1000 pg/mL for E1, E2 and E3 respectively. Such sensitivity was successfully used to report samples that could not be quantified before with immunoassays.

The assay also generated acceptable precision and accuracy in repeated use meeting the needs of a routine steroid hormone analysis. In validating the method certified reference serums and individual patient samples with known estrogens concentrations were measured and good correlation was obtained.
Conclusions:
A method for routine high-sensitivity analysis was successfully developed for estrogen analysis in human plasma. The sample preparation is easy and very efficient to ensure low pg/mL limits of quantification as well as ruggedness and accuracy. Linearity of the assay allows to measure all kind of samples, not only the low-level ones. This method could be helpful to support clinical research.

Novel Aspect:
Development of a highly sensitive routine platform for estrogens analysis in clinical pathology laboratories.
ICP-MS immunoassay using metal-doped nanoparticles for multiplex detection of HIV-1 p24 and HBsAg.

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ICP-MS immunoassay using metal-doped nanoparticles for multiplex detection of HIV-1 p24 and HBsAg.

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Keywords: ICP-MS immunoassay; Nanoparticles; Determination of viruses; Multiplex detection; Clinical analysis.

Introduction: Because pathogen species can interact within the host and coinfection is quite common, it is crucial to detect viruses simultaneously in clinical analysis for accurate and fast diagnosis. So far, we have developed ICP-MS immunoassay using synthesized nanoparticles for multiplex detection and high sensitivity through particle tagging and signal amplification. And, the method has been demonstrated in a wide range of biotargets from small molecules to large cells.

Methods: In this work, we expand the ICP-MS immunoassay to HIV-1 p24 and HBsAg selected among the representative viruses. For their detection, metal-doped magnetic nanoparticles and silica nanoparticles were synthesized for target extraction and tagging as probes, respectively, and sandwich-type conjugates of selected proteins representing the targets were formed through immunoreaction. Then, the quantitative determination was performed using ICP-MS.

Results: For target extraction and multiplex detection, Fe3O4 magnetic nanoparticles were doped with Cs, and silica nanoparticles were doped with Gd/RhBITC for HIV p24 and Eu/RhBTIC for HBsAg, and then both were immobilized with the antibodies of HIV-1 p24 and HBsAg for immunoreaction, confirmed by a confocal laser fluorescence microscope and SEM. After the formation of sandwich-type conjugates, the concentration of both targets was simultaneously determined through a ratiometric detection of the doped metals using ICP-MS. The calibration curves showed a linear range of 0.78-8.0 ng/mL and 10-100 pg/mL with a detection limit of 0.61 ng/mL and 11.54 pg/mL, respectively. The multiplex detection of HIV-1 p24 and HBsAg spiked in diluted human serum produced recovery rates of 85-95%.

Conclusion: So far, we have demonstrated ICP-MS immunoassay for a multiplex detection of biomarkers, CRP (cardiovascular disease), AFP (tumor), and NSE (heart disease). In this work, we successfully expand its application to simultaneous determination of representative viruses, HIV-1 p24 and HBsAg in human serum for the first time, which implies great potential for clinical application.

Novel Aspect: In this work, we successfully demonstrated ICP-MS immunoassay using magnetic nanoparticles to determine viruses simultaneously for the first time with high sensitivity.
Introduction:
Tranexamic acid (TXA) is an antifibrinolytic agent routinely used in clinical settings to stop or reduce blood loss during surgeries. Attainment of TXA blood plasma concentration within the therapeutic range is difficult because it presents high interpatient variability besides, persistently high TXA concentrations have been associated with seizures. Therefore, a quantitative point-of-care method based on SPME coupled to mass spectrometry was developed and validated.

Methods:
TXA was monitored directly from plasma of 6 patients undergoing cardiac surgery using biocompatible SPME (BioSPME) fibers. The coating, 4 mm-long and 25 µm thickness, used HLB® particles (≤5 µm) embedded in a polyacrylonitrile polymer. A microfluidic open interface (MOI) was used to couple these fibers directly to MS. The developed point-of-care method was cross validated against a SPME-LC-MS/MS method using Passing Bablok regression and Bland-Altman plot.

Results:
BioSPME-MOI-MS/MS revealed that the point-of-care method herein developed was able to accurately reproduce the profiles of 6 cardiac surgical patients as the profiles did not show statistical difference to those generated by the bioSPME-LC-MS/MS method. Statistical cross validation by means of the Bland-Altman plot and the Passing Bablok regression yielded for the former that 92% of the data pairs compared were distributed randomly within the acceptable range of the limits of agreement, demonstrating that the method had no biased tendencies for the results. The latter supported that the two methods demonstrated good statistical agreement achieving a slope of 0.984. Given that the expected concentration range of tranexamic acid is between 80 – 500 µg/mL, the method produced an acceptable linear dynamic range of 50-1000 µg/mL. One of the main advantages of this approach involves a single sample turn-around time of 15 min while in a high-throughput mode (96-well plate), the speed of analysis is below 30 seconds.

Conclusions
Bio-SPME-MOI-MS/MS proves to be a reliable and feasible tool for rapid therapeutic drug monitoring. Affording total times of analysis per sample as low as 30 seconds in its high-throughput mode configuration, the currently presented method offers comparable results to the gold standard HPLC-MS/MS method with acceptable RSDs, while only necessitating a fraction of the time needed for implementation of the HPLC-MS/MS method.

Novel Aspect:
High throughput therapeutic drug monitoring achieved in a fraction of the time via point of care SPME-MOI-MS/MS.
References
Introduction:
Profilin 1, encoded by the PFN1 gene as one of the recently identified ALS genes, is an evolutionarily highly conserved protein[1]. Mutant PFN1 proteins form aggregate in vivo and in vitro which can be a cause of motor neuron death[2]. Mass spectrometry-based techniques can open new insights into characterization of biomarker proteins in neurodegenerative disorders. Hence, we have investigated PFN1G118V aggregation and its inhibition using mass spectrometry.

Methods:
Proteins in aggregates were identified by bottom-up proteomic analysis using LC-MS/MS and MALDI-TOF. The effect of purified natural compounds was investigated by western blotting in transiently transfected (PFN1G118V and PFN1WT) cells. Moreover, we expressed PFN1WT and ALS-linked PFN1G118V in E. coli and purified them using anion-exchange chromatography. Recombinant PFN1G118V was incubated with compounds at various ratios and analyzed by mass spectrometry.

Results:
The primary result of bottom-up proteomic analysis on the insoluble fraction of spinal cord lysates of hPFN1G118V transgenic mice revealed the identity of PFN1 along with other up- and down-regulated proteins in aggregates. Additionally, our data on the insoluble fractions of transfected and non-transfected cells showed that only mutant PFN1 forms aggregate compared with wild type and non-transfected cells. In order to block the aggregation of mutant PFN1, we tested natural compounds derived from a medicinal plant and observed that treatment with our ROG-102 compound decreased the aggregation of PFN1G118V in vitro. To test whether this compound can directly interact with PFN1G118V and inhibit its aggregation, we sought to assess it in a cell-free system. The non-covalent interaction of recombinant PFN1G118V with the selected compounds being investigated kinetically by LC-MS/MS in non-reduced form and in digested form. Additionally, our results have been used for the binding site determination.

Conclusions:
In conclusion, our mass spectrometry results can be a significant step on the road of detailed deciphering the neurodegenerative process. The identification of proteins in the aggregates will shed light on cellular protein interactions with mutant PFN1. Moreover, anti-aggregation properties of natural compounds may be highly valuable for drug discovery and therapeutic development for ALS.

Novel Aspect:
We were able to show the anti-aggregative activity of a natural compound on mutant PFN1. This novel finding has the potential as new therapy to treat motor neuron disease.

References
DETECTION AND QUANTIFICATION OF CARBOHYDRATE DEFICIENT TRANSFERRIN BY MALDI-COMPATIBLE PROTEIN CHIPS PREPARED BY AMBIENT ION SOFT LANDING

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Keywords: Ambient ion landing, Protein chips, Immuno-affinity enrichment, MALDI, Carbohydrate Deficient Transferrin

Introduction:
Transferrin is the most important iron-transport carrier in the human body. Severe alcohol consumption leads to alterations in glycosylation of transferrin (CDT). Mass spectrometry can provide fast detection and quantification of transferrin isoforms since they have different molecular masses. In this study we use antibody chips prepared by ambient soft ion landing in combination with MALDI -TOF for detection and quantification of transferrin isoforms.

Methods:
Protein chips were prepared by functionalization of indium tin oxide glass using anti-transferrin antibody. Several microliters of patient serum were applied on the antibody-modified spots and after incubation, washing and matrix deposition, the transferrin molecule forms were detected by MALDI-TOF mass spectrometer. The CDT values obtained by MALDI chip method were compared with the results obtained by capillary electrophoresis (CE).

Results:
Functionalized MALDI chips were successfully used for enrichment and detection of CDT from human serum. A cohort of samples from 186 patients was analyzed using functionalized MALDI chips and the results were compared with data obtained by CE. Out of the 186 samples, 44 were positively identified as belonging to alcoholic patients, while 142 were negative by MALDI chip approach.

Conclusions:
Functionalized MALDI chips modified by anti-transferrin antibody prepared by ambient soft ion landing were successfully used for detection and quantification of CDT from human sera. The correlation of the data obtained by the CE and the chip-based MALDI was r=0.986, 95% confidence interval.

Novel Aspect:
MALDI chips prepared by ion soft landing are suitable for quantification of CDT.

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446 - ACETYLCHOLINE AND ACETYLCHOLINESTERASE IN A NON-NEURONAL TISSUE (CORNEAL EPITHELIUM)

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Keywords: Acetylcholine, Acetylcholinesterase, Corneal Epithelium Cells, Diurnal, Twilight and Nocturnal Mammals

Introduction:
Acetylcholine (ACh) and Acetylcholinesterase (AChE) are integral parts of the central and peripheral nervous system. The substances have in addition been observed in tissues outside the nervous system such as corneal epithelial cells (CEC). ACh and AChE were analysed in CEC of diurnal, twilight and nocturnal mammals by tandem HPLC/MS-MS and fluorometric methods, respectively.

Methods:
CEC from 6 different species (cattle, sheep, pig, horse, goat and cat) were obtained from local slaughterhouses. The CEC was rubbed off the eyes with a glass slide and homogenized using a pellet mixer for 2 min in mobile phase or MilliQ-water. For analysis of ACh, a HPLC/MS-MS method was used. For analysis of AChE, Amplite™ Fluorometric Acetylcholinesterase Assay Kit from AAT Bioquest® Inc was used according to the manufactures recommendations.

Results:
The method for analysing ACh in CEC in this study was a modified version of the method described in [1]. Endogenous quaternary ammonium compounds (including ACh) were confirmed to be found in the CEC from all species as described in references [1,2]. The concentration of ACh and AChE in the CEC were varying between the different species. Diurnal species revealed a concentration of ACh in the range 0.18 to 2.80 µg ACh/mg CEC, in contrast to twilight and nocturnal species where ACh was close to zero, similar to previous observations [3]. Twilight and nocturnal species revealed a high concentration of AChE in the CEC, in contrast to diurnal species that revealed a low concentration. For AChE the concentration was varying from 0.5 mUAChE/mg CEC in cattle and horse to 20.4 mUAChE/mg CEC in cat.

Conclusions:
Diurnal animals (cattle, horse, goat and sheep) revealed high amounts of ACh in the CEC, in contrast to cell samples from twilight and nocturnal animals (pig and cat) that contain low amounts of ACh in CEC. For AChE, the picture is reversed: diurnal animals revealed low amounts of AChE and twilight and nocturnal revealed high amounts of AChE in CEC.

Novel Aspect:
To our knowledge, this is the first time Acetylcholine and Acetylcholinesterase has been measured in CEC from different mammals.

References

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INTRODUCTION

LC-MS/MS ID is nowadays recognized as the gold standard method to quantify steroids and clinical laboratories are adopting MS for steroids analysis to overcome inaccuracy of immunometric Methods.[1] Costs of LC-MS/MS are now competitive, but this technique is still less automatized, and methods are less standardized.[2] In setting up this new method we focused our attention on characteristics that facilitate routine application also with different instruments.

METHODS

We set up a two dimensional chromatography. The first column (C18 Luna 5 μm 2 cm x 2mm) was used as sampling column and flushed with a polar eluent to eliminate polar compounds and concentrate steroids; the analytical column (C18 luna 3μm 5 cm x 2 mm) separated androgens, progestogens, glucorticoids that were quantified with a SCIEX 6500QTRAP in positive mode. Different eluents [3] and a PFP 3 μm 5 cm x 2 mm analytical column allowed quantification of estrogens in negative mode.

RESULTS

This method allows the simultaneous determination of testosterone, androstendione, progesterone, 17OH progesterone, dihydrotestosterone, cortisol and cortisone, or estrone and estradiol, in a 12 min run time with high sensibility. Samples are simply diluted with the internal standard mix in methanol and water, centrifuged to eliminate proteins, and injected. It was validated calculating precision, accuracy and sensibility for each compound. To assess the method flexibility we tested it with different biological samples (urine, serum, tissue homogenates, follicular fluids), and established that the minimum sample dilution to avoid matrix effect and preserve chromatographic performance is 1:5 with an injection volume range from 10 to 100 μL. Using the SCIEX 6500QTRAP we dilute urine and serum samples 1:10 with an injection volume of 25-50 μL. The method is already used in our hospital (AOU-Careggi- Firenze)) for routinely analysis of urinary free cortisol and of cortisol/cortisone ratio and It will be soon adopted for serum steroids.

CONCLUSIONS

The method fit the routine characteristics being simple, fast and robust. The sensitivity reached allows an accurate determination of androgens also in women and pre-puberal subjects. The efficiency of the proposed chromatography confers flexibility necessary to the scope. In fact, the possibility to change sample dilution or injection volume, without affecting performance, are useful to adapt the method to different sample and different instruments.

NEOLOGISTIC

The proposed LC-MS/MS system allows the analysis of steroids in many biological matrices with minimal sample preparation, maintaining high level of detection performances.

REFERENCES

Today’s clinical laboratory is focused on the delivery of accurate results at the right time to the right user. To reach this goal, all components of the laboratory, from the technologists on the bench to the analyst handling the LIS (laboratory information system) need to work in an integrated and coordinate way. The large number of methodologies used in the clinical laboratory can make the integration challenging and standardization processes are becoming the answer to the challenges brought by this diversity. Among the methodologies, mass spectrometry, whether single stage or tandem, often coupled with liquid or gas chromatography, is becoming one of the most widespread techniques in the clinical laboratory, because of its sensitivity, specificity, rapid turnaround time, and low sample volume requirement. Mass spectrometry is therefore used in many areas, from newborn screening to therapeutic drug monitoring, endocrinology, infectious diseases, in addition to being used as a research tool in metabolomics applications.

We have evaluated standardization processes for the mass spectrometry area in our laboratory, in which we have two opposite settings: 1) newborn screening, high throughput/rapid turnaround time/low sample volume and 2) biochemical genetics, low throughput/high complexity/rapid turnaround time. From our preliminary observations, we have seen that standardization of the platform used may be beneficial in the first setting, while in the second setting, standardized analytical/instrument methods could improve the workflow, independently from the platform.

In both cases, automated data transfer to the LIS is a key factor in delivering test results rapidly and accurately. The implementation of standardization should take into account the laboratory setting in order to optimize its efficiency. Each laboratory should identify “why standardize?” and “what to standardize?” before identifying “how to standardize”. This will fulfill the mission of the clinical laboratory by improving operational efficiency without compromising delivery of care.
MASS-SPECTROMETRY APPROACH FOR RAPID INTRAOPERATIVE IDENTIFICATION OF BRAIN TUMOR TISSUES - A WAY FOR ONLINE ANALYSIS

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Keywords: ambient mass-spectrometry, clinical application, brain tumor, neurosurgical support, microextraction.

Introduction:
It is known that the lipid metabolism reprogramming is one of the hallmarks of cancer so mass-spectrometry-based method of lipid profiling is a promising approach for precise intraoperative tumor identification. In this research we propose the online method of tissue profiling based on express and simple capsular extraction followed by ESI with an identification algorithms based on a search through a reference database containing mass spectra of different tumors.

Methods:
The online microextraction system consists of disposable capsules compatible with any standard ESI source. Tissues, dissected by the surgeon were placed into the capsules which are then connected to the ESI interface for analysis. Features were extracted from mass-spectrometry data and aligned for further analysis. Significant features were detected by shrinkage discriminant analysis and classifiers were developed with lasso-regression.

Results:
It was demonstrated that it is possible to prepare large amount (up to 5 per minute) of capsules with samples immediately during the operation because of simplicity of placing sample into the preassembled capsules so almost all the delay between receiving the tissue from surgeon and analysis depends only from transportation time between operational suite and laboratory room. The minimum amount of sample is very small (less than 1 cubic mm) so proposed method could be used during neurosurgery intervention where amount of resection could be very small. The data obtained with proposed microextraction method are in good correlation with our previous results obtained with spray-from-tissue ionization source. The rapid identification of the tumor type is allowed by the search inside the collected reference database containing mass spectra of samples that are also characterized by histopathological Methods:

Conclusions:
Application of microextraction capsules significantly simplifies the manipulations with samples by excluding any special sample preparation steps. Rapidity of method is also very important during the surgery as the minimization of surgery duration improves patient’s postoperative prognosis. Developed data processing algorithms and classifiers provide the reliable differentiation of extracted brain tissue by their lipid profiles.

Novel Aspect:
The online mass-spectrometry profiling of brain tissues could be used as intraoperative technology for characterization and identification of removed tissues.
589 - DEVELOPMENT AND VALIDATION OF A SENSITIVE LC-LINEAR ION TRAP METHOD FOR THE DETERMINATION OF TRACE DI-IODOTHYRONINES IN HUMAN BLOOD SERUM

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Keywords:
Thyroid hormone metabolites, serum, tandem mass spectrometry, MS3, validation

Introduction:
In addition to the thyroid hormones (TH)3,5,3'-triiodothyronine (T3) and L-thyroxine (T4), thyroid hormone metabolites (THM) to exert their own distinct biological actions[1]. By incorporating MRM/MS3 switched scanning and extract de-lipidation, we have modified and validated out tandem mass spectrometry method[2] for 3,5-diiodothyronine (3,5-T2) and 3,3'-diiodothyronine (3,3'T2) in human blood serum, and applied it to measure T2 concentrations in healthy individuals.

Methods:
The method includes mixed mode cation exchange extraction (SPE) of 150µL serum, followed by de-lipidation using Waters Ostro sorbent (both using a 96-well plate format). The liquid chromatography-mass spectrometric method uses switched mode (MRM/MS3) acquisition using a linear ion trap (LIT).

Results:
Endogenous 3,5-T2 and 3,3'T2 were detected in commercially obtained serum, in addition to T3,3,5',3'-triiodothyronine (rT3) and T4. Inter- and intra-day accuracy and precision for exogenously spiked TH/THM were in the range 70-100% and less than 20% RSD respectively for low, medium and high concentrations. Excellent linearity was obtained across a range from 0.01 nM to 250 nM. Sera from healthy individuals contained concentrations of T3 and T4 (65.6-181.5 nM) within expected reference ranges (1.3 - 3.1 and 65.6 - 181.5 nM respectively) [3] whilst concentrations of 3,5-T2 and 3,3'-T2 were significantly lower (0.02 – 0.05 and 0.1 – 0.25 nM respectively). Limits of quantification (LOQ) for T2 isomers were 0.01 nM. Matrix effects for all analytes were 30% or less.

Conclusions:
The use of switched MRM/MS3 scanning enables quantification of endogenous TH/THM with widely differing concentrations in a single analysis, with excellent inter- and intra-day accuracy and precision. Due to enhanced selectivity and low chemical noise, MS3 scanning delivered 4-5 times lower LOQ compared with traditional MRM for T2 isomers. In healthy individuals both 3,5-T2 and 3,3'-T2 appear to be minor metabolites (derived from T3 and T3/rT3 respectively).

Novel Aspect:
The direct SPE extraction of serum without preliminary protein precipitation, combined with solid phase de-lipidation and switched MRM/MS3 scanning.

References
Introduction:
Acute respiratory distress syndrome (ARDS) is a severe form of lung injury characterized by an increased inflammatory burden and changes in the alveolar-capillary membrane permeability with extravasation of fluid and protein into the alveoli[1]. Despite the clear association between plasmatic hypoalbuminemia and poor outcome, there are no data on albumin metabolism in ARDS patients.

Methods:
113C-Leucine, as albumin precursor, was administered to 13 ARDS adults and to 8 patients without lung disease (controls). Albumin was isolated from serial plasma and tracheal aspirate (TA) samples, and its kinetic parameters were derived from the 13C enrichment curve obtained by isotope ratio mass spectrometry[2]. As index of alveolar-capillary permeability the TA/plasma albumin ratio was measured.

Results:
In patients with ARDS the fractional synthetic rate was significantly higher than in control patients [15.1 (12.3-22.7) vs 11.9 (8.9-12.9) %/day; p=0.04]. Median albumin secretion time and half-life were 0.7 (0.3-1.9) vs 0.6 (0.3-1.0) h, and 43.3 (35.7-64.9) vs 42.7 (33.3-76.0) h in ARDS and in control patients respectively, no significant differences were observed.
In patients with ARDS the TA to plasma albumin ratio measured in the early phase after the diagnosis of acute lung injury was 7-fold times higher than the level found in control patients (p<0.001). In the ARDS group the TA/plasma albumin ratio was significantly correlated with the PaO2/FiO2, as index of lung dysfunction (r=-0.732, p=0.016).

Conclusions:
In this study, stable isotopes have been used to better understand albumin metabolism during a disease state like ARDS. Albumin synthesis rate in ARDS patients was higher than in control adults. Furthermore, the comparison between TA/plasma albumin ratio of ARDS and control patients revealed a dramatic increase in alveolar-capillary membrane permeability during the genesis of ARDS.

Novel Aspect:
This study will give new insight into albumin turnover and compartmental distribution in patients with ARDS.

References
Keywords: metabolic profiling, gangliosides, Alzheimer's disease, monosialoganglioside GM1, membrane lipidomics

Introduction:
Alzheimer's disease (AD) is the most common cause of dementia (60-80% of cases) in elderly population worldwide [1]. The current body of research supports the concept that protein aggregation initiates the onset of AD [2]. However, in spite of the large number of studies, a mechanism of pathogenesis and potential treatment for AD remains elusive. We explored the distribution of membrane lipids (gangliosides) as they supposedly trigger protein aggregation [3].

Methods:
We applied ultra-high performance liquid chromatography (UHPLC) and selected reaction monitoring (SRM) as well as high resolution/accurate mass (HR/AM) orbital ion trap mass spectrometry to characterize membrane lipid composition (GM1, GM2, GM3, GD1a/b, GD2, GD3, GT1b, GQ1b) in human clinical samples (i.e. serum, cerebrospinal fluid - CSF, brain tissue) and samples from animal model of aging (Sprague Dawley, Wistar). We compared 1 month and 11 months old animals (n=26).

Results:
Our results show optimization of ganglioside extraction protocol and development of UHPLC-SRM assay. We tested several solvent systems to achieve approx. 70% extraction efficacy in biological matrix. UHPLC was optimized and suitable analytical column and mobile phase additives chosen to allow for separation of mono-, di-, tri- and quatrosialo species. Gangliosides present in biological samples were identified using HR/AM mass spectrometry. Consequently positive and negative ion mode SRM assays were utilized for determination of gangliosides. The dominant brain gangliosides were GM1, GD1a/b and GT1b and similar composition was found in CSF. In serum the most abundant species are GM3, GM2, GD1a/b, GD2 and GT1b. We have used custom synthesized isotopically labelled internal standards for absolute quantification in biological samples.

Conclusions
New analytical method established to determine 9 major gangliosides in brain tissue, CSF and serum samples with nanomolar LODs. The differences in ganglioside composition were demonstrated in specific brain structures (i.e. cerebral cortex, cerebellum, hypothalamus, hippocampus, brain stem). The composition of gangliosides in brains reflected CSF and partially also serum concentrations. The age dependent changes in composition of major gangliosides were evaluated.

Novel Aspect:
Novel analytical approach to study distribution of important membrane lipids, which are supposedly playing role in Alzheimer's disease pathogenesis.

References
Introduction:
In complex biological samples similar small molecules often exist as different structural isomers or similar phase II conjugates such as glucosides or glucuronides. This often results in multi reaction monitoring (MRM) transitions that are too general for reliable assignment of selected unknowns or suspects in a triple quadrupole instrument. However, by letting data dependent acquisition techniques guide selection of MRMs, a reliable and sensitive method was developed.

Methods:
Benzoxazinoid-rich complex biological samples were fractionated using consecutive SPE and HPLC to concentrate benzoxazinoid metabolites. Our established HPLC-MSMS (ABSciex Qtrap 4500) benzoxazinoid quantification method was adapted for data dependent acquisition (DDA/IDA) using mainly enhanced precursor scan (EMS) and MRM in different forms as survey scans and enhanced product ion (EPI) scans as dependent scans.

Results:
24 different peaks were deduced as interesting based on their spectral properties and varying similarities to known benzoxazinoids or metabolites thereof. Figures will show selected examples of these such as the spectrum of the glucuronide of the lactam HBOA, known from rye. This spectrum contains the fingerprint of HBOA (164/136/118/108) and of the glucuronic acid (193/175/113) as well as the neutral loss of 18 u from the precursor ion (340) typical for carbohydrates. 340/164 and 340/108 were reliable MRM transitions for this ion, however general in their nature, and aided the discovery of other interesting peaks at other retention times. 340/118 and 340/136 were also reliable but much more selective. Other examples are the MRMs 354/164 and 354/108 that gave a peak at Rt=10.9 min. However, the spectrum varied slightly from sample to sample. Based on spectral data obtained by DDA, the MRMs 354/165 and 354/118 were introduced, revealing the presence of two distinct peaks differing by only 0.05 min in retention time.

Conclusions:
Highly selective MRM transitions were produced that enabled reliable detection and assignment of selected unknowns and suspects in complex biological matrices. Using this DDA-guided method for selection of MRM transitions, it was possible to distinguish even between compounds with similar retention times, precursor ions, and even fragment ions.

Novel Aspect:
Using DDA techniques for selection and evaluation of MRM transitions for semi-quantitative methods for selected unknowns and suspects.
Introduction:
UDP-glucuronosyltransferase 1A1 (UGT1A1) catalyzes glucuronidation of bilirubin. Mutations in the UGT1A1 gene cause bilirubin glucuronidation deficiencies, leading to diseases with hyperbilirubinemia or jaundice, Crigler-Najjar syndrome type I, type II and Gilbert syndrome. Since all the patients carrying these mutations showed hyperbilirubinemia, the enzyme activities of the mutated enzymes were determined.

Methods:
We applied the liquid chromatography-mass spectrometry (LC-MS) method to evaluation of UGT1A1 activity toward bilirubin. Chromatographic separations were performed using an Ultimate 3000 HPLC system (Dionex). Mass spectrometry was performed on an LTQ Orbitrap Discovery mass spectrometer (Thermo Scientific). The electrospray ionization source was used.

Results:
We determined the remaining UGT1A1 activity of the models of wild-type and mutant-type UGT1A1. The UGT1A1 activities of the models were considered as the normal and diseases with hyperbilirubinemia UGT1A1 activity, respectively. We applied LC/MS to evaluation of mutation effects on the UGT1A1 activities towards bilirubin. Bilirubin has two sites capable of glucuronidation and forms bilirubin mono- and di-glucuronides. LC/MS can detect a small amount of their glucuronides with accuracy. We confirmed the reduced substrate-glucuronidation activity of mutated UGT1A1 enzymes.

Conclusions:
We aimed to establish a highly sensitive method to determine the UGT1A1 activities. The activities varied according to wild-type and mutant-type UGT1A1. We tested here all the mutations found in patients with hyperbilirubinemia. The obtained results allow us to discuss the relationship between remaining activity and clinical phenotype of the patients with hyperbilirubinemia.

Novel Aspect:
We established a highly sensitive method to determine the UGT1A1 activities and evaluated wild-type and mutant-type UGT1A1 activities.
Keywords: Clinical mass spectrometry, UPLC-MS/MS, Chemometrics, Design of experiments, APRT deficiency,

Introduction:
Patients with APRT deficiency have excessive renal excretion of 2,8-dihydroxyadenine (DHA), causing chronic kidney disease. Treatment with allopurinol or febuxostat reduces DHA excretion and kidney stones formation. A chemometric approach was used for optimization of a UPLC-MS/MS assay for simultaneous quantification of DHA, adenine biomarkers and the pharmacological agents in human urine and plasma for clinical diagnosis and pharmacotherapy monitoring.

Methods:
Design of experiments (DoE) was used for optimization of UPLC-MS/MS assay for quantification of DHA, adenine, adenosine, inosine, hypoxanthine, xanthine and the drugs allopurinol, its active metabolite, oxypurinol, as well as febuxostat in human plasma and urine. Different sample preparation methods were tested prior to analysis of samples from controls and patients with APRT deficiency, before and after treatment with allopurinol or febuxostat.

Results:
A UPLC-MS/MS assay for simultaneous quantification of DHA and the main purine metabolites in human plasma and urine was developed by chemometric approach. D-optimal design was used to reveal both quantitative and qualitative significant factors influencing retention time and peak area for the analytes. Significant factors were optimized with CCF-design and related to sensitivity utilizing PLS regression. There was a strong interaction effect between several variables, indicating that these variables cannot be independently controlled to reach optimum settings. The assay was used to measure both plasma and urine concentration of the analytes in samples from healthy controls and APRT patients, before and after treatment. The DHA concentrations in plasma from 3 APRT patients were 456, 459 and 741 ng/mL off drug treatment and 130, 61 and 27 ng/mL while on allopurinol or febuxostat therapy. DHA was not detected in plasma from healthy controls. Highly significant changes were observed in the urinary excretion of DHA and adenine with drug therapy.

Conclusions:
Optimizing UPLC-MS/MS assay by DoE, only a fraction of experiments was used compared to what would have been required by changing one separate factor at a time (COST) approach. Our data suggest that the UPLC-MS/MS assay will greatly facilitate clinical diagnosis of patients with the rare hereditary purine metabolism disorder, APRT deficiency. At the same time the clinical assay can be used for monitoring pharmacotherapy and treatment adherence among these patients.

Novel Aspect:
Implementation of UPLC-MS/MS assay to facilitate clinical diagnosis and therapeutic drug monitoring of patients with the rare kidney disorder APRT-deficiency.
A BIOMPHALARIA GLABRATA PEPTIDE THAT STIMULATES EXTREME BEHAVIOUR MODIFICATIONS IN AQUATIC FREE-LIVING SCHISTOSOMA MANSONI MIRACIDIA

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Keywords: Schistosoma mansoni, Biomphalaria glabrata, miracidia, kairomone, proteomics

Introduction:
The human disease schistosomiasis is caused by the parasite Schistosoma mansoni, which requires an intermediate host, the freshwater snail Biomphalaria glabrata. An excellent olfactory sense allows the parasite to locate an appropriate host. Towards identifying the kairomone biomolecule(s) that helps S. mansoni miracidia detect its host, we have performed an integrated analysis of the snail-conditioned water (SCW), through chromatography and bioassays of behaviour, followed by mass spectrometry.

Methods:
The lyophilised SCW crude extract was resuspended in 0.1% TFA and fractionated using HPLC, and the fractions were collected and lyophilised for bioassays, with behaviour analysed by TrackMate plugin1 and MTrackJ2 for FIJI software3. The LC-MS/MS analysis of metabolites and peptides were carried out using UHPLC-QToF and NanoHPLC-ESI-Triple TOF, respectively. Data analysis of was performed using Agilent MassHunter Qualitative software and PEAKs studio. CD experiments were performed in a Chirascan CD spectrophotometer (Applied Photophysics).

Results:
Using an in vivo bioassay in conjunction with behaviour analysis, we first determined a raw mucus extract and filtered SCW could stimulate behaviour changes in S. mansoni miracidia, which was in alignment with previously classified miracidial responses4,5. The lyophilised crude extracts from SCW were HPLC-fractionated and collected for subsequent bioassays, and a single HPLC fraction demonstrated behaviour modification bioactivity. Metabolites and proteins present within the active HPLC fraction and two adjacent fractions were identified using LC-MS/MS. We found 16 metabolites, all of which did not match to any known compound within the METLIN6 or HMDB7 metabolite databases. Also in the active HPLC fraction, a total of 24 peptides were identified that matched to precursor proteins in the genome-derived B. glabrata protein database8. The activity analysis of these 24 peptides identified a single peptide with alpha helical characteristicss that stimulates extreme behaviour modifications in miracidia, and it can be produced within numerous snail tissues.

Conclusions:
We have reported the identification of a peptide, which is secreted from adult B. glabrata and triggers extreme behaviour modifications in the S. mansoni miracidia. Its precursor appears to be unique to this snail, thus providing an ideal species-specific attraction cue for S. mansoni miracidia. This finding contributes greatly to our understanding of a key part within the parasite's life-cycle, and may help towards establishing novel control interventions for schistosomiasis.

Novel Aspect:
This information will be helpful for developing approaches to manipulate this parasite’s life cycle, and opens up new avenues for exploring other parasitic diseases.

References
A.13 LIFE SCIENCES - SINGLE CELL

873 - ANALYSIS OF SINGLE MAMMALIAN CELLS WITH SCOPE-MS WITH MPOP

Harrison Specht (1) - Guillaume Harmange (1) - David Perlman (1) - John Neveu (2) - Christopher Adams (3) - Nikolai Slavov (1) - Bogdan Budnik (2)

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Keywords: single cell proteomics

Harrison Specht, Guillaume Harmange, David H Perlman, John Neveu, Chistopher M Adams, Nikolai Slavov, Bogdan Budnik

Introduction:
Quantifying proteins in single cells has a long history. For decades, scientists and physicians have used antibodies and fluorescent proteins to identify or quantify a few different proteins per cell. These methods have enabled new discoveries, clinical applications, and even spawned new fields based on measuring just a few different proteins per cell. We introduced a new technique SCoPE-MS1 that allows measurements of hundreds of proteins in single mammalian cells. The biggest limitation of that technique was throughput of sample preparation procedure and losses during clean up steps. To improve throughput and minimize losses we introduce a new sample preparation protocol for single cell proteomics.

Methods:
A major limitation to applying quantitative mass-spectrometry to a small amount of sample, such as single mammalian cells, are the losses during sample cleanup. To relieve this limitation, we developed a Minimal Proteomic sample Preparation (mPOP)2 method for culture-grown mammalian cells. mPOP obviates cleanup, and thus eliminates cleanup related losses while simplifying and expediting sample preparation for analysis of single cells.

Results:
SCoPE MS technique with mPOP preparation procedures were applied for several types of single cells analysis. As previously, new preparation procedures were tested for differentiation of Jurkat vs U cells to prove that new preparation protocols did not aggravate the ability to accurately quantify proteomes of the single cell. This new technique was applied to analysis of human activated Jurkat cells, mouse brain neurons and astrocytes. Obtained data shows that the new method was able to do quantitative analysis of hundreds of mammalian cells and show clustering of different types of cells according to their unique proteome properties.

Conclusions
Using mPOP with cell-sorting and liquid handling, we can lyse thousands of single cells in minutes, and quantify more than 700 proteins across 100 single cells in less than 12 hours. Likewise, mPOP enables protein measurements in 10, 100, and 1000 cell samples with unprecedented depth and throughput.

Novel Aspect: (Limit of 150 characters)
SCOPE MS with mPOP preparation procedure unlocks our ability to quantify hundreds of cells per day, with depth up to a thousand proteins. This type of analysis will allow researchers to create better models of protein-protein interaction and build new models of biological systems as well as new biomarkers of particular cell types.
464 - FUNCTION OF PIWIL3 IN MAMMALIAN OOCYTES.

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Keywords: Affinity purification crosslinking mass spectrometry (AP-XL-MS), single-cell sequencing, PIWI

Introduction:
PIWIs are argonaute proteins that interact with small non-coding RNAs (PIWI interacting RNAs; piRNAs). PIWI-piRNA complexes serve to target transposons for degradation, and are crucial guardians of genome integrity. PIWIL3 is specifically and highly expressed in unfertilized human oocytes, but a homologue is absent in mouse and rat, hence conventional animal systems cannot be used to study the critical function of PIWIL3 before fertilization[1].

Methods:
We performed an in-depth analyses of bovine oocytes by mass spectrometry. From manually dissected oocytes, proteins were extracted by repeated freeze-thaw cycles and sonication. In parallel, we (i) performed AP-XL-MS on the bovine PIWIL3 complex, and (ii) profiled the oocyte proteome by LC-MS/MS on a Q-Exactive HF mass spectrometer, with pre-fractionation at high pH. We also followed the expression of PIWIL3 by microinjection and immuno-labeling techniques.

Results:
The bovine system is the only large mammal system that expresses a PIWIL3 homologue. Herein, we investigated the expression, distribution, interaction, and argonaute function of PIWIL3 in bovine oocytes. By microinjection of PIWIL3-EGFP mRNA, we followed the localised expression of PIWIL3 in oocytes and early-stage fertilized embryos. From extremely limited oocyte material, we identified a total of 3897 proteins, which revealed the functional state of oocytes prior to fertilisation. In parallel, we identified a novel PIWIL3 interaction network involving two known regulators of argonaute function, which was independently verified by immuno-staining confirming intracellular co-localisation. Additional experiments involving single-cell sequencing and pi-RNA sequencing further support a critical role for mammalian PIWIL3 in protection of genome integrity prior to fertilization and generation of a new genome.

Conclusions
By a combination of AP-XL-MS and pi-RNA sequencing, we shed light on the role of PIWIL3 in mammalian oocytes prior to fertilization, and assign PIWIL3 to an interaction network in which its function may be further characterised.

Novel Aspect:
We showcase here the extreme sensitivity of AP-XL-MS approach in mapping protein complexes from limited starting material.

References
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Picoliter pressure-probe-electrospray-ionization mass spectrometry for in situ monitoring single plant cell metabolite changes under stress conditions

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Keywords: pressure probe ESI, single cell, organelle, metabolomics.

Introduction:
Cytoplasmic streaming occurs consuming the ATP energy formed during glycolysis, respiration and photosynthesis, moving organelles and metabolites all around plant cells. CPP is an instrument that can measure the water status and several properties of intact cells (i.e., volume)[1]. The CPP-mass spectrometer combination (picoPPESI-MS)[2,3] allows to measure water status, cytoplasmic streaming velocities and metabolites in cells sequentially.

Methods:
picoPressure Probe for water relations. Osmometer for cell turgor.
Internal Electrode Capillary picoPressure Probe Electrospray Ionization (picoPPESI) as ions source, with Orbitrap mass spectrometer (Exactive Plus, Thermo Fisher Scientific Inc., MA) as MS analyzer.

Results:
Plants were subjected to water stress. Water status (turgor pressure; water potential) slightly decreased and they recovered by osmotic adjustment in 12 h. The cytoplasmic streaming velocity was maintained during osmotic adjustment. PicoPPESI-MS indicated that several phosphate-metabolites (m/z 78.96 [HPO3-], 96.97 [H2PO4-], and 259.02 [Glu-6-phosphate]) were transiently accumulated in the cells in response to the treatment. Furthermore, not only metabolites yielded by glycolysis occurred in cytosol (m/z 87.01 [Pyruvic ac.], 179.06 [Hex], 259.02 [Glu-6-phosphate], and 341.11 [Hex2]) but also those from Krebs cycle in the mitochondrion (m/z 115.00 [Fumaric ac.], 117.02 [Succinic ac.], 133.01 [Malic ac.], 145.02 [α-Ketoglutaric ac.], 173.01 [Aconitic ac.] and 191.02 [Citric ac.]) accumulated in response to the stress treatment. Because these metabolic pathways are essential for ATP hydrolysis, ATP production and the osmotic adjustment, is obvious their contribution to maintain cytoplasmic streaming providing energy.

Conclusions:
Tacking into account the metabolic pathways mentioned above and the results obtained by picoPPESI-MS monitoring the cell metabolites, ATP hydrolysis, ATP production and the osmotic cell adjustment are essential for keeping cytoplasmic streaming providing the necessary energy.

Novel Aspect:
PicoPPESI mass spectrometry system has a potentiality to monitor metabolic changes in situ at cell level as well as at cell level.

References
A TOF-SIMS Tandem MS Imaging Method to Probe the Composition of Cellular Organelles

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Keywords: TOF-SIMS tandem MS imaging, 3D imaging, HEK cell, organelle, subcellular imaging

Introduction: (Limit of 400 characters)
A goal of cellular imaging is to ascertain the composition of organelles, e.g., lipid profiling or pharmaceutical efficacy. Most subcellular imaging is accomplished by stable isotope labeling [1], but such analyses are void of molecular specificity. We employed a TOF-TOF imaging capability [2] to achieve molecular specificity and conjectured that an ER-Tracker stain would yield characteristic molecular ions with which to image the ER and ER tubules.

Methods: (Limit of 400 characters)
HEK cells, transfected to express GFP-Kv2.1 fluorescent ion channels, had a high number of endoplasmic reticulum (ER) tubules near the plasma membrane (PM). The cells were stained with ER-Tracker Blue-White DPX which selectively labels the ER. Control specimens were neither transfected nor stained. Precursor ions, defined by monoisotopic selection, were fragmented by keV-CID. A lateral resolving power of ≈ 80 nm was achieved in the TOF-SIMS tandem MS imaging.

Results: (Limit 900 characters)
We observed, by simultaneous MS imaging and tandem MS imaging, atomic and molecular moieties characteristic of the ER-Tracker stain localized to ER and ER tubule structures. The ions used for tandem MS imaging of the ER and ER tubules were shown irrefutably via the product ion spectra to arise solely from the ER-Tracker stain. 2D imaging revealed intersection of some ER tubules at the PM. 3D visualization to a depth of ≈ 40 nm revealed additional ER tubules just under the PM. Some ER-Tracker was observed in the PM indicating ER tubule contact with the PM to form ER-PM junctions. We were also able to confirm the presence and position of the PM by imaging characteristic lipids, lipid fragments and fatty acids. The observed tubule features had measured diameters in the range of approximately 500 nm to 2 μm corresponding well with previous studies [3] and our present total internal reflection fluorescence (TIRF) observations. More than a dozen control cells were analyzed, and neither atomic nor molecular moieties characteristic of the ER-Tracker were observed.

Conclusions (Limit of 400 characters)
TOF-SIMS tandem MS imaging was employed with a molecular stain to isolate and observe ER tubules and ER-PM junctions in transfected HEK cells. Our next aim is to visualize the endoplasmic reticulum within entire cells and to assess the lipid composition at different locations within this organelle. By extension, with organelle-specific imaging, we can apply this method to aspects of pharmaceutical efficacy and metabolism.

Novel Aspect: (Limit of 150 characters)
Molecular markers together with TOF-SIMS tandem MS imaging enables the identification of endogenous and exogenous components in specific cellular organelles.

References:


Introduction:

In the last few decades, the pharmaceutical industry has transformed people’s lives. However, the development of new drugs is becoming increasingly and a paradigm shift in the drug discovery workflow is required to reduce attrition and transform conventional drug screening assays into translatable analytical techniques for the analysis of drugs in complex environments, both in-vitro and ex-vivo. The ability to visualise unlabelled compounds inside the cell at physiological dosages can offer valuable insight into the compound behaviour both on and off-target.

Methods:

LiveCell MS is a semi-automated methodology that allows the collection of intracellular content using a modified CQ1 imaging system developed by Yokowaga. The instrument is equipped with a confocal microscope that allowed bright field imaging as well as fluorescence imaging with 4 lasers (405, 488,561 and 640nm). Sampling was performed using the tips developed by Professor Masujima (reference below). The tip, holding the cellular contents, was then used for static nanospray of the contents into an Orbitrap Fusion Lumos (Thermo Scientific) and the resulting data processed using Compound Discoverer (Thermo Scientific).

Results:

In this study, we show the applicability of the LiveCell MS technology to drug discovery, as it is crucial to identify compound and its metabolites when incubated in a mammalian cell at a therapeutic dose. We report on the validation studies performed using the LiveCell MS platform. In the first of these studies we assess the dynamic range of the assay by directly comparing the results obtained with a state of the art metabolomics assay using bulk samples with our single cell technology.

In the second validation study we explore the effects of amiodarone, an anti-arrhythmia drug, in a macrophage model (rat-derived NR8383 cell line) identifying the drug intracellularly and the detectable endogenous metabolites.

Conclusions

We demonstrate that dosed compound can be identified in a single cell after sampling using the modified CQ1Endogenous metabolites can also be identified, that can further understanding of the drug’s mechanism. This technique has direct relevance for assessing compound effects on disease relevant cells and it’s low sample requirement makes it applicable to studying rare cell types. The use of high content imaging system enables the effect of compounds on live cells to be studied and suitable time points selected for sampling cell contents.

Novel Aspect:
Identification of drug compounds and endogenous metabolites using an automated sampling technique and well asin combination with high-resolution mass spectrometry.

References

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1351 - DRUG UPTAKE AND METABOLISM IN SINGLE LIVER CELLS

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Keywords: single cell, drug metabolism, nanospray, high-resolution mass spectrometry

Introduction: (Limit of 400 characters)
Cells, even within a genetically identical culture, possess individual differences that cannot be detected by analyzing cell populations. These individual differences can be crucial to cellular health and survival. Herein, we present a method that combines automated live single cell sampling with high resolution mass spectrometry that enables the detection and quantification of drug and drug metabolites in individual liver cells.

Methods: (Limit of 400 characters)
Amiodarone was used as a test compound. HepG2 and cryopreserved human hepatocytes were used as liver cellular models. Cells were incubated with 10 μM amiodarone for 24h at 5% CO2 and 37 °C. Live cells were visualized in real-time during automated single cell sampling using a confocal microscope modified with a XYZ stage robot (Yokogawa Electric Corporation). Platinum-coated glass capillaries (cellomics tip, HUMANIX) were used for cell sampling and nanospray ionization. A Bruker static nanospray source and a 7T SolariX FT-ICR-MS were used for mass spectrometry analyses.

Results: (Limit 900 characters)
CT-10 tips (10 μm I.D.) were chosen to sample whole hepatocyte cells, which were maintained at 5% CO2 and 37 °C inside the sample vessel of the confocal microscope until sampled. Before automatic live cell sampling, series of slice images (z-stacks) were acquired to determine the volume of each cell. The cellular metabolism was quenched immediately after cell sampling. Prior to mass spectrometry analysis, an organic solvent mixture spiked with internal standard was loaded into the tip. Direct infusion of single cell contents resulted in complex mass spectra, with varied ion abundances and many interfering background ions. The resolving power of the SolariX FT-ICR-MS enabled unambiguous amiodarone identification and, operated in CASI mode, an improvement of at least 10-fold in sensitivity. The internal standard was used for both lock-mass calibration and intensity normalization. Concentrations of amiodarone and its major metabolite (N-desethylamiodarone) were integrated over total cell spray times and multiplied by the flow rates to afford the amount of each compound within each cell. Single cell concentrations were determined by normalizing these amounts to individual cell volumes. Single cell measurements agreed well with determinations of a hypothetical average cell obtained from cell lysates. Single cell measurements were, nonetheless, more variable than technical replicate analysis of cell lysates.

Conclusions: (Limit of 400 characters)
Pioneering work conducted by Masujima and colleagues had previously demonstrated the applicability of cellomics tips for both live single cell sampling and direct infusion into the mass spectrometer by nanospray ionization without prior sample processing [1,2]. In the current work, we confirmed the feasibility of the approach and addressed many technical challenges currently associated with single cell analysis: limited sample amount, high dynamism of cellular metabolic state, low sampling throughputs, high complexity of ion mixtures and varied abundances. With this method, absolute compound quantification in the actual cells was achieved.

Novel Aspect: (Limit of 150 characters)
A new method for determining compound uptake, accumulation and metabolic stability inside individual liver cells.

References
Introduction:
Metabolomics with single-cell resolution opens new frontiers in the study of cell heterogeneity [1] and establishment of functional differences during embryonic development [2]. Mass spectrometry (MS) is the technology of choice for metabolomics, but sensitivity limitations in detection hinder the application of traditional metabolomics to single embryonic cells. Here, we present a single-cell MS approach that enables the extraction and detection of metabolites from single cells from live Xenopus laevis embryos with high technical and biological reproducibility. We conduct in situ, minimally invasive sampling of identified embryonic cells to peer into metabolic pathways active during early embryogenesis. To enhance metabolite coverage, we integrate anionic and cationic analyses using a custom-built capillary electrophoresis electrospray ionization (CE-ESI) MS platform.

Methods:
Single dorsal D1 and ventral V1 cells were identified in the 8-cell Xenopus laevis embryo using cell fate maps, pigmentation, and morphology criteria. Our CE-MS system [3] consisted of a microprobe to aspirate ~10 nL portion of the cell’s cytoplasm, which was immediately extracted in 40 % acetonitrile/40% methanol and water. Metabolites were detected in a custom-built CE-ESI system coupled to a high-resolution quadrupole-time-of-flight mass spectrometer (Impact HD, Bruker).

Results:
Our CE-ESI-MS platform provided 60 amol sensitivity and a quantitative range of 4–5 orders of magnitude. Trace-sensitive detection by this platform allowed us to detect ~250 cationic and ~150 anionic metabolite features in the positive and negative ion-mode. We identified 70 of the cationic features with high confidence as metabolites based on accurate mass, tandem MS, and migration time information against reference standards. Likewise, 50 of the anionic features were putatively assigned based on accurate mass match against data in Metlin [4]. These quantitative metadata enabled us to compare metabolic activities between cells along the dorsal-ventral developmental axis of the vertebrate embryo using multivariate and statistical analysis. Surprisingly, we observed clustering of metabolites based on their cell type of origin. Moreover, pathway analysis revealed the arginine-proline metabolism pathway (p-value 2.24 x10-12) and the glycine-serine-threonine metabolism pathway (p-value 5.13 x10-10) to be of significance, suggesting the importance of these pathways during early development.

Conclusions:
We developed a unique single-cell MS technology that enabled the metabolic characterization of identified single cells in the live vertebrate embryo of Xenopus laevis. Quantitative data from this study revealed previously unknown metabolic cell heterogeneity between the dorsal-ventral axis of the 8-cell embryo, raising the possibility that metabolites also contribute to cell fate specification.

Novel Aspect:
Enabling in situ sampling and detection of cationic and anionic metabolites from single-cells during early development to uncover cell heterogeneity.

References
Single Cell Mass Spectrometry: From Qualitative to Quantitative Studies

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University of Oklahoma (1)

Keywords: Live single cell, ambient mass spectrometry, metabolomics, microscale bioanalysis, Single-probe

Introduction: (Limit of 400 characters)
Cell is the basic unit of life. Cells are traditionally studied through population analysis, and averaged results are obtained. Understanding individual cells is critical for advances in many areas ranging from cell biology to clinical treatment. Due to its high sensitivity and broad detection range, MS is a promising technique for single cell analysis. (1-4) We have developed novel techniques using microscale probe to study live single cells under ambient conditions.

Methods: (Limit of 400 characters)
We have developed a microscale sampling and ionization device (the Single-probe) that can be used for SCMS studies. (5-7) The Single-probe is fabricated by combining a laser-pulled dual-bore quartz needle (tip size smaller than a cell) with a silica capillary and a nano-ESI emitter. In SCMS experiments, cells are attached to an XYZ-translational stage for sample movement. Cell insertion is monitored using digital microscopes as the Z-stage is lifted precisely.

Results: (Limit 900 characters)
Qualitative studies. Cancer cell lines were used as model systems, and they were cultured and treated with anticancer drugs. We have successfully detected anticancer compounds, drug metabolites, and cell metabolites inside single cancer cells. (5-7)

Semi-quantitative studies. Cancer stem cells (CSCs) and non-stem cancer cells (NSCCs) were used for comparison studies. Metabolomic analyses indicated that CSCs possess higher abundances of unsaturated lipids, and the level of desaturation can be altered using inhibitors.

Quantitative studies. To quantify the amount of anticancer drugs in cells, sampling solvent containing the deuterated drug was delivered into a target cell to extract intracellular compounds. Both target and deuterated molecules were simultaneously detected, and abundances of target molecule were estimated from their relative ion intensities, the concentration of the internal standard, solvent flow rate, and data acquisition time. Standard HPLC/MS analysis of cell lysates were done for comparison.

Conclusions (Limit of 400 characters)
Using the Single-probe SCMS techniques, we conducted live single cell studies including qualitative, semi-quantitative, and quantitative analyses. Broad ranges of molecules were detected. Experimental data were comprehensively analyzed to extract metabolomic information and reveal mechanisms of anticancer drugs and cell metabolism. Drug uptake amounts by single cancer cells were estimated. Our techniques can be potentially applied in clinical studies.

Novel Aspect: (Limit of 150 characters)
Multi-functional microscale device was coupled to MS for live single cell studies. Experiments and advanced data analysis were combined to obtain metabolomic information.

References
Keywords: microbial MALDI-MSI, MALDI-2, high resolution, pathogenic bacteria, sample preparation

Introduction:
Standard protocols to visualize the metabolic exchange in microbial communities by MALDI-MSI are based on analyzing colonies directly from agar [1]. However, this comes with notable drawbacks such as a lacking inactivation step and a limited reproducibility [2]. Here, we introduce a robust membrane-based workflow that enables steam inactivation and highly-resolved imaging. Examples of competing strains including probiotics (e.g. E. coliNissle) are shown.

Methods:
E. coli, S. aureus, P. aeruginosa and B. subtilis were investigated as prominent gram-negative and gram-positive samples. Bacterial colonies were co-cultured on LB agar, using mixed ester cellulose membrane filters. Steam inactivation was performed in a home-made chamber. Synapt G2-S QTOF and QExactive plus orbitrap mass spectrometers, equipped with MALDI-2 ion sources (from Waters [3] and Spectroglyph [4], respectively), were used for data acquisition.

Results:
Inactivation of pathogenic bacteria and metabolic quenching is an indispensable prerequisite for a wider use of microbial MALDI-MSI. With our protocol, this requirement was – for non-sporulating strains – achieved by steam inactivating of membrane-grown colonies for 5 s. Following heat-induced dehydration, the 3-dimensionality of the cultures was reduced to a 2D space. Following matrix application, sample flatness fostered reproducible MALDI analyses and use of laser-induced postionization (MALDI-2), boosting the signals of numerous metabolites in positive and negative ion mode. Differentiation and identification of chemical identities was supported by the high resolving power (280k @m/z 200) and tandem MS capability of the QExactive plus.

Making use of these advantages, we recorded metabolic profiles from several single and co-cultured microbial communities at high lateral and high mass resolution. Examples for the microbial interaction on a molecular level of probiotic E. coliNissle and pathogenic E. coli will be demonstrated.

Conclusions:
With the emerging interest in microbial MALDI-MSI, a reliable protocol for handling of pathogenic samples is still challenging. Our new membrane-based culturing protocol solves this issue for non-sporulating gram-negative and gram-positive bacteria. The use of MALDI-2 in combination with a high-resolving orbitrap increased chemical coverage, a critical step for a better understanding of the complex chemical communication in bacterial communities.

Novel Aspect:
Advanced protocol for microbial MALDI- and MALDI-2 imaging, visualizing the metabolic exchange in (co-) cultured colonies with high sensitivity and mass accuracy.
References:
Introduction:
Iodinated contrast agents (ICAs), e.g., iopromide and iodixanol, are excreted via the kidneys. The fast and complete excretion of the unmetabolized CA is crucial with respect to toxicity issues. Rats were treated with iopromide or iodixanol with a dosage of 4 g iodine/kg bodyweight. They were sacrificed after 24 or 48 hours, kidneys were embedded and cut into 10 µm cryosections. To investigate the accumulation and clearance of the ICAs, different bioimaging methods were used.

Methods:
Micro X-ray fluorescence (µXRF) and laser ablation-inductively coupled plasma-mass spectrometry (LA-ICP-MS) were used to investigate the elemental distribution, especially of iodine, in rat kidneys. Matrix-assisted laser desorption/ionization-MS (MALDI-MS) generated information on the distribution of different small molecules in the range of m/z 100-1600 with MS/MS identification.

Results:
Significant differences in the accumulation and distribution of iopromide and iodixanol were detected with the three imaging methods when comparing kidney sections of rats, which have been treated with either ICA. In kidney sections treated with iodixanol and analyzed with LA-ICP-MS, the iodine was concentrated in the cortex region. The signal intensities decreased after 48 h. For iopromide, the iodine was accumulated in the medulla region. It was possible to quantify the different concentrations in kidneys spatially resolved. With µXRF the same distribution and the decrease in intensity after 48 h were investigated.

Iodixanol was detected and identified with MS/MS via MALDI-MS. Additional information about small molecules were possible to analyze and to visualize different regions in tissue sections.

Conclusions:
It is possible to investigate the difference in clearance and accumulation in rat kidneys with the different bioimaging Methods: Due to the lower iodine concentration in medulla or pelvis, iopromide has a faster clearance. Iodixanol causes a higher urine viscosity, accordingly the iodine amount is higher and it accumulates in the cortex region. Additionally, MALDI-MS can be used to determine the phospholipid distribution in different regions of kidneys.

Novel Aspect:
Three different bioimaging techniques are used to investigate the distribution of iopromide and iodixanol in rat kidneys.
Introduction:
Qualitative and quantitative analysis of fatty acids can be difficult based on lack of fragmentation shown by [M-H]-species. Ionization as metal adducts has proven to be a powerful tool for structure elucidation. Induction of charge-remote fragmentation results in generation of meaningful fragments, enabling identification and quantification of structural isomers.

Methods:
To study desorption and fragmentation behavior desorption electrospray ionization (DESI) analysis was performed on mixtures of fatty acids and metals spotted onto PTFE targets. Tandem MS experiments were performed to identify the underlying fragmentation mechanistic. Suited metals were evaluated for direct ionization of fatty acids adducts directly from tissue sections and their fragment ions were used to create a targeted imaging approach.

Results:
Lithium adducts showed mixed fragmentation pattern along the fatty acid chain including formation of radical ions while silver adducts showed a preferred cleavage in allyl position to C=C bond through formation of cyclic intermediates.
The identified fragmentation pattern were subsequently used to identify fatty acids present in tissue sections. Washing steps to remove salts from the tissue sections prior spray-deposition of salt solutions improved the sensitivity of the method. Fatty acids present in tissue sections were identified by DESI-MS/MS experiments. Unique fragments enabling differentiation of structural isomers, e.g. arachidonic acid and eicosatetraenoic acid, were subsequently used to create multiple-reaction-monitoring (MRM) transitions for a targeted imaging approach performed on a triple-quadrupole mass spectrometer. The methodology was then used to map the spatial distribution of structural fatty acid isomers across various rodent tissues and human tumor sections.

Conclusions:
Ionization of fatty acids as metal adducts has been demonstrated to be a powerful tool for structural identification of fatty acids. Incorporated into a targeted MSI approach it enables to study distribution of fatty acids and potentially of closely related molecules, e.g. eicosanoids and prostaglandins in tissues. This is particularly interesting for the study of inflamed tissues and tumors studying the underlying disease mechanisms involving free fatty acids.

Novel Aspect:
In this study we present the use of charge-remote fragmentation for identification and targeted analysis of fatty acids in MSI experiments.
Antibiotic resistance causes 25 000 deaths every year. The rate of the spread and the threat that antibiotic resistance pose on a global scale makes this a field of great interest for research. Transfer of plasmids (mobile DNA) between bacterial cells through conjugation is one of the major contributors to the spread of antibiotic resistance. A larger understanding of the mechanisms behind this process and how to possibly hinder it could help to quench the development observed around the world. In this study, the changes in lipid composition in the cell membrane of three mutated E. coli strains that have previously been found unable to transfer plasmids were analysed using a J105 - 3D Chemical Imager.

Methods:
The J105 (Ionoptika Ltd, UK) is an unconventional time-of-flight secondary ion mass spectrometry (ToF-SIMS) instrument specifically developed for analysis of organic materials [1]. A 40 keV CO2 gas cluster ion beam (GCIB) is coupled to the J105 enabling imaging as well as depth profiling of native, underivatised biological samples and detection of intact lipids and other higher mass species [2]. Using the J105 coupled to a GCIB has allowed analysis of the outer membrane of mutated E. coli and how the lipid composition has changed in the membrane due to the mutations. Maximum autocorrelation factor (MAF) analysis was used as a tool to identify species of interest by mapping the largest differences within the data set.

Results:
A previous study found that the stress response in E. coli exposed to carbon starvation were changes in the lipid composition by elongation, cyclopropanation, and increased cardiolipin formation [3]. In our study, three different mutated E. coli strains unable to transfer plasmids were analysed and compared to wild type E. coli. Here we have analysed mutants that have been shown to have impaired ability to directly transfer DNA (an important process in antibiotic resistance development). The sub-set of mutants selected for SIMS analysis possess mutations that are expected to influence lipid and protein composition in the cellular envelope of the bacteria and are thus ideally targets for surface sensitive MS analysis. The preliminary results from this study show extensive lipid changes in the mutated bacterial cell membranes, some of which are closely related to those previously observed in carbon starved E. coli cultures. Changes in elongation and cyclopropanation of unsaturated fatty acids were observed along with differences in the production of cardiolipins. Such effects can influence membrane fluidity and protein incorporation and also the functional properties of pili used to initiate conjugation. Lipid structure can also influence the fusion pore formation required for plasmid transfer during conjugation.

Conclusions
The ToF-SIMS GCIB/J105 system has here proven to be a good method for analysis of the surface and the subsurface of the cellular envelope in mutated E. coli. MAF analysis was able to identify a large number of species which showed changes in the cell membrane of mutated strains relative to wild type E. coli. Some of these species have previously been identified as related to stress response in E. coli exposed to carbon starvation.

Novel Aspect:
Linking lipid composition changes in the cell membrane in mutated E. coli to the bacteria’s inability to transfer plasmids.
References
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Keywords: dual isotope labelling; MALDI imaging; metabolic pathway; structural isomers

Introduction:
Regardless of major advances in mass spectrometry imaging (MSI), there are three intrinsic limitations associated with MSI including intricate molecular identification, low molecular coverage and incapability to obtain ‘true’ spatial distribution due to isobaric and particularly isomeric ions interference[1].

Methods:
We have developed a novel approach that integrates in vivo dual isotope labeling of precursor metabolites with MSI for identification of spatially localized metabolite and metabolic network map reconstruction. In a proof-of-concept study, lemna plants were fed with tyrosine (unlabeled Tyr; Tyr2H4 and Tyr13C915N) and tomato fruits with phenylalanine (unlabeled Phe; Phe2H5 and Phe13C915N), they were then analyzed with LC-MS and MALDI imaging.

Results:
We have identified 60 and 28 tyrosine and phenylalanine derived metabolites in lemna and tomato fruits respectively, in which half of them were detected due to the increased concentration as a result of precursor feeding. With the identified metabolites, we were able to construct a detailed metabolic network map based on their labeling patterns and known biochemical reactions. Significantly, 20-30% of the identified metabolites were found to contain at least one structural isomer that has different distribution pattern. The noteworthy feature of this approach is that it enables distinguishing the localization of different structural isomers, hence providing the ‘true’ distribution of molecules of interest.

Conclusions:
This approach offers a number of advantages: (i) it improves metabolite identification; (ii) it increases metabolite coverage via precursor feeding; (iii) it allows construction of a detailed metabolic network map by tracking the specific pathway and most importantly, (iv) it enables precise localization of structural isomers, hence providing the ‘true’ distribution of molecules of interest.

Novel Aspect:
(i) combined dual isotope feeding, LC-MS and MALDI imaging to address several key issues in MSI
(ii) developed an R package for automatically and efficiently fishing out the dual isotope-labeled metabolite

References
Keywords: gene function; MALDI imaging; mutants;

Introduction:
Plants are highly differentiated multicellular organisms which consist of multiple specialized organs and tissues with unique anatomy features; anatomical differentiation parallels different gene expression and metabolite profiles[1]. As such, information on metabolite distribution has been regarded as important readouts of tissue-level gene function[2].

Methods:
We used various geneticstools including RNA interference (RNAi), virus induced gene silencing (VIGS) and agroinfiltration for mutating the genes in tomato and Nicotiana benthamiana, and characterized the gene functions using LC/GC-MS and MALDI imaging.

Results:
In this study, we proposed mass spectrometry imaging (MSI) as a potential member in functional genomics toolbox. Three exampleswere provided showing how MALDI imaging can benefit gene functional annotation. In particular, ultra-high mass resolution MALDI imaging (R=1400,000 at m/z 400) was used to visualize and compare the steroidal glycoalkaloids pathways in wild type and mutant tomato fruits. One defining feature of VIGS and agroinfiltration is that the spread of phenotype is partial in most plants, which results in two or more phenotypes heterogeneously distributed over the same sample, as demonstrated in the second and third examples.MALDI imaging allows directly compare the multi-phenotypes over the sample.

Conclusions:
MSI offers at least two unique advantages for gene functional annotation: (i) it enables detection of previous undetected gene-related metabolites due to the dilution effect in bulk metabolomics analysis; (ii) it provides tissue specific gene-to-metabolite correlation. MSI is therefore an attractive functional genomics tool; the physiological role of a gene can be inferred by comparing WT and mutant using MSI.

Novel Aspect:
(i) Applied different genetics tools to mutate genes of interest, MALDI imaging and LC/GC-MS were then used to compare the WT and mutant to infer the gene function.

References
Introduction:
Latest developments in the field of MALDI imaging has led to a significant impact in the pharmaceutical and cosmetics field. MALDI imaging of biological tissue requires high selectivity which is provided by tandem MS and/or accurate mass measurements. Highly selective MALDI imaging is now enabled on high resolution Orbitrap instruments by means of an Atmospheric Pressure MALDI source.

Methods:
An AP-MALDI UHR ion source (Masstech Inc.) coupled to a LTQ/Orbitrap Elite high resolution mass spectrometer (Thermo Scientific) was used for the development of targeted and untargeted imaging Mass Spectrometry experiments. Cryosections of biological tissue were coated with matrices using a HTX-TM sprayer. Time-Of-Flight Secondary Ion Mass Spectrometry (TOFSIMS) was used as a complementary MS imaging modality.

Results:
Several applications of the AP-MALDI Orbitrap setup are presented based on samples from various types and sizes for the detection of targeted and untargeted molecules. The instrument allows for the localization of endogenous or exogenous molecules in various types of tissue. MALDI images are correlated with other imaging techniques in a multimodal imaging workflow including e.g. optical microscopy and TOFSIMS.

Conclusions:
We show the characteristics and applications of a Masstech AP-MALDI UHR ion source as a part of a multimodal imaging workflow. With a laser spot sizedown to 10 micron lateral resolution, when associated to a LTQ/Orbitrap mass spectrometer.

Novel Aspect:
Multi-modal MS imaging workflow including MALDI HR-MS or -MSn imaging capabilities down to 10 micron lateral resolution.
ELUCIDATION OF NATURAL PRODUCT BIOSYNTHESIS IN AMAZON WOOD SEXTONIA RUBRA BY IN SITU TANDEM MS IDENTIFICATION WITH 100 NM-SCALE IMAGING

Gregory L Fisher (1) - Tingting Fu (2) - David Touboul (3) - Serge Della-Negra (4) - Emeline Houel (5) - Nadine Amusant (6) - Christophe Duplais (5) - Alain Brunelle (3)

Physical Electronics, Marketing & Applications, Chanhassen (1) - National Physical Laboratory, NiCE-MSI, Teddington, Middlesex (2) - CNRS, Institut de Chimie des Substances Naturelles, Gif-sur-Yvette (3) - CNRS, Institut de Physique Nucléaire, Orsay (4) - CNRS, UMR EcoFoG, AgroParisTech, Cayenne (5) - Cirad, UMR EcoFoG, AgroParisTech, Kourou (6)

Elucidation of Natural Product Biosynthesis in Amazon Wood Sextonia rubra by in situ Tandem MS Identification with 100 nm-Scale Imaging

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Keywords: TOF-SIMS tandem MS imaging, Amazon tree wood, natural products, biosynthesis

Introduction: (Limit of 400 characters)
The secondary metabolites of rubrenolide and rubrynolide, having xylophage toxicity and antifungal properties [1], are reproduced in heartwood parenchyma cells before active or passive transfer to other cells and tissues. There are thought to be several biomolecular precursors en route to the bioactive metabolites. Our goal was to identify biosynthetic precursors and to verify their coincidence with rubr(y)nolide via TOF-SIMS tandem MS imaging [2].

Methods: (Limit of 400 characters)
Specimens culled from transition zone sections of Amazon Sextonia rubra were maintained at -18°C from harvest until final preparation of the analytical surface by diamond knife ultramicrotome. Biosynthetic precursor ions, defined by monoisotopic selection, were characterized by in situ analysis of the wood specimens using keV-CID; a lateral resolution of ≈400 nm was measured. Purified rubr(y)nolide was characterized by both TOF-SIMS tandem MS and LC-MS/MS.

Results: (Limit 900 characters)
TOF-SIMS tandem MS imaging was successfully applied to perform in situ characterization and localization of secondary metabolites and their biosynthetic precursors. MS/MS imaging significantly increased the confidence of the molecular identifications and the localization of the low abundance bioactive metabolite to anatomical structures. TOF-SIMS tandem MS images demonstrate the localization of rubr(y)nolide principally in and around ray parenchyma cells, in agreement with previous work on the biosynthesis of wood metabolites. Notably, the in situ tandem MS imaging reveals a contribution of oil cellisin producing the secondary metabolites. We were able to demonstrate the presence of numerous precursors and to confirm or derive their structure using the tandem MS product ion spectra, thus contributing in the exploration of natural product biosynthesis.

Conclusions (Limit of 400 characters)
We are employing complementary structural analysis methods to obtain unambiguous identifications. Nevertheless, by TOF-SIMS tandem MS imaging alone, we have garnered unassailable evidence for previously unreported $\gamma$-lactone and obtusilactone molecules localized with rubr(y)noline suggesting a common biosynthetic origin. These observations underpin the promise of TOF-SIMS tandem MS imaging for studies of plant metabolism and biosynthesis.

Novel Aspect: (Limit of 150 characters)
TOF-SIMS tandem MS imaging provides in situ identification and high resolution visualization of low abundance molecules for the study of metabolism and biosynthesis.

References:

Introduction: ToF-SIMS has been successfully used for analysing lipids behaviour in biological samples like breast cancer tissue. Basal cell carcinoma (BCC) is one of the most increasing cancers worldwide and it is the most common malignancy in white people. Although the mortality is low as BCC rarely metastasises, this malignancy causes considerable morbidity and places a huge burden on healthcare services worldwide. Furthermore, people with BCC are at high risk of developing other malignancies.

Methods: Samples were collected from patients with basal cell carcinoma, by Mohs surgery. The tissue was cryo-sectioned for ToF-SIMS analysis and H&E staining of consecutive tissue slices was performed. ToF-SIMS was performed using an Ionoptika J105 instrument using a 40 keV (CO2)6000+ ion beam.

Results: A set of basal cell carcinoma samples, removed by Mohs micrographic surgery and pathologically identified as having an aggressive subtype, have been analysed using time-of-flight secondary ion mass spectrometry (SIMS). The SIMS analysis employed a gas cluster ion beam (GCIB) to increase the sensitivity of the technique for the detection of intact lipid species. The GCIB also allowed these intact molecular signals to be maintained while surface contamination and delocalized chemicals were removed from the upper tissue surface. Distinct mass spectral signals were detected from different regions of the tissue (epidermis, dermis, hair follicles, sebaceous glands, scar tissue, and cancerous tissue) allowing mass spectral pathology to be performed. The cancerous regions of the tissue showed a particular increase in sphingomyelin signals that were detected in both positive and negative ion mode along with increased specific phosphatidylserine and phosphatidylinositol signals observed in negative ion mode. Samples containing mixed more and less aggressive tumor regions showed increased phosphatidylcholine lipid content in the less aggressive areas similar to a punch biopsy sample of a nonaggressive nodular lesion.

Conclusions ToF-SIMS imaging with a GCIB distinguished cancerous and non-cancerous tissue from multiple patients along with different grades of high and low aggressive cancer.

Novel Aspect: ToF-SIMS analysis using 40 keV GCIB was used to analyse basal cell carcinoma samples, removed by Mohs micrographic surgery.

For information please contact: scientific@ims2018.it
Keywords: operational, casework, fingerprints, blood, MALDI

Introduction:
Nine years of intense and pioneering research into the extraction of forensic intelligence from fingermarks have resulted in the recent implementation of MALDI MSI methods in police casework in Europe. Here insights are provided into the MALDI MSI protocols in relation to the analysis of crime scene fingermarks as yet another application of MALDI MSI. This topic is discussed in the context of some casework undertaken together with highlights and limitations.

Methods:
Fingermarks were subjected to different treatments depending on the intelligence required. For profiling, they were subjected to MS and MS/MS spot analyses via either the MALDI MS Qstar Pulsar i mass spectrometer (Applied Biosystems, CA) or the Synapt G2 HDMS (Waters Corp., UK) QTOF. For imaging, they were matrix spray-coated using the automated Suncollect system (Sunchrom, Germany). All protocols were developed by using at large the same matrix (α-CHCA).

Results: Results from three example of casework undertaken will be discussed in the context of harassment, murder and sexual assault. Results highlights success and limitations various degree of success in the implementation of MALDI MSI Methods: Overall, it was possible to 1) extract intelligence on the contextual consumption of alcohol and cocaine from a 28 day old mark, in the harassment case, exceeding the information gathered by the police through conventional investigations [1]; 2) reconstruct the fingermark ridge pattern from a two year old mark found on a tape wrapped around the victim's neck whose body was dumped in a lake; 3) assist in a case of child abuse by investigating condom lubricants presence - results reassured the mother who did not press charges against her husband. MALDI MS and Ion mobility MS/MS in profiling and imaging mode were used to provide such intelligence.

Conclusions: MALDI MSI is compatible with prior application of crime labs fingermark enhancement techniques and can be used under full operational conditions. Detection and mapping of molecular intelligence as well as fingerprint pattern reconstruction are possible even in harsh environmental conditions. It is not always possible to determine the reasons of the occasional limited success due to lack of knowledge on the molecular information originally available.

Novel Aspect:
Unprecedented implementation of MALDI MS based methods in police casework involving fingerprints

References

1033 - DESI MASS SPECTROMETRY IMAGING OF THE DISTRIBUTION OF A FLUORESCENCE MARKER IN MALIGNANT GLIOBLASTOMA BRAIN TUMOURS

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Keywords: Glioblastoma, DESI-MS, Fluorescence, Imaging, MSI

Title: DESI mass spectrometry imaging of the distribution of a fluorescence marker in malignant glioblastoma brain tumours

Introduction: The use of fluorescence guided surgery (FGS), using 5-aminolevulinic acid (5-ALA), is important to establish safe maximal resection and better progression-free survival for glioblastoma (GBM) patients. 5-ALA acts as an orally administered fluorescent pro-agent of protoporphyrin IX (PpIX).1 Accumulating in glioma cells, 5-ALA is metabolized to PpIX, affording intraoperative fluorescence visualization of the tumour, (λex405 nm - blue, λem705 nm - red).

Methods: The distribution of PpIX was investigated using Desorption Electrospray Ionization MS Imaging (DESI-MSI) in preclinical and human GBM tissue samples. The fluorescence signature of PpIX was obtained using a fluorescence microscope (Olympus BX51 with QImaging Retiga 6000 camera) with excitation at 405 nm. Mass spectrometry images of the PpIX; parent ion at m/z 563.26 and CID MS/MS fragments, were obtained on a Waters Xevo G2-XS Q-ToF in positive ion mode.

Results: The distribution and detection levels of PpIX in human glioblastoma tissue is reported, with the goal of understanding where 5-ALA, and consequently PpIX, is localized relative to the tumour margins. DESI-MSI analysis of various PpIX dosed tissue homogenates, reveal detection of PpIX at the same concentrations required to observe fluorescence during surgery. Further, we describe the distribution of PpIX in samples from patients who have undergone tumour resection following the administration of 5-ALA at 20 mg/kg, 2 hours prior to the time of surgery. DESI-MS images and fluorescence images of PpIX are co-registered alongside Hematoxylin and Eosin (H&E) staining sections to determine the effectiveness of fluorescence to define tumor margins, with mass spectrometry confirming the molecular source of the fluorescent signal.

Conclusions: The preclinical data confirm that PpIX, the fluorescent porphyrin that results from 5-ALA administration, is detectable by DESI-MSI. By co-registration of fluorescence and mass spectrometry images, we demonstrate that PpIX is detectable to the same concentrations required for fluorescent imaging. This work also measures the relative co-localization of molecular PpIX with the fluorescence signal in the tumours and its ability to define tumour margins.

Novel Aspect: For the first time, the distribution of a fluorescence guided surgical molecule has been mapped in malignant glioblastoma tumour samples using MSI approaches.

References
Keywords: MALDI-MSI, Cancer diagnosis, Method development, Organic synthesis, Metabolomics

Introduction

The diagnosis of malignant glioma, one of most aggressive brain tumor, is still a challenge in the biomedical and surgical imaging procedures. An emerging cancer hallmark is the reprogramming metabolism where the energy metabolism is adjusted to enhance the energy supply and therefore the biosynthetic pathways for the uncontrolled cell growth. The main metabolic phenotypes in a tumor cell are an overproduction of lactate, so called Warburg effect, and several mutations of mitochondrial enzymes. Due to this, an alteration of cellular metabolism occurs causing the cancer progression [1]. Imaging of cancer metabolism is an optimal diagnostic tool for an accurate glioma classification and localization of tumor in surgical biopsies.

In this regard, Matrix Assisted Laser Desorption Ionization Mass Spectrometry Imaging (MALDI-MSI), a multi-component imaging technique able to detect and localize onto the tissue many compounds at the same run, could perform a visualization of the cancer metabolism directly on the glioma tissue. Unfortunately the detection of metabolites is still challenge because of low concentrations, matrix interference and fragmentations phenomena. It is known that the chemistry of matrix serves fundamental functions in desorption and ionization. For this reason the search for new MALDI matrices is required and then improve the limit detection of metabolites without any matrix background. For the first time, the 1,8-bis(dimethylamino)naphthalene (DMAN) or Proton Sponge was used in MALDI-MS for the detection of small weak acidic compounds with any matrix interference [2]. Unfortunately it was observed that DMAN is vacuum stable and then not suitable for the long-time MALDI-MSI procedures. Indeed in order to increase the vacuum stability the DMAN was structural modified generating two derivatives 1,8-Di(piperidinyl)-naphthalene (DPN) [3] and 3-(4,5-bis(dimethylamino)naphthalen-1-yl)furan-2,5-dione (4-maleicoanhydrido proton sponge or MAPS) [4],[5].

Regarding the para-substituted derivative MAPS, it was shown that this novel MALDI-MSI matrix is able to detect and localize compounds as small as chloride directly onto glioma tissues [6]. This work wants to show the potentiality of the MAPS as MALDI-MSI matrix in the discovery of novel biomarker candidates in the glioma diagnosis and as synthetic precursor of a new proton sponge based matrices generation.

Methods

Fresh-frozen glioma sections of five patients were covered with a matrix suitable for the detection of small molecules, Maleic Anhydride Proton Sponge (MAPS), using Image PrepTM as sprayer. Afterwards the glioma sections were analyzed by MALDI-ToF (ultrafleXetrem; Bruker) using a spatial resolution of 70 μm. The ionic spatial signatures on the tissue were generated using FlexImaging (Bruker) software. The chemical identification of ions was performed using High resolution MALDI-FT-ICR (solarix; Bruker) Imaging. Furthermore, in order to investigate the tissue microheterogeneity, clustering analysis of MALDI-MSI data was performed using Cardinal® as statistical software [6]. The cluster analysis was compared with histological characterization of H&E stained glioma sections, using QuPath assoftware.
Two proton sponge derivatives, 4-(N-phenyl)maleimido proton sponge (PMPS) and 4-(N-methyl)maleimido proton sponge (MMPS), were synthesized and sprayed onto mouse brain sections using an airbrush. MALDI-MSI experiments were performed in reflectron negative mode using 100 μm of spatial resolution and compared with MAPS.

Results
Several metabolic pathways of glioma metabolism, e.g. aerobic glycolysis, tricarboxylic acid cycle, and aminoacids metabolism, were visualized by MALDI-MSI procedures.
In the aerobic glycolysis, ionic maps of intermediates such as glucose, glucose 6-phosphate, and lactate were observed, confirming the Warburg effect as general biomarker of cancer tissue. MALDI-MSI of citric acid cycle metabolites, succinate and fumarate, were detected and different spatial signatures were observed in five patients thus suggesting mitochondrial enzymatic aberrations. It is very interesting to note that the spatial distribution on the tumor region of amino acids such as glutamine, which has a crucial role in the cancer progression. Moreover based on metabolite’s distribution and through an unsupervised cluster analysis, specific clusters were found in the five patients showing a correlation with tumor type and grading. Furthermore the intra-tumor differentiation, shown by the clustering analysis, was confirmed by PCA.
For the first time the 4-(N-phenyl) proton sponge (PMPS) and 4-(N-methyl) proton sponge (MMPS) were synthesized, characterized and applied as matrix in the MALDI-MSI of mouse brain tissue section. Small metabolites were detected and imaged on the tissue. Indeed several tissue-specific ionic maps were obtained showing a correlation with the histological features.

Conclusions
MALDI-MSI of glioma metabolism showed the detection of patient survival correlated biomarkers by phenotypically spatial signatures e.g. IDH1 or IDH2 mutations. Moreover it was shown the heterogeneity in metabolite distribution, confirming the tissue microheterogeneity that is one of main reasons of drug resistance. Moreover a new generation of proton sponge were synthesized and applied in MALDI-MSI showing very good performance as matrices. In conclusion the para-substituted proton sponges are valid candidate as matrix for tissue profiling by using MALDI-MSI procedures.

Novel Aspect
MAPS and its derivatives could contribute to the cancer diagnosis by MALDI-MSI procedures. Moreover the generation of new proton sponge derivatives could permits to gain more insight into the MALDI of metabolites. In conclusion this study is an emerging contribution for the clinical tumor diagnosis and also an outlook for a more detailed investigation of the ion formation mechanisms in MALDI that occur in complex systems such as biological tissue.

References
THE EFFECT OF PROTEIN BINDING OF DRUG SUBSTANCES IN DESORPTION ELECTROSPRAY IONIZATION MASS SPECTROMETRY IMAGING

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Keywords: Protein-drug interactions, Mass Spectrometry Imaging, Desorption Electrospray Ionization

Introduction:
Mass Spectrometry Imaging (MSI) is increasingly used as a tool in pharmaceutical R&D for analysing the distributions of drugs in tissue [1]. However, some drug substances are detected with greatly compromised sensitivity compared to traditional bioanalysis, due to strong binding to blood proteins such as albumin [2,3]. The study characterizes the protein binding of drugs in MSI and different approaches to improve the MSI detection of protein bound drug substances.

Methods:
Cryo-sections were made of liver homogenates spiked with different drug substances, representing different degrees of protein binding. For each cryo-section, one half was treated with different denaturants in order to break the protein binding. The sections were analyzed by Desorption Electrospray Ionization (DESI)-MSI, and the treated and non-treated tissue was compared.

Results:
DESI-MSI was applied to characterize protein binding, and significantly reduced signals were observed in tissue homogenates spiked with a strongly protein bound drug substance like Roflumilast, compared to cryo-sections of blocks of gelatin spiked to the same concentration. This effect was less pronounced for drug substances like codeine known to have a lower degree of protein binding. Different denaturants are being tested on similar cryo-section in attempts to alleviate the protein binding and improve the DESI-MSI sensitivity (work still in progress). Some of the denaturants appear to be more efficient than others in breaking drug-protein bonds.

Conclusions
The use of denaturants provide an increase in the free fraction of drug which can be detected by DESI-MSI. Although the results were obtained by DESI-MSI, it is likely that similar findings would be made with other MSI methods such as MALDI-MSI. The use of agents that denature proteins thereby breaking the drug-protein binding is worth considering as a sample preparation step in MSI in imaging of drug substances which are known to have strong protein binding.

Novel Aspect:

References:
Title: MS for MS; a mass spectrometry-based study into Multiple Sclerosis

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Keywords: Mass spectrometry imaging; quantitative LC-MS/MS; Lipidyzer; Multiple Sclerosis; Lipids

Introduction (400/400 characters)
Multiple Sclerosis (MS) is a chronic inflammatory disease of the central nervous system (CNS) in which infiltrating immune cells form MS lesions, resulting in extensive demyelination, axonal damage and neurodegeneration. Lipids are important mediators in immune response and a main constituent of the CNS, but their phenotype and contribution to MS pathogenesis remain elusive. Here we study lipids by applying multiple mass spectrometry based approaches to human MS.

Methods (394/400 characters)
Human post-mortem brain tissue from 3 MS patients and two healthy controls were collected and sectioned for both high resolution MALDI-TOF-MSI and MALDI-FTICR-MSI, as well as for quantitative LC-DIMS-MS/MS. To confirm the location of MS lesions, we performed immunohistochemical staining (IHC), using anti-myelin (PLP) and immune cell markers (LN3). The MSI data was interrogated in a histology-directed manner, and by using multivariate statistical Methods:

Results (860/900 characters)
Interrogation of the quantitative LC-MS/MS data, acquired using the SCIEX Lipidyzer platform after homogenizing tissue sections, resulted in the finding that several ceramides (dLCER(16:0), LCER(18:1), LCER(18:0)) were considerably higher in concentration in the MS patient tissues, compared to the healthy control tissues. Based on mass matching, m/z features corresponding to different ceramides, both lactosyl-, and hexosylceramides (HCER), were detected specifically in the MS lesions in the MALDI-MSI analyses. The specific detection of overexpressed LCER lipids in MS confirms the findings from a previous study on an experimental autoimmune encephalomyelitis (EAE) mouse model of MS, where astrocyte-derived LCER lipids were shown to contribute to the EAE disease phenotype [1]. Besides the LCER and HCER lipids, additional lipid features were found to be specific to the MS lesions, and using spatial segmentation of the tissues the MS lesions were clearly discerned from surrounding tissues.

Conclusions (342/400 characters)
In this proof-of-principle study on the investigation of the MS lipidome by using several MS techniques, we were able to confirm, for the first time, the presence and increased abundance of lactosylceramides inside the lesion of human MS patients. This study provides a solid basis into the further investigation of the role and location as well as function of these lipids in the context of MS pathogenesis.

References

Novel Aspect (120/150 characters)
The combination of off-tissue quantitative LC-DMS-MS/MS and in-situ high-resolution MALDI-MSI for the study of lipids in human MS samples.
SELECTIVE MS IMAGING OF HYDROPHOBIC/HYDROPHILIC COMPOUNDS WITH A NOVEL AU COATED BLACK SILICON SUBSTRATE

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Keywords: black silicon, Au, LDI-MSI, tissue imprinting

Introduction:
Silicon based substrates such as DIOS and NIMS proved their value in MS studies by successful detection of small molecules with reduced background noise and fragmentation, despite their unsafe fabrication method [1] [2]. Au nanoparticles were used in laser desorption ionization MS analyses with increased resolution and sensitivity [3] [4]. We developed Au coated black silicon substrates (BSi) with hydrophobic/hydrophilic selective regions for MSI applications.

Methods:
The BSi substrates were created by dry etching with SF6 and O2 plasma mixture [5]. Au layer was deposited by sputtering and the hydrophobic or hydrophilic surfaces were created with CHF3 or O2 plasma, respectively. Substrates were analysed by SEM, AFM, UV-VIS spectroscopy and mean contact angle. Animal tissue samples were imprinted on the substrates by touching. The MSI acquisition was executed with a MALDI TOF/TOF UltrafleXtreme spectrometer from Bruker Daltonics.

Results:
Physicochemical characterization of the AuBSi substrates showed needle-like array structure (<900 nm height) that absorbs ~95% of visible light, specifically 98% at 355 nm (the wavelength of the MALDI Nd:Yag laser). The hydrophobic regions of the substrates have a mean contact angle (CA) of ~165°, because of the CHx terminal groups, while the hydrophilic regions have a mean CA of ~45°, due to the HO groups. The AuBSi substrates revealed their efficacy by promoting the ionization/desorption processes of standard compounds as well as metabolites in tissue samples. The detected Au cluster peaks allowed an accurate mass calibration process throughout the full mass range. Many imprinted endogenous molecules were detected in the MS images. Therefore, the selectivity of the substrate was demonstrated by the specific adhesion of molecules: hydrophobic or hydrophilic compounds adhered to hydrophobic or hydrophilic regions, respectively. This may simplify untargeted analysis (molecule assignment process) of polar analytes from a broad mass range.

Conclusions
Selective imaging of adhered compounds from imprinted tissues is possible with our novel selective AuBSi substrates. Compounds adhered to the AuBSi substrate based on polar interactions between tissue and surface: hydrophobic analytes were found in the hydrophobic region and hydrophilic analytes were found in the hydrophilic region. Thus, knowing the polarity of the detected molecules simplifies the putative identification process.

Novel Aspect:
For the first time selective AuBSi substrates were entirely fabricated by dry etching and sputtering. No sample preparation was needed for selective imprinting of molecules.

References
APPLICATION OF ISOTOPE-LABELING IN WHOLE-BODY DESI-MSI STUDIES FOR INTRA-ANIMAL COMPARISON OF DRUG ADMINISTRATION ROUTES

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Keywords: Drug distribution analysis, DESI-MSI, whole-body imaging, stable isotope labeling, drug administration

Introduction:

The distribution of an administered drug in the organism is highly dependent on the administration route as well as the drug formulation. Therefore, assessing the distribution and metabolism of drug substances or drug candidates is of great importance in drug development, and mass spectrometry imaging has demonstrated many advantages, in particular due to its unique selectivity which enables specific imaging of several exogenous and endogenous compounds simultaneously.

Methods:
The antidepressant drug amitriptyline was intravenously (IV) dosed to a mouse and immediately after, a similar amount of a deuterium labeled version of the drug was intraperitoneally (IP) dosed to the same animal. Whole-body cryo-sections were made at t=5, 15, 30 and 60 min, according to a previously published protocol [1], and the two drug substances were simultaneously imaged with high sensitivity by DESI-MS/MS using the Displaced Dual-mode imaging approach [2].

Results:
The approach provides a unique way to compare administration routes in the very same animal, thereby eliminating uncertainties caused by animal-to-animal variation. Already after 5 minutes, the IV dosed drug was detected throughout the animal, while the IP dosed drug (deuterium labeled) was primarily found in the abdominal cavity. After 15 minutes, the IP injected drug appeared highly similar to the 5 minutes IV injection, while the IV injected had started accumulation in the stomach region. At later survival times, the differences of the two administration routes became less pronounced.

The deuterium label was partly preserved in metabolism to the primary metabolite nortriptyline, and the approach thus also enabled assessment on how the drug metabolism depends on the administration route. Interestingly, even at early survival times, the two administration routes provided highly similar metabolite distributions.

Conclusions
The presented method does not only enable IV and IP comparison, but could also be used for comparison of other administration routes such as subcutaneous or oral administration, and in the case of the latter, two different oral formulations could be tested in the same animal. While autoradiography has higher sensitivity, this study demonstrates how much more information MSI can provide with its unique selectivity, even within different isotopologues of the same drug substance.

Novel Aspect:
Highly selective MS imaging of different isotopologues of a drug and its metabolites provides unprecedented intra-animal comparison of administration routes.

References
Introduction:
In recent years, there has been a growing interest in the investigation of single cells and unicellular organisms. Single-cell measurement science has numerous applications across the biomedical and life science fields. Especially development of ambient MS techniques along with high performance MS systems provides powerful tools [1] which push the boundaries of system sensitivity so that only a minimal amount of cell material is needed to perform an analysis.

Methods:
Unicellular marine diatoms (Coscinodiscus sp.), freshwater algae (Haematococcus sp.) and various plant cells were analyzed in LDI mode via AP-SMALDI10-Orbitrap-MS/MSI Q-ExactivePlusMS system (TransMIT/Thermo Fisher Scientific, Germany). Cells were picked up from their media and transferred on a wet membrane filter or placed in a thin hydrogel film. All substrates were moistened/rehydrated with a suitable medium in order to ensure the cell viability.

Results:
The nanostructure of the Coscinodiscus sp. silica frustule acts as UV laser energy absorber and provides an intense ion signal in LDI mode[2] without MALDI matrix. Especially pigments characteristic to algae such as chlorophyll a (m/z 892), fucoxanthin (m/z 658) or beta-carotene (m/z 536) were present in the spectra together with their common fragments. The identity of these signals was confirmed with standards. Furthermore amino acids, fatty acids and different groups of lipids were found in the MS spectra. In the same way an AP-SMALDI-FT MS analysis of Haematococcus pluvialis cells showed strong signals of its typical red pigment, the antioxidant astaxanthin (m/z 596). Moreover, PLS-D analysis of the MS spectra showed a clear separation between groups of nutrient-depleted cultures of Haematococcus pluvialis cells (green phase) and repleted cultures (red phase). Using the very same approach it was also possible to show distinctive patterns of old and young cells of Coscinodiscus granii.

Conclusions:
Our LiveScan method requires only little sample preparation. Fresh and vital algae cells isolated from their aqueous substrata were placed on a wet membrane filter/hydrogel and analyzed directly. The obtained MS spectra covered different groups of compounds including amino acids, fatty acids, pigments and potential signaling molecules in high intensity. Characteristic patterns of the acquired spectra enable the differentiation of cell species.

Novel Aspect:
LiveScanMS-MSI represents a simple, fast and high throughput method for analyzing single cells in liquid phase under ambient conditions.

References
EVALUATION OF EMBEDDING STRATEGIES FOR FRAGILE AND SMALL SPECIMEN IN MSI ANALYSIS

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Keywords: MSI, fragile specimen, tissue embedding

Introduction:
Sectioning of fragile and small specimen can be challenge for mass spectroscopy imaging (MSI) analysis. However, embedding multiple small specimen allows simultaneous sectioning. An optimum embedding medium should meet several requirements, e.g. no altering the tissues, no negative effects for MSI and be sectionable of multiple specimen. The comparison of polymers with the freezing methods for embedding are critical factors in obtaining the best MSI result.

Methods:
5 different polymers (Gelatine, N-(2-Hydroxypropyl)-methacrylamide (HPMA), Sodium-Carboxymethylcellulose (CMC), (Hydroxypropyl)-methylcellulose (HPMC), Polyvinylpyrrolidone (PVP) were evaluated for their MSI suitability. All embedding media were evaluated for ease of sectioning, adherence to tissues and matrix interference, analyte delocalization and post-DESI histological evaluation.

Results:
Preliminary results displayed that Gelatine, HPMA, CMC, HPMC and PVP modified HPMC solution are able to provide easy sectioning whilst CMC with addition of alcohol and PVP were not suitable as they were not fully frozen under usual sectioning conditions and were not further investigated.

Adherence of the embedding medium to the tissue is important as the tissue sections must not fall out or curl up during sectioning. CMC and PVP modified HPMC are well adapted for this purpose while HPMA and HPMC are mostly suited for larger samples but not for small samples like intestines.

The last significant criteria is matrix interference. Gelatine shows chemical background from e.g. choline and arginine and tissue damage from hot gelatine solution. CMC showed adduct shift towards Na-adducts and smearing. HPMA, HPMC and PVP modified HPMC showed no matrix interference in MADLI and DESI-MSI.

Conclusion:
We found that the PVP modified HPMC is meeting all the criteria of an optimal embedding media, easy sectioning, high adherence to tissues and non-observed matrix interference.

We also found that snap freezing with isopropyl alcohol is a very promising method for preventing analyte delocalisation, block fracturing and small tissues thawing before fully frozen.

Novelty:
This study compares different polymers and freezing methods to develop a comprehensive tissue embedding protocol for MSI analysis.

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939 - VISUALIZING MOLECULAR DISTRIBUTIONS ACROSS/UNDER THE HUMAN SKIN BY 2D-DESI MS IMAGING OF TAPE-STRIPPED STRATUM CORNEUM

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Keywords: DESI, Mass Spectrometry Imaging (MSI), Natural Moisturizing Factor (NMF), Tape-stripping, Stratum Corneum (SC)

Introduction:
We have developed a new method combining DESI MS imaging (MSI) [1] and tape-stripping[2] techniques to visualize planer/depth profiles of natural moisturizing factor (NMF). NMF is a mixture of hygroscopic small molecules important to maintain hydration and barrier homeostasis of stratum corneum (SC) [3]. Differences of NMF content/composition are good index of skin conditions, whereas fine molecular distributions could not be obtained by conventional Methods:

Methods:
SC samples were collected by tape-stripping from the face or the inner forearm of healthy volunteers (one 30s female and one 30s male). Polymer-based adhesive sheets(25 × 70 mm, rectangular, in-house product) were used for SC collection. A high-resolution mass spectrometer equipped with a 2D-DESI source(Synapt-G2-SI, Waters) was used for DESI MSI analysis. Data processing was conducted by HDI-Imaging software® (Waters).

Results:
After the careful optimization of DESI experimental conditions, the developed visualizing method allowed to detect important 16 NMF-related molecules simultaneously. As a proof-of-principle experiments, planar distribution of NMF molecules across the half face was visualized. SC samples were collected from one side of the face on 8 adhesive sheets and measured with 500μm spatial resolution. It was found that the distribution/conversion of NMF molecules had large diversity. For example, serine (Ser) and pyrrolidone carboxylic acid (PCA), which are both abundant NMF [3], showed completely different distribution profiles (PCA distributed unevenly opposed to Ser). We also successfully visualized the changes of NMF depth profiles when the skin was treated with 2% sodium lauryl sulfate (SLS). SCs were collected before/after the treatment and measured by DESI-MSI. PCA was significantly eluted by the treatment, while the elution was prevented when a hand cream containing a film-forming ingredient was applied before the SLS treatment.

Conclusions:
NMF molecular distributions across the face and depth profile in the arm skin were successfully visualized for the first time. Because of the multi-target simultaneous analysis capability of the developed DESI-MSI method, molecular kinetics such as enzymatic conversion could be assessed. We believe that the new method is a promising tool for skin biological research and will provide useful insights for the development of new skincare products.

Novel Aspect:
The first application of DESI MSI to tape-stripped SC to visualize molecular distribution of NMF across the human face and depth profile.

References:
719 - DISTRIBUTION OF LIPIDS AND ANTI-TB DRUGS IN MOUSE LUNG TISSUE

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Keywords:
ALDI imaging, anti-TB drugs, high resolution

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Introduction:

The rise of multi drug-resistant Mycobacterium tuberculosis strains creates an ever increasing need for novel anti-TB drugs. The development of anti-TB drugs however is impeded by the shortcomings of current preclinical animal models. In preparation for studies on novel anti-TB drugs, an AP-MALDI imaging method was established in this study to map the distribution of phospholipids and multiple anti-TB drugs in mouse lung tissue.

Methods:

MS imaging experiments were carried out on a Q-Exactive™ HF Hybrid-Quadrupole-Orbitrap mass spectrometer (Thermo Fisher Scientific, Bremen), coupled to the AP-SMALDI10 ion source (TransMIT GmbH, Gießen) [1]. Mass spectra with high mass accuracy (<2 ppm) and high mass resolution (R=240,000 @m/z 200) were acquired. Measurements were performed on lung tissue of BALB/c mice treated with anti-TB drugs. Different matrices were applied using a pneumatic sprayer system.

Results:

Sample preparation including matrix application and cryosectioning was optimized for several anti-TB drugs. MS imaging measurements of mice lung sections were performed with pixel sizes between 10 and 40 μm. These experiments showed high spatial details in phospholipid images, which corresponded well with histological features. Phospholipid patterns differed substantially between different tissue regions. In addition, the distribution of several anti-TB drugs could be shown in mouse lung tissue.

Conclusions

The investigated anti-TB drugs differ significantly in their physicochemical properties, for example polarity. Therefore, a sectioning protocol was adapted to avoid leaking of the drug from tissue. Different matrices and solvents had to be used for different drugs. Several drugs were detectable in treated mice, but showed different limits of detection. In conclusion, the MALDI imaging workflow must be optimized for each drug.

Novel Aspect:

We established a high resolution MALDI MSI method to analyze distributions of lipids and anti-TB drugs in mouse lungs with a spatial resolution close to the cellular level.

References

451 - SILVER-DOPED NANO-DESI MSI FOR PROSTAGLANDIN IMAGING

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Keywords: nano-DES I MSI, quantification, prostaglandin, silver, uterine tissue

Introduction:
Silver ions are known to form weak charge transfer complexes with alkenes, and the addition of Ag+ to a double bond can therefore increase ionization efficiency and detectability of a compound. This work demonstrates nanospray desorption electrospray ionization mass spectrometry imaging (nano-DESI MSI) of endogenous prostaglandin species (PG) by utilizing the complexation between Ag+ and PG to enable the generation of unique PG ion images from thin tissue sections.

Methods:
Nano-DESI MSI was performed by connecting a custom built source to a Q-ExactiveOrbitrap mass spectrometer. 10 ppm Ag+ was added to the solvent, which also contained acetonitrile and methanol (9:1), and deuterated PG species to facilitate quantification. Mouse uterine tissue sections of ~ 2 mm in diameter were imaged with higher spatial resolution using oversampling. The generated quantitative ion images of PG had an approximate pixel size of 30 µm x 10 µm.

Results:
The inclusion of silver into the solvent increased the sensitivity for [PG+Ag]+ ions by ~ 30 times compared to conventionally monitored [PG-H]- ions. Additionally, it was found that the natural isotopic pattern from the two stable Ag+ isotopes was beneficial when corroborating the identity of analytes as silver ion adducts and that the exclusive binding of Ag+ to the alkene moiety enabled direct identification of double bond containing metabolite species.

Quantitative nano-DESI MSI revealed the localization of PG species to the luminal epithelium and glandular epithelium of mouse uterine tissue sections at day 4 of pregnancy prior to embryo attachment. Interestingly, the amount of both PGE2 and PGF2α were found to be three times as high in these cellular regions. The cellular regions of the glandular epithelium are only 100-300 µm in diameter, and were resolved in this study by use of oversampling. Three additional PG species were identified in the tissue with similar localizations displaying the great utility of silver-doped nano-DESI MSI.

Conclusions:
The presented results successfully demonstrate quantitative nano-DESI MSI of [PG+Ag]+ species directly from biological tissue without additional sample preparation. The described approach will be extremely valuable for future studies investigating the role of PG species in biological systems.

Novel Aspect:
First study detailing the specific localization and quantity of prostaglandin species in biological tissue sections.

References:
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Calcium and Lipid Imaging of Regenerated Bony rays of Zebrafish Caudal Fin using Time-of-Flight Secondary Ion Mass Spectrometry

Keywords: ToF-SIMS, MS imaging, zebrafish, bone, small molecule analysis

Introduction:
We observed regenerated caudal fins of zebrafish using time-of-flight secondary ion mass spectrometry (ToF-SIMS). The spatial distribution of the small molecules including mineralized ions, lipids and fatty acids in the regenerated area of the fin was found to be different from the spatial distribution in the existing part.

Methods:
ToF-SIMS images in this study were obtained using ToF-SIMS 5 (ION-TOF GmbH) equipped with liquid metal ion gun (LMIG) and gas cluster ion beam (GCIB) sources [1,2]. The caudal fins were sputtered with Ar clusters and etched to 20-25 μm from the surface in the depth direction, then ToF-SIMS images of the bony ray and the inter-ray mesenchymal tissue were obtained using rastering of a Bi3+ ion.

Results:
ToF-SIMS analyzed both positive and negative ion modes and about 1000 specimen-related spectra were obtained from zebrafish caudal fins. Since the ToF-SIMS method detect mineral ions very well, it provided high spatial resolution ion images including Ca+, Mg+, K+, and PO2- ions to intuitively identify the precise location of the truncated site and the regeneration shape of the bony rays of the zebrafish caudal fin at 4 days-post-amputation (dpa) and 7 dpa [3]. Whereas the calcium and PO2- ions were be found to contain more in the regenerated area than in the existing area of the caudal fin at 4 dpa, there were little difference between the two areas of the caudal fin at 7 dpa. For phosphocholine ion in the caudal fins at 4 dpa and 7 dpa, the regenerated area had a higher signal intensity than the existing area.

Conclusions:
Several ion images of regenerated bony rays in the zebrafish caudal fin indicated that the regeneration of bony rays might precede the regeneration of other tissue materials. Consequently, ToF-SIMS imaging is a simple and easy approach to understanding the spatial distribution of small molecules in the regenerated caudal fin of a zebrafish.

Novel Aspect:
The zebrafish caudal fin is not only a very appropriate bio-specimen for mass spectrometry due to its thickness of less than 200 micrometers but also a very important organ for tissue regeneration studies.

References:
131 - TOF-SIMS IMAGING OF RAT AND DROSOPHILA BRAIN TISSUE SECTIONS PREPARED BY USING THE NEW SAMPLING METHOD

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Keywords: ToF-SIMS imaging, Tape-supporting sampling, Brain, Rat, Drosophila

Introduction:
Brain imaging using ToF-SIMS has been reported to produce the distorted biomolecular distributions due to the cholesterol-induced matrix effect, delocalization of the analytes, and/or inadequate tissue adherence during sample preparation. In this talk, we propose new sample preparation and drying methods that can be used to obtain accurate biomolecular images of brains at room temperature, instead of frozen-hydrate analysis using liquid-nitrogen.

Methods:
The rat or Drosophila brains prepared by using a double-sided adhesive tape on a pre-cooled (−20 °C) stainless steel plate was freeze-dried in a load-lock chamber of ToF-SIMS for about an hour and moved directly to the main chamber. All measurements (depth profiles, images) were obtained using a TOF-SIMS V instrument. The 25-keV Bi3+(current~0.1 pA, cycle time: 200 μs) was used as an analysis ion beam and the 5-keV Ar1500+(current~3nA) was used as a sputtering ion beam.

Results:
We obtained the depth profiles of rat brain tissue (corpus callosum) as a function of sampling and drying Methods: In the white matter of the rat brain prepared by using the thaw-mounting method, cholesterol migrated to the sample surface regardless of room temperature-drying or freeze-drying. However, freeze-drying of the sample prepared by using the tape-supporting method did not cause the migration of cholesterol to the sample surface. In the frozen-hydrate analysis, cholesterol also did not migrate to the sample surface of the rat brain prepared with the thaw-mounting method or tape-supporting method [1]. Regarding the Drosophila brain imaging, the use of conductive double-sided adhesive carbon tape has been shown to enable the successful collection of small and fragile samples to surfaces compatible with ToF-SIMS analysis [2]. In addition, the tape-supporting method can effectively preserve the distribution of biomolecules on the rat and Drosophila brains in the same manner as that of the frozen-hydrated analysis (below −100 °C) [1,2].

Conclusions:
Accurate and undistorted images of biomolecules in the rat and Drosophila brains can be obtained from brain sections prepared by the tape-supporting and freeze-drying method without performing frozen-hydrate analysis. We expect that this new sample preparation method will enable scientists using ToF-SIMS to easily and accurately detect important biomarkers for degenerative diseases.

Novel Aspect:
Our protocol circumvents cholesterol migration to the surface, delocalization of small molecules, and surface water interference.

References
Introduction
Multimodal imaging is a growing scientific field beneficial for explanation of many biological and chemical processes. Matrix-assisted laser desorption/ionization mass spectrometry imaging (MALDI MSI) together with laser scanning confocal microscopy (LSCM) conducted immunohistochemistry (IHC) allow to study drug distributions and related cellular responses. [1] In our study, 3D cell lines (spheroids) were employed as improved model for drug testing [2].

Methods
Spheroids were treated with the drug perifosine for selected time intervals, transferred to gelatine, frozen, sectioned and placed to conductive MALDI glass slides. Dried sections were covered with sublimated DHB matrix and measured using MALDI MSI. The matrix was washed away by organic solvents and samples were processed using standard IHC protocol with selected antibodies. Later, the analysis on LSCM and its correlation to MALDI MSI was carried out.

Results
The study of perifosine penetration by MALDI MSI revealed that perifosine does not penetrate the spheroid core even after 24 hour induction, it concentrates only in outer 150-µm cell layer. A specific characteristic of this drug is also its diffusion from the tissue to gelatine medium during sample preparation. Viability assays by IHC were conducted for markers of cell proliferation and apoptosis with parallel staining of all cell nuclei. We detected elevated signals of apoptotic marker in spheroid core and proliferative cells on spheroid edges. This corresponds to general knowledge that spheroids from certain size physiologically develop necrotic core, due to the lack of oxygen and nutrients in deeper cell layers [3]. Unfortunately, none of these spheroid physiologies were changed when the spheroids were treated by perifosine, meaning perifosine does not have any negative effect on cell viability.

Conclusions
MALDI mass spectrometry imaging and immunohistochemistry analysis of colorectal carcinoma spheroids showed no correlation between perifosine and dead cell presence. Apoptotic cells were found in spheroid core, however this was caused only by natural sample physiology. Perifosine was also not capable of penetrating the spheroid deeper than 150 µm, suggesting a poor drug efficacy.

Novel Aspect
The analysis of the same tissue section by both MALDI MSI and immunohistochemistry, enabling correlation of both drug distribution and cell viability.

References
Introduction:
Traumatic Brain Injury is mainly due to direct mechanical damage to the brain tissue leading to an axonal disruption and widespread neural dysfunction. Monitoring lipid changes post injury could help in a possible therapeutic approach. In fact, lipid biomarkers have been studied in several brain injury models using mass spectrometry as demonstrated by Nielsen et al which identified NAPE as a biomarker of dead neurons in mice cerebral ischemia injury model.

Methods:
Lipid 3-Dimensional MALDI mass spectrometry imaging was applied on injured brain harvested from rat subjected to controlled cortical impact model (CCI) injury model. Images were conducted using high resolution MALDI-LTQ-XL Orbitrap. Data visualization and processing was performed on SciLS lab MVS software along with 3D image reconstruction, principal component analysis, and unsupervised spatial segmentation.

Results:
MALDI images were performed on serial sections from an injured rat brain at 3 days post injury and the 3D reconstruction was performed after performing unsupervised spatial segmentation on all sections followed by image alignment. The unsupervised spatial segmentation showed two unique clusters that co-localized within the injured cortical tissue and not expressed in any other regions of the brain. These clusters both departed from the non-injured cortical tissue on the opposite hemisphere of the brain in the PCA analysis, thus confirming the first finding of lesion specific clusters. In 3D manner, we visualized and assigned specific m/z values solely expressed in the injured tissue. At the same time, certain m/z ions showed a particular expression within the injury site and the ventricular system of the brain.

Conclusions:
Our results can help in further monitoring and identifying lesion-specific m/z in a 3D manner to obtain a better understanding and visualization of molecular and cellular events occurring post-injury. We have also showed injury-elevated m/z in common with the ventricular system of the brain thus suggesting a possible trafficking of molecules between the injured brain area and the spinal cord.

Novel Aspect:
Applying 3D MALDI-MSI on traumatic brain injury for the first time. High spectral resolution allowed to differentiate between m/z ions with a difference of up to 0.1 mDa.
References:


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**Introduction:**
MALDI MSI visualizes distributions of a broad variety of biomolecules in tissue. µXRF is an non-destructive elemental imaging method with high analyte specificity, and therefore a favourable choice for a multimodal imaging approach targeting elemental and molecular distributions. A methodology is presented allowing µXRF and MALDI MSI analysis from the same undecalcified tissue section to visualize such distributions in tissue.

**Methods:**
Chicken feet were purchased from a local market, digits were removed and embedded in a hydrogel containing NaCMC and gelatin. Cryosectioning of the phalanx was performed with a non-disposable tungsten carbide knife according to the Ullberg’s method. An in-house built µXRF spectrometer (Atominstitut, TU Wien) was used for XRF analysis and MALDI MSI experiments were carried out on a Waters Synapt G2 after applying matrix (dithranol) by sublimation.

**Results:**
In this proof-of-principle study, we established and tested sample preparation strategies, allowing multimodal analysis of lipids (sphingomyelin and phosphatidylcholines) and elements relevant for bone structures as calcium, phosphorous and sulphur in the very same sample section of a chicken phalanx without tissue decalcification. The results of the investigation of such parameters as adhesive tapes supporting tissue sections, and sequence of the imaging experiments are presented. We show specific lipid distributions in skin, cartilage, muscle, nail, and intact morphology of bone by calcium and phosphorous images. Combination of molecular and elemental imaging was achieved, thus, providing now for the first time the possibility to gather MALDI MSI and µXRF information from the very same sample without any washing steps omitting therefore analytical artifacts that inevitably occur in approaches using consecutive tissue sections.

**Conclusions:**
Conversely to most previous multimodal (multisensor) investigations, our imaging experiments are carried out on the very same samples without any additional steps, thus, avoiding analytical artefacts. We showed that we are able to cut difficult samples properly and visualize lipid distributions together with biologically relevant elements like Ca, P or S. We performed imaging experiments with similar lateral resolution facilitating the correlation of the images and gaining morphological information that was not possible from either analytical method independently.

**Novel Aspect:**
Combining MALDI MSI and µXRF can benefit in researches where elemental and lipid interaction play an essential role.
INTRODUCTION: Since the pioneer work of J. J. Thomson on magnetic deflection of charged particles, mass spectrometry (MS) is the most progressing clinical tool, continuously providing new applications in medical research [1, 2]. Mass spectrometry imaging (MSI) recently emerged as an option in the prospection of new biomarkers and has opened many doors for clinical analysis, especially in the oncological area [2]. In hepatocellular carcinoma (HCC), MS can be used from disease surveillance in early stages into constant evaluation of effective treatments [3, 4, 5], but until now, MSI was not used on HCC molecular and elemental imaging.

METHODS: Samples were collected from patients underwent orthotopic liver transplant (OLT) within two distinguish groups: i) liver end-stage patients solely with hepatitis C virus (HVC) chronic inflammation (named INF); ii) liver end-stage patients with hepatitis C virus (HVC) chronic inflammation and HCC. DESI: performed in a Q-Exactive®, a hybrid Quadrupole-Orbitrap mass spectrometer within a m/z 100−1200 with a step sized of 200 μm, a scan rate of 740 μm s−1, and a pixel size of 200 μm × 200 μm. Treatment was performed in BioMAP software. LA-ICP: performed in quadrupole-based ICP-MS (PerkinElmer ELAN DRC-e) coupled with a laser ablation system (New Wave UP 213), mass-to-charge ratios (42Ca+, 63Cu+, 55Mn+, 31P+ and 64Zn+). The used conditions were a spot size of 65 μm, scan speed of 50 μm s-1, a frequency of 15 Hz, and a laser intensity of 55%. Treatment was performed in LA-iMageS software. COMEP 026/98. Results: In the red-dashed-line the cirrhotic microenvironment (INF) and in the blue-dashed-line the hepatocellular carcinoma (HCC) (Figure 1). Co-localization generated by LA-ICP-MS and DESI approach enable the visualization of topological shifts between micronutrients abundance and metabolites relative abundance association, in intact tissues of HCC and its microenvironment (Figure 2). This study reveals spatially relative abundance association between m/z 303.23 and 42Ca in INF scenario; m/z 792.48 and 31P in HCC scenario, for example. Conclusions: The integration of these imaging stages provides identification of elemental microdomains and their secondary metabolism alterations, allowing direct observations on HCC heterogeneity, microenvironment diversity and tumor molecular characterization. Shedding light on the understanding of HCC metabolic regime, to improve comprehension on pathogeneses progression. Novel Aspect: New mass spectrometry insight for application in medical research on tracking microenvironment/cancer (HCC) shifts related to micronutrients abundance and its correlation on metabolic macromolecular regime.

REFERENCES
COMBINING MASS SPECTROMETRY IMAGING & MICROPROTEOMICS TO INVESTIGATE INTRATUMOR HETEROGENEITY

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Keywords: MALDI, imaging, microproteomics, laser capture microdissection, intratumor het

Introduction:
MSI simultaneously records the distributions of hundreds of molecules directly from tissue. When combined with multivariate analysis it can reveal regions of tissue with distinct molecular signatures, including tumor subpopulations. The small pixel sizes and direct tissue analysis of MSI limits the achievable depth of proteome coverage. Here, we demonstrate how MSI can be combined with high sensitivity microproteomics, even of the same tissue section.

Methods:
Protein MALDI MSI data of patient tissues was combined with histopathological analysis to isolate the MSI data from tumor regions. Five independent multivariate tests were used to identify tumor subpopulations, and subpopulations associated with phenotype identified. Regions of tissue were isolated by laser capture microdissection and analyzed by LC-MS/MS using an Orbitrap Fusion.

Results:
MALDI MSI has been applied to patient tissues of gastric cancer, breast cancer, and soft tissue sarcomas. Following histopathological annotation and automated feature identification and extraction, the resulting datacube of MS images of only tumor regions, was subject to five independent multivariate methods to identify tumor subpopulations characterized by distinct molecular signatures. Associations with clinical data identified those subpopulations linked with patient phenotype (survival and metastasis).

In order to dig deeper into proteome changes in small, localized regions of tissue we have developed an SP3 based microproteomics approach utilizing 10-plex TMT labels and high-pH fractionation, to combine high relative quantitation precision with in-depth proteome profiling. We demonstrate the performance of this approach and demonstrate how it may be seamlessly combined with MALDI MSI of the same tissue section.

Conclusions
MALDI MSI combined with microproteomics can be used to identify and investigate phenotypic tumor subpopulations.

Novel Aspect:
Intratumor heterogeneity; seamless integration of MALDI MSI and LC-MS/MS, of the same tissue section.

References
Introduction:
The uptake of nanoparticles (NP) such as cerium oxide or zinc oxide NP via inhalation may pose a health hazard for humans. Therefore, knowledge about their distribution in the lung and their biological effects is needed. Here, we combined elemental and molecular imaging techniques to study the distribution of NP and changes of phospholipid distribution in the lung.

Methods:
Different doses of nanoparticles were intratracheally instilled into rat lungs. The lungs were removed and cryosections were prepared. Laser ablation inductively coupled plasma mass spectrometry (LA-ICP-MS) experiments were performed to reveal the distribution of NP in the tissue. To analyze phospholipid distribution in positive and negative ion mode, matrix assisted laser desorption/ionization mass spectrometry experiments were carried out.

Results:
To analyze the impact of nanoparticles on lung tissue, MALDI-MS experiments were performed. Different phosphatidylcholines were detected in the positive ion mode and verified via MS/MS experiments by characteristic fragment of m/z 184. In case of lung inflammation increasing signal intensities of PC(32:1) and PC(36:4) were detected. In the negative ion mode phosphatidylglycerols and phosphatidylinositols were detected and were confirmed via MS/MS experiments. Nanoparticle distribution patterns as well as the distribution of naturally occurring elements such as phosphorus were depicted by means of LA-ICP-MS. Accumulations of nanoparticles could be detected in alveolar areas of the lung.

Conclusions:
The combination of elemental and molecular mass spectrometry by MALDI-MS and LA-ICP-MS shows the impact of nanoparticles on lung tissue. Depending on elemental composition, concentration, and period of incubation lung inflammation and fibrosis can occur. Alterations in phospholipid distribution were detected by MALDI-MS.

Novel Aspect:
Results show that combining MALDI-MS and elemental imaging techniques can provide new insights into local changes caused by nanoparticles in tissues.
Introduction:
Bowel cancer is the second leading cause of cancer mortality in the United Kingdom. Mutations in the APC and KRAS genes are found in these tumours at rates of over 70% and 40%, respectively. In order to better understand the effects of these mutations on cellular metabolism, MALDI, DESI, and SIMS mass spectrometry imaging were used at multiple research sites to identify and map the localization of key metabolites in genetically engineered murine tissue.

Methods:
Murine samples were prepared and sectioned onto glass or ITO coated slides and stored at -80°C until analysis. MALDI-MSI was carried out on a RapifleX ToF-ToF (Bruker Daltonics) or Synapt G2-Si Q-IMS-ToF (Waters). DESI-MSI was carried out on a Synapt or Xevo Q-TOF (Waters). SIMS analysis was carried out on an OrbiSIMS (IONTOF, Thermo Scientific). Data were converted to imzML by imzMLconverter and analysed in MatLab (2017a, MathWorks) via SpectralAnalysis.

Results:
Colon and small intestine from sixteen animals were embedded into blocks of four containing four different genotypes each (wild type, KRAS-mutated, APC-mutated, and dual KRAS/APC-mutated). DESI and MALDI data were collected at multiple sites to discern variations in data seen between different instruments and to enhance method development to produce robust, reproducible practices. Preliminary analysis of these data indicates that the use of MSI followed by multiple computational analysis techniques including NMF, PCA, k-means clustering, and t-SNE neural networking allows for the segmentation of anatomical regions as well as grouping of phenotypic variation by genotype. Work is ongoing to utilize database matching and MS/MS studies to identify metabolites within these segmented data to correlate metabolic changes to genetic type. Lastly, further analysis of these datasets will allow guided analysis of additional samples via high resolution MSI to examine metabolite localization in a cellular context.

Conclusions:
Preliminary analysis indicates that both anatomical and phenotypic segmentation can be generated using a combination of computational analysis techniques. Current work is underway to identify metabolites within these segmented regions and determine how the APC and KRAS mutations may be effecting metabolic processes. Future work can integrate MSI results with proteomics and transcriptomics to inform additional studies.

Novel Aspect:
Multimodal, multisite analysis of bowel cancer murine models by MSI followed by multiple computational analysis methods to map metabolites using database matching.
INTERFERENCE FOR THE MALDI-MSI ANALYSIS OF IN-VIVO THYROID BIOPSIES

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Keywords: Fine needle aspiration, thyroid lesion, haemoglobin interference, MALDI-MSI proteomics, thin prep.

Introduction: Fine Needle Aspiration biopsy (FNAB) is the gold standard exam to determine the malignant nature of thyroid nodules[1]. Contamination of FNAB samples with red blood cells is problematic for proteomics analysis, given that large amounts of haemoglobin (Hb) suppress other protein signals[2]. Hence, it is paramount to standardise the sample preparation of ex-vivo and in-vivo thyroid FNABs for proteomic MALDI-MSI analysis, in order to minimise Hb interference.

Methods: Human FNABs were collected and deposited onto conductive glass slides from both ex-vivo(n=9), surgically removed thyroid specimens, and in-vivo(n=12) thyroid specimens for intact proteins MALDI-MSI analysis. Three protocols were compared using ex-vivo biopsies collected from the same thyroid: a) conventional air-dried smear; b) cytological smear immediately fixed in ethanol; c) ThinPrep (TP) cytological preparation using a ThinPrep2000 system.

Results: The spectral profiles of both ex-vivo and in-vivo conventional air-dried smears were characterized by high inter-patient variability related to the abundance of the Hb peaks. In particular, the strong vascularization of some thyroid nodules is reflected in FNABs with a high content of Hb. The amount of Hb was markedly decreased in TP preparation with respect to both conventional air-dried and fixed smears. On the other hand, the absolute intensity of other protein signals, suppressed with the other two methods, were significantly increased in TP samples. Furthermore, the management of Hb interference of ex-vivo and in-vivo TP samples was comparable, indicating the opportunity to use in-vivo TP specimens for MALDI-MSI proteomic analysis and biomarker discovery. The MALDI-MSI approach combined with virtual microdissection permitted to extract specific protein signatures from different histotypes of both benign and malignant thyroid cell clusters.

Conclusions: The Thin Prep procedure for thyroid FNABs samples preparation combined with MALDI-MSI proteomic analysis allow us to obtain high-quality spectra, follicular cells specific protein profiles and to manage the haemoglobin interference. The application of this reproducible technique to in-vivo cytological samples can help cytopathologists in the diagnosis of thyroid nodules combining both morphological and proteomics information.

Novel Aspect: This study represents the first example of MALDI-MSI applied to ex-vivo and in-vivo thyroid FNABs, prepared using the ThinPrep preparation, for proteomic analysis.

References

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HIGH SPATIAL RESOLUTION IMAGING OF AGED HUMAN RETINA TISSUE

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Keywords: imaging mass spectrometry, retina, retinoids, lipids

Introduction:
The human retina is made of morphologically distinct layers responsible for the absorption of photons and for sending signals necessary for vision to the brain. Imaging mass spectrometry (IMS) is a powerful tool for examining spatially disparate molecular signatures. We have applied IMS to human retina tissue to examine the spatial distribution of analytes in retina cell layers and to detect differences with age and disease.

Methods:
Fixed and frozen human retina tissue from older donors (80-893 years) was sectioned to 12-14 µm thickness and placed on ITO slides for IMS experiments. MALDI matrix was applied by sublimation and images were acquired using a modified Bruker Solarix 9.4T FTICR instrument. Data were analyzed with FlexImaging software. Registration between optical images and MALDI images was aided by autofluorescence microscopy and custom registration software.

Results:
Fixed tissue sections provided superior morphology with reduced analyte signal intensity in contrast to frozen sections that had poorer morphology but provided higher signal intensity. Ion maps unique to many retina cell layers were obtained with 15 µm spatial resolution. Autofluorescence microscopy facilitated registration of IMS and microscopy via explicit mapping of IMS pixels to their MALDI laser ablation marks, allowing confident identification of retina cell layers in the IMS data. Molecular signatures were identified for the high density of Mueller cells within the fovea, for basal laminar deposits, and for the retinal pigment epithelium (RPE). The bisretinoid metabolite, A2E, was detected in RPE cells of peripheral retina. A series of BMP lipids were detected throughout the RPE and photoreceptor outer nuclear layer. LC-MS/MS analysis is being used to identify novel compounds within the basal laminar deposits and within the fovea region. Unique signals were also observed within the inner and outer segments of photoreceptor cells.

Conclusions:
IMS provides a powerful tool to examine molecular changes in distinct retinal cell layers with age and extracellular deposits in disease. Specific lipid and retinoid metabolites have been localized to the RPE and fovea regions of older human retinas.

Novel Aspect:
Novel analytes identified in distinct retinal layers using high resolution IMS of human retina sections with characteristic aging features.
Keywords: skin, thermal desorption, ambient mass spectrometry, imaging, biomolecule

Introduction:
Skin plays a vital role in protecting human being against the attack by pathogenic microorganisms. Biomolecules such as neurotransmitters, amino acids and other metabolites are also released from skin through numerous glands underneath it. Although these biomolecules on skin are possibly potential disease biomarkers, current analytical techniques are incapable to efficiently identify them on skin, except blood drawing. In this study, thermal desorption-electrospray ionization/mass spectrometry (TD-ESI/MS), a novel ambient mass spectrometric technique[1], was used to characterize the skin compounds including neurotransmitters, amino acids, lipids and metabolites without doing any sample pretreatment. Since TD-ESI/MS owns the feature of rapidness, high-throughput sample analysis, molecular imaging of skin can be presented on specific chemical compounds. Herein, ambient mass spectrometry imaging was demonstrated for visualizing the distribution of biomolecules on whole body skin.

Methods:
The TD-ESI/MS system consists of sampling probe, heated oven, electrospray device, and mass analyzer. A stainless steel probe was used to gently scrape through the skin surface for ca 1 cm for sampling. It was then inserted into a heated oven to thermally desorb the analytes on it. The analytes were carried by a nitrogen stream down to a plume consisting of acidic methanol/water and ionized by reacting with the charged species (e.g. H+, H3O+, etc) in the ESI plume. Any residual sample on the probe was efficiently removed by burning it with a high-temperature gas torch for a few seconds. It took less than 30 seconds to complete an analysis.

Results:
In typical case, approximate 2,000 samples were collected on skin within an hour and the analysis of these samples took less than 12 hours. The imagings were obtained using commercial available imaging software. Taking caffeine or medicine as an example, the analyte ion signal was mostly detected on the upper body especially forehead, fingertips and backside of the body. This may due to different sweating rates in different skin parts as well as the distribution of veins and capillaries over the body. Not only medicines and their metabolites but regular metabolites such as neurotransmitters, amino acids, fatty acids, cholesterol, diglycerides, triglycerides and squalene were also detected and their whole body skin imagings show that the distribution of metabolites on skin also depend on the structure and chemical property of the compounds.

Conclusions:
Numerous biomolecules on the human skin was rapidly detected by insitu probe sampling and TD-ESI/MS analysis within a few seconds per sample. The ambient molecular imaging of squalene and cholesterol on whole body skin was also successfully established. In summary, we combined in situ probe sampling and TD-ESI/MS analysis, an noninvasive skin imaging technique for efficiently perform molecular imaging on the skin with high-throughput analysis was developed.

Novel Aspect:
Thermal desorption-electrospray ionization/mass spectrometry was used to perform whole body skin ambient imaging on potential biomolecules.

References:
Aldosterone specific visualization in primary aldosteronism using imaging mass spectrometry

Introduction:
Visualization of steroid hormones using MALDI-IMS is one of the active subjects for endocrine studies. Unfortunately, it is difficult to detect steroid hormones due to the low polarity. To overcome the problems, on-tissue chemical derivatization (OTCD) was developed [1], however the problem of structural isomers in steroid hormones is still remain. Herein, we introduce aldosterone specific visualization method using OTCD and tandem MS.

Methods:
Frozen tissue was sectioned and thaw-mounted on conductive glass slides. Girard’s reagent T (GirT) was used and the GirT solution was sprayed on the tissue surface with an artistic air-brush. After one hour reaction at room temperature, α-CHCA was applied using two-step matrix application method. MALDI-IMS analysis was performed using LTQ XL linear ion trap mass spectrometer equipped (Thermo Scientific, Bremen, Germany).

Results:
We first demonstrated discrimination of aldosterone and cortisone which is a structural isomer of aldosterone using authentic standard sample. Aldosterone and cortisone were successfully discriminated by MS3 analysis. To confirm the utility of our method, next two experiments were performed in rat sodium diet model and human primary aldosteronism (PA), which is one of the causes of secondary hypertension. According to our experimental result, aldosterone and structural isomer of cortisone were clearly visualized separately. In PA patient tissues, we successfully visualized these hormones individually and compared with immunohistochemistry of aldosterone synthase. Our finding in this study was the expression level of aldosterone production enzyme and produced aldosterone were different in distribution.

Conclusions:
Although some literatures described MALDI-IMS approach for steroid hormone imaging, structural isomers have not been fully considered. In this study, we have developed aldosterone specific visualization method. After validation of the method in rat adrenal gland, we successfully visualized aldosterone producing region in PA. We found that combination of different imaging results (MALDI-IMS and IHC) were valuable to elucidate diseases.

Novel Aspect:
Combination of GirT and MS3 analysis allowed us aldosterone specific imaging in human adrenal gland, and it will provide more reliable information in diagnosis of PA.

References:
MALDI-MS IMAGING IN THE SEARCH FOR PROTEOMIC INDICATORS OF RESPONSE TO THERAPY IN MEMBRANOUS NEPHROPATHY

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Keywords: clinical proteomics, high spatial resolution, membranous Nephropathy, MS imaging, pathology

Introduction: Membranous Nephropathy (MN) is the most frequent cause of nephrotic syndrome in adults and the disease course is characterised by the “rule of third”, with one third of patients experiencing complete remission and the remaining experiencing relapses or progression [1]. Moreover, the therapeutic approach is not standardised, leading to further heterogeneity in terms of outcome [2], and a marker that can reliably predict the response of a patient a priori is lacking.

Methods: MALDI-MS imaging of renal biopsies taken from patients who differentially responded to immunosuppressive treatment (Ponticelli regimen) was performed. Initially, FFPE specimens (n=13) were analysed using an ultraflexXtreme™ MALDI-TOF-MS instrument, employing a pixel width and raster setting of 40 μm and 50 μm, respectively [3]. Analysis of additional specimens (n=7) was performed using arapifleX MALDI Tissuetyper™, employing a pixel width and raster of 10μm.

Results: Following Receiver Operative Characteristic (ROC) analysis, three signals were determined to have a statistical significance when comparing the two patient groups (m/z 1111, 1198 and 1303). The signal at m/z 1303 displayed the greatest discriminatory power and was observed to be of higher intensity in the glomeruli of the non-responding patients. The corresponding tryptic peptide was putatively identified as macrophage migration inhibitory factor (MIF) and validated using immunohistochemistry, depositing on podocyte and parietal epithelial cells (PEC). We also generated specific profiles of the mesangial cells and podocytes of the glomeruli whilst employing a 10μm pixel. This high spatial resolution imaging was then used to visualise the spatial localisation of those additional discriminatory signals detected (m/z 1111 and 1198) within the glomerular and tubular structures. These signals were putatively identified as tryptic peptides deriving from sonic hedgehog protein (SHH) and α-smooth muscle actin (SMA), respectively.

Conclusions: Despite much effort being made in recent years to understand the pathogenesis of MN, a biomarker able to predict the outcome of these patients following therapeutic treatment is still lacking. Here, we highlight three proteins that could differentiate between these MN patients (MIF, SHH and α-SMA) and thus represents promising starting point in the search for protein markers to provide complimentary support in the routine prognostic assessment of MN patients.

Novel Aspect: This work represents the first study to employ high spatial resolution MALDI-MSI as a means of detecting tissue proteins with the potential to predict response to therapy in MN.

References:

The research leading to these results has received funding from MIUR: FIRB 2007 (RBRN07BMCT_11), FAR 2014–2016 and in part by Fondazione Gigi & Pupa Ferrari Onlus.
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Keywords: chronic kidney disease, clinical proteomics, diabetic nephropathy, hypertension, MS imaging

Introduction: Diabetic nephropathy (DN) and hypertensive nephrosclerosis (HN) represent the most common causes of chronic kidney disease and many patients progress to end-stage renal disease [1]. Whilst both diseases are treated primarily through the management of hypertension, the latter group of patients have a more favourable outcome. Separating these two entities would allow for more appropriate resource allocation to those patients at highest risk of progression.

Methods: MALDI-MS imaging of renal biopsies taken from patients with DN and HTN was performed. These patients were also stratified into disease sub-groups on the basis of clinical parameters. FFPE specimens (n=18) were analysed using arapifleX MALDI Tissuetyper™, employing a pixel width and raster of 10 μm and 20 μm, respectively. Subsequent identification was performed using a Dionex UltiMate 3000 rapid separation (RS) LC nano system coupled with an Impact HDTM UHR-QqToF.

Results: Unsupervised Principal Component Analysis (PCA) highlighted that DN and HN could be clearly distinguished on the basis of their tryptic peptide profiles, with a cluster of signals having an altered intensity between the two disease groups. However, there was also some heterogeneity noted within each disease group. Upon further investigation, this heterogeneity was shown to be associated with the severity of each disease as defined by both clinical parameters and histological evaluation. Receiver Operative Characteristic (ROC) analysis highlighted a small panel of signals whose differential intensity was associated with the different stages of each disease. These signals were then correlated with a protein identification list obtained by complimentary nLC-ESI-MS/MS analysis, resulting in a putative cluster of proteins able to discriminate between the different stages of DN and HN.

Conclusions: A sensitive and specific protein marker that can reliably differentiate between diabetic and hypertensive causes of CKD would allow for targeted monitoring guidelines for clinicians. The cluster of putative proteins presented here represents a valuable starting point for a future study employing a larger cohort of patients and, if verified, would allow for more appropriate disease intervention and potentially improve the outcome of patients with DN and HN.

Novel Aspect: This complementary proteomics strategy may provide specific markers for diabetic and hypertensive causes of CKD and highlight those patients at highest risk of progression.

References:

High-resolution AP-SMALDI MS imaging of parasites

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Keywords: High-resolution MS imaging, neglected tropical diseases, parasites, 3D surface imaging, atmospheric pressure MALDI

Introduction:
Biomolecular analysis by atmospheric-pressure (AP) MALDI MS imaging (MSI) at high lateral resolution is a key issue for the investigation of small biological objects such as multicellular or protozoan parasites. Analysis of non-planar surfaces or tissue sections of parasites can be useful for, e.g., understanding invasion mechanisms. 3D-surface MSI allows describing the biochemistry, chemical communication and drug response of small objects.

Methods: An AP-SMALDI AF ion source (TransMIT GmbH, Giessen, Germany) on a Q Exactive HF X orbital trapping mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) was used to image two-dimensional planar sections and three-dimensional non-planar surfaces at a lateral resolution of 10 µm and below. High-speed acquisition up to 10 pixels per second was used at reduced mass resolution. Full pixel mode enhanced signal intensities for low-abundant compounds.

Results: Schistosoma mansoni, a dioecious worm parasite causing schistosomiasis in humans and animals, was obtained from the Institute of Parasitology. 100 to 300 µm thick and approx. 1.5 mm long worms were investigated as couples or as individuals. Contact surfaces (the teguments) were differentially analyzed at the phospholipid and peptide levels, indicating molecular markers for males and females, and for their mating state.

Samples of apicomplexan protozoan cell pellets and monolayers of bovine umbilical vein endothelial cells (BUVEC) were studied at the lipidome level to find markers of infection. More than 20 lipid biomarkers for infection were determined for each of the parasites Besnoitiabesnoiti, Eimeriabovis and Toxoplasma gondii.

Pixelwise autofocusing, provided by the 3D imaging ion source, was essential to reliably analyze the samples. Height adjustment in the micrometer range allowed obtaining topography-independent, concentration-related signal intensities from the surface of parasites and non-flat tissue sections.

Conclusions:
High-resolution methods were developed for pinpointing biochemical features of parasites such as schistosomes or coccidia. Molecular communication between paired male and female schistosomes was characterized by 3D surface imaging of their contact zone, the tegument.

Semi-quantitative analysis was enabled by pixelwise autofocusing, resulting in topography-independent signal intensities.

Novel Aspect: Molecular 3D surface imaging of endogenous compounds of parasites at 5 to 10 µm lateral resolution
References:

AN UNTARGETED APPROACH TO ELUCIDATE THE METABOLISM AND TISSUE DISTRIBUTION OF PHOSPHOROTHIOATE LINKED OLIGONUCLEOTIDES BY HIGH RESOLUTION MASS SPECTROMETRY

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Keywords: MALDI-FTICRMS imaging, oligonucleotides, tissuedrugdisposition, target site ADME, metaboliteidentification

Introduction:
Oligonucleotides linked with phosphorothioate bonds are a class of therapeutics under investigation in various pharmaceutical companies. Investigating the metabolism and disposition to target or non-target tissue by mass spectrometry could be a major advantage for a successful development. We developed two complementary analytical methods: To identify oligonucleotide metabolites an untargeted highly sensitive qualitative method using capillary flow liquid chromatography coupled to high resolution mass spectrometry was used, and for in situ imaging of tissue distribution a complementary method based on MALDI-FTICR MS. Both methods utilize a diagnostic fragment derived from the phosphorothioate backbone. This combined approach was successfully applied in a rat study with oligonucleotides of different sequence with and without conjugation to N-acetylgalactosamines (GalNAc).

Methods:
Metabolite identification was achieved by capillary flow liquid chromatography with column switching coupled to a Fusion Orbitrap mass spectrometer. Data-dependent scans were performed in parallel to an untargeted MS2 experiment (AIF) and a high resolution full scan. In situ tissue imaging was performed by MALDI-MS Imaging using a 7 Tesla SolariX XR FTICRMS instrument (Bruker). Matrix (α-Cyano-4-hydroxycinnamic acid) was applied using an iMatrixSpray device. Negative polarity and 300 laser shots were applied (2000 Hz) at 50 μm lateral resolution. The quadrupole was used in full transferring mode followed by CID (60eV). Full scan MS was acquired in the ICR cell. Tissue distribution of oligonucleotides was also evaluated using Immunohistochemistry.

Results:
The detection and identification of oligonucleotides and their metabolites was achieved on a Fusion Orbitrap by combining full scan MS with two parallel MS2 experiments, one data-dependent scan and an untargeted MS2 experiment (AIF) applying high collision energy. In the AIF scan, a diagnostic fragment originating from the phosphorothioate backbone (O2PS-: m/z 94.936) was formed efficiently upon collisional activation. Based on this fragment an accurate determination of oligonucleotide metabolites was achieved, independent of their sequence in an untargeted but highly selective manner. The method was effectively applied to investigate uptake and metabolism of oligonucleotides in rat liver and rat kidney homogenates down to the subnanomolar range. For MALDI-FTICR MS Imaging, oligonucleotides were detected indirectly and independently of their sequence using the quadrupole in an unselective transferring mode followed by strong collisional activation. We could identify in the ICR cell the diagnostic fragment (O2PS-: m/z 94.936). Both methods were applied in a mechanistic multiple-dose rat study. Oligonucleotides with different sequence were used both as unconjugated compounds or conjugated with N-acetyl galactosamine which is a high-affinity ligand for the asialoglycoprotein receptor on the hepatocyte cell surface. Liver and kidney were investigated for metabolites and were evaluated in situ by immunohistochemistry and MALDI-FTICRMS Imaging. In the liver, unconjugated oligonucleotides were demonstrated mainly in Kupffer cells, whereas GalNAc-conjugated oligonucleotides accumulated in both Kupffer cells and hepatocytes. In the kidney, all oligonucleotides tested accumulated in the cortex primarily in proximal tubules. Interference with nucleotides of endogenous RNAs or DNAs was not observed. Phosphorothioate instead of phosphate linkage is a common modification for therapeutic oligonucleotides. Therefore, this approach might be generally applicable to investigate tissue distribution of this evolving therapeutic modality.
Conclusions:
It could be shown that the distribution and metabolism of oligonucleotides linked with phosphorothioate bonds can be analyzed in an untargeted manner by mass spectrometry. The metabolism of oligonucleotides was elucidated by LC-MS/MS whereas in situ imaging of tissue distribution was achieved on a MALDI-FTICR MS instrument. Both approaches harness the phosphorothioate backbone of these oligonucleotides as a diagnostic fragment (O2PS-: m/z 94.936) as this is not present in endogenous nucleic acids.
Both methods proofed their applicability in a mechanistic multiple-dose rat study for which several oligonucleotides of different sequences were dosed.

Novel Aspect:
Untargeted approach to investigate metabolism and tissue distribution of therapeutic phosphorothioate linked oligonucleotides by high resolution LC-MS and MALDI-FTICRMS Imaging.

References:
A molecular snapshot of clear cell renal cell carcinoma: multimodal MALDI-MSI and nLC-MS/MS of N-glycans and proteins

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Introduction
Renal cell carcinoma (RCC) is the most frequent form of kidney cancer and approximately 80% of cases are defined as clear cell RCC (ccRCC)[1]. Understanding molecular alterations associated with the onset and progression of the disease could provide a valuable starting point for the development of new target therapies. Therefore, combined high resolution mass spectrometric approaches were used to investigate the glyco-proteome of different ccRCC grades[2-3].

Methods
FFPE tissue samples from patients with a proven diagnosis of ccRCC were analysed by MALDI-MSI. A 9.4T MALDI-FTICR-MS platform was used to investigate the spatial distribution of proteolytic peptides, whereas released N-glycans were analysed with a MALDI-TOF/TOF-MS platform from a consecutive tissue section. Finally, bottom-up proteomicusing nLC-ESI-MS/MS, was performed on homogenized tissue to assign putative identifications to the molecules of interest.

Results
Considering the possibility of tumour grade-heterogeneity within a single lesion, traditional histology and molecular information obtained by MALDI-MSI were used to better define signals specific for each region of the tissue. MALDI-MSI analysis highlighted peptides signals able to discriminate between grade one and grade four lesions (e.g. m/z 944.71, m/z 1032.78, m/z 1325.99, m/z 1428.92). Nevertheless, preliminary data on the characterization of glycosylation offered an additional layer of information regarding the glyco-phenotype of different areas of the tissue. Finally, nLC-ESI-MS/MS analyses provided a list of putative identifications (protein and glycoproteins) for those signals that differed significantly among the four grades. In particular, a putative identification was assigned to the four aforementioned signals and were tryptic peptides of H2, H3, H4 and Vimentin, respectively. The identification of other signals of interest and their released glycans is currently under investigation.

Conclusions
Proteomics represents a highly specific approach in the investigation of molecular changes associated with tumourlesions. Furthermore, the study of aberrant glycosylation is bringing consistent advantages in the
understanding of ccRCC progression. All those findings represent a first attempt to highlight molecular differences among ccRCC grades and could provide further insights into the molecular mechanisms implicated in ccRCC progression.

Novel Aspect
The combination of high resolution MALDI-MSI and nLC-ESI-MS/MS is a highly effective approach for studying the proteome and the glycome of different tumour grades.

References


Acknowledgments
The research leading to these results has received funding from the MIUR: FIRB 2007 (RBRN07BMCT_11), FAR 2014–2016; and in part by Fondazione Gigi & Pupa Ferrari Onlus.
Introduction:
Investigating the local distribution of drug and biomarker in target organs provides useful information, which accelerates drug discovery and development. Imaging mass spectrometry (IMS) is a powerful technique to detect the localization of compounds of interest in biological tissue without any labeling. In the present report, we demonstrate the utility of IMS to investigate drug and biomarker distribution in an amiodarone-induced phospholipidosis model.

Methods:
Rats were orally administered amiodarone (AMD) for 7 days at 150 mg/kg/day. After the final dose, lungs, spleens and mesenteric lymph nodes were collected. Prepared cryosections (5 μm/section) were coated with α-cyano-4-hydroxycinnamic acid using iMLayer (Shimadzu) and analyzed using the IMS system (Q-Exactive (Thermo Scientific) equipped with MALDI ion source AP-SMALDI 10 (TransMIT)). Mass images were generated using Million ver.3.2 software (TransMIT).

Results:
AMD, its metabolites (N-desethyl amiodarone and mono-oxidized amiodarone), and representative phospholipids, which are considered biomarkers of phospholipidosis, were detected in lungs, spleens and mesenteric lymph nodes in a single IMS analysis. Detection intensity of all the compounds was relatively higher in foamy macrophage infiltration areas than in non-infiltration areas. For the spleen, the distribution pattern of representative phospholipids markedly differed between AMD-administered rat and vehicle-administered rat.

Conclusions:
These results indicated that AMD and its metabolites were stored in macrophages, and this is quite consistent with the mechanism of amiodarone-induced phospholipidosis. They also indicate that IMS is a useful technique for evaluating the distribution of drugs and biological components in the elucidation of toxicity mechanisms.

Novel Aspect:
IMS analysis revealed the distribution pattern of AMD, its metabolites, and representative phospholipids in an amiodarone-induced phospholipidosis model.

Reference
Introduction:
Overexpression of Myc and ErbB2 are frequent inducers of human breast cancers with distinct metabolic profiles [1]. Such metabolic deregulation is one of the hallmarks of cancer [2]. Mass spectrometry imaging (MSI) provides the potential to understand metabolic changes between genetic phenotypes, and within tumor regions; to expose therapeutic vulnerabilities. We performed a multimodal MSI study of genetically engineered mouse models of breast cancer.

Methods:
Mouse normal mammary glands, Myc- and ErbB2-induced mammary gland tumours were snap-frozen and cryosectioned at 10 μm. MALDI MSI was performed on a Waters Synapt G2-Si at 45 μm pixel size using 9-AA or DHB matrix. DESI MSI was performed on a Waters Xevo G2-XS at 75 μm pixel size with 95:5 MeOH:water as the spray solvent. Data were analyzed using PCA, NMF, k-means clustering and deep learning t-SNE. Database matching was performed to tentatively assign ions of interest.

Results:
Commonly applied analysis techniques PCA, NMF and k-means clustering proved complementary in revealing heterogeneity between subtypes and within individual tumours in an unsupervised manner. A novel deep learning t-SNE method enables visualisation of diverse chemical environments in a dataset that would be prohibitively large for traditional t-SNE Methods: Consideration of spectral factors and loadings from multivariate analysis led to the identification of peaks that delineate chemical heterogeneity. Database matching was used for tentative identifications of molecules including lipids, fatty acids and small metabolites. Information provided by multivariate segmentation was broadly consistent across techniques. MALDI and DESI were found to be highly complementary, with a number of molecules uniquely identified by each technique alone. Future work will integrate MALDI and DESI MSI into a data guided approach for high spatial and mass resolution SIMS imaging using 3D OrbiSIMS.

Conclusions:
We show the power of a multimodal MSI pipeline to delineate and define molecular heterogeneity within and between Myc- and ErbB2-induced models of breast cancer. MALDI and DESI MSI are shown to be complementary in
their coverage, demonstrating the importance of multimodal MSI. This work provides a blueprint for the design and application of further multimodal and multiscale MSI studies.

Novel Aspect:
A multimodal, multiscale MSI pipeline for the study of metabolic deregulation in genetically engineered mouse models of breast cancer is described.

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630 - NEW FRONTIERS OF MALDI-IMS AND LIPIDOMICS: AN UNTARGETED APPROACH TO SKIN LIPIDS MAPPING

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Keywords: MSI, lipidomics, chemometrics, skin, bioimaging

Introduction:
Skin is large and integrated metabolic organ highly specialized in the synthesis of lipids, and skin untargeted lipidomics aim to facilitate the understanding of possible mechanisms of diseases with cutaneous pathological phenotype or pharmacokinetics of topically administered drugs [1,2]. Skin lipids present a specific distribution that might be exploited for the structural characterization of skin, thus obtaining a mapping that would guide any future detection of anomalies in the lipid profile. To this end, development of innovative high-resolution and high-sensitivity analytical approaches characterized by speed, robustness, and high metabolite coverage is essential to empower the information associated with the skin lipidome. In this context, in addition to the powerfulness demonstrated by mass spectrometry in “omics” studies, mass spectrometry imaging (MSI), a molecular analytical technology suited for simultaneously measuring multiple analytes (including lipids, drugs and their metabolites) directly from intact tissue sections, adds a new dimension and holds potential for bringing lipidomics to the spatial molecular characterization level [3]. However, such an aim represents a remarkable computational challenge due to the size and analytical complexity of a typical MALDI MSI lipidomic analysis that, especially when high spatial resolution is used to characterize the tissues, increases the problem to the scale of Big Data. Therefore, a smart combination of bioinformatics and chemometric tools for image analysis and data mining is needed to effectively support MALDI MSI skin lipidomics, but adequate and user-friendly solutions comprising all of the steps required for this aim (from peak picking, to lipid identification via multivariate statistical analysis) are still lacking.

Here we propose a novel and semi-automatic workflow to map skin lipids that, starting from multivariate statistics, leads to skin lipids mapping and biomarkers discovery in an untargeted manner under orientation of histological structures by MALDI MSI.

To prove the efficacy of the proposed workflow, both minipig, a representative preclinical species for skin studies, and human skin samples where analysed with unprecedented spatial resolution (10 μm and 25 μm) under positive as well as negative ionization mode. Data were then processed by means of multivariate statistical analysis with the aim of identifying differences among skin features.

As a result, a relatively comprehensive set of specific lipids markers for epidermis, hair follicles, and for the first time, for sebaceous glands is identified. Structure identification of identified markers is confirmed by post-MALDI MSI H&E stained optical images and validated by additional samples.

Methods:
Both minipig and human skin samples were collected and stored in -80°C before sectioning. The samples were cryosectioned at a thickness of 6 μm (minipig) or 10 μm (human) by a Leica Cryostat (Leica Biosystems Inc., Buffalo Grove, IL, USA) at -20 to -25°C. Sections for MALDI MSI were applied of matrix (DHB for positive and 9- AA for negative ionization mode) by a TM sprayer (HTX Technologies, Carrboro, NC, USA) upon optimization of spray conditions. MALDI MSI was performed using a Solarix 7T Fourier transform ion cyclotron resonance mass spectrometer (BrukerDaltonics, Billerica, MA).

Images were acquired at spatial resolutions of 10 μm and 25 μm in positive and negative ionization mode. Mass spectra were reacquired in full scan mode (m/z 200-1500). Allion images were generated using FlexImagingv4.0.
software (BrukerDaltonics, Billerica, MA) from the raw data. Regions of interest (ROIs) were defined under the guidance of hematoxylin and eosin (H&E) staining as: epidermis, EP, papillary dermis, PD, reticular dermis, RD, hair follicle, HF, sebaceous glands, SG, sweat glands, SWG. At least three different skin portions were chosen for each ROI. Average spectra from the different ROIs were exported from FlexImaging v4.0 software. LipoStar software [4] (Molecular Discovery, London, UK), adapted for handling MALDI MSI datasets, was used for peak detection, samples alignment as data matrix (rows: samples or ROIs, columns: m/z values), supervised (partial least squares discriminant analysis, PLS-DA) and unsupervised (principal component analysis, PCA) multivariate statistical analysis and lipid identification.

Results: (Limit 900 characters without spaces)
The skin has three main layers: the epidermis, dermis, and hypodermis. Epidermis (EP) is the surface layer in contact with the exterior, while the dermis (D) is divided into two further substructures: papillary dermis (PD), immediately adjacent to the EP, and reticular dermis (RD). Additional skin appendages are present, namely: Hair follicles (HF), sebaceous glands (SG), and sweat glands (SWG). In order to investigate differences among substructures, tissues were analysed at high spatial resolutions (10 μm and 25 μm) under positive as well as negative ionization mode. Data were pre-processed as described in Methods, and the obtained aligned data matrix underwent both supervised and unsupervised multivariate statistical analysis.

PCA analysis shows that groups of lipids can be identified to distinguish the different layers and structures at a spatial resolution of 25 μm for both minipig and human skin samples. For instance, a lipid was identified to be specifically localized in the epidermis and the external root sheath of hair follicles. Considering that the external root sheath consists of epithelial cells and finally merges with epidermis, this identification is consistent with histological interpretation. Another lipid was found to be specifically expressed in hair follicles that contain a shaft. Moreover, in human skin samples, SWG and SG substructures were characterized, and by means of PLS-DA it was possible to identify lipids specifically located on those substructures. Noteworthy, a list of lipids specifically located in SG was identified for the first time.

At the higher resolution of 10 μm, it was possible to identify lipids discriminating EP from PD and substructures of SG in minipig and human skin. Lipid specific for each substructure were tentatively annotated with LipoStar software and post-MALDI MSI H&E stained optical images were collected to confirm the structure identification. When isobars were identified, an additional inquiry to a database of lipids previously identified in skin samples allowed for a priority match assignment.

The obtained list of skin substructure-specific lipids provided a lipid mapping that will be a valuable help in future skin lipidomic investigations.

Conclusions
In this work we presented a novel high-throughput cheminformatics workflow to efficiently map skin lipids and ultimately achieve untargeted lipidomics with MALDI MSI. With the aim of mapping skin lipids, both minipig and human skin samples were analysed at 10 μm and 25 μm spatial resolution in positive as well as negative ionization mode. These two spatial resolutions allowed distinguishing among substructures of different size. Starting from the selection of different ROIs according to H&E staining, multivariate statistical algorithms (PCA, PLS-DA) were applied to enlightening analogies/differences among the different skinsubstructures (stratum corneum, epidermis, dermis, hair follicles, and for the first time, sebaceous glands). In the present work we focused our attention on finding substructure-specific lipids, that is, lipids that exclusively belong to a specific substructure. A first annotation of those lipids was also provided, resolving isobars conflicts by means of a database of lipids previously identified in skin.

Big challenges still remain, and these should guide the design of more sophisticated and fully integrated (from peak detection to pathway analysis, via lipid identification) workflow. However, by designing a workflow to successfully bringing lipidomic studies to spatial molecular characterization, we believe that these results will pave the way to establish a data-driven histology interpretation of MALDI MSI analyses and ultimately achieve functional elucidation to support drug and biomarkers discovery.

Novel Aspect:
With the aim of performing for the first time an untargeted lipidomic analysis of skin by means of MALDI MSI, a novel cheminformatic workflow was designed to fish for lipids that are specifically localized in given skin substructures. Both minipig and human skin samples were analysed by MALDI MSI at unprecedented spatial resolution (10 µm and 25 µm) in positive and negative ionization mode. As a result, a relatively comprehensive set of specific lipids markers for epidermis, papillary dermis, hair follicles, and for the first time, for sebaceous glands is identified. To the best of our knowledge, this is the first time that histology interpretation of MALDI MSI analyses is achieved in a completely data-driven fashion. These results will guide further pathway analyses to achieve unprecedented functional interpretation, to comprehensively map skin lipids and ultimately support drug and biomarker discovery.

References

Introduction:
Flax (Linum usitatissimum L.) is used for fiber, oil, and nutraceutical production. Fusarium oxysporum f. sp. lini is the pathogen that causes fusarium wilt in flax. Epidemics of the disease can result up to 100% loss in yield. Some of flax genotypes are resistant to F. oxysporum, but the mechanisms of resistance are still unclear. Comparative analysis of metabolic changes in flax plants of resistant and susceptible cultivars in response to the pathogen infection is essential for understanding the role of metabolites in resistance and can be useful for development of antifungals.

Methods:
Seven-day-old seedlings of one susceptible (AP5) and two resistant (Dakota and #3896) flax cultivars were inoculated with spore suspension of F. oxysporum pathogenic isolate #39 from the phytopathogen collection of the All-Russian Research Institute for Flax. For control plants, sterile water was used instead of F. oxysporum spore suspension. After 48 hours from inoculation, root tips were collected. After that, roots were embedded in gelatin and gently frozen on dry ice. The frozen tissue was then sectioned into 25-μm slices using a cryostat MK-25M (adapted for work with blades Leica DB80LS) at −20°C. The sections were thaw-mounted onto ITO slides. Matrix (40 mg/mL DHB in 50:50 water:acetonitril) was applied using an airbrush (iwata micron cm-b2). A MALDI-Orbitrap mass spectrometer (Thermo Scientific Q-Exactive orbitrap with MALDI/ESI Injector from Spectroglyph, LLC) that was equipped with an 355nm laser (spot diameter of 20 μm) was used in positive ion mode for imaging and MS/MS[1]. Spectra were obtained in mass range of m/z 100–1000, a mass resolution of 140,000. The tissue region to be imaged and the raster step size were controlled using the Spectroglyph MALDI Injector Software. To generate images, the spectra were collected at 35-μm intervals in both the x and y dimensions across the surface of the sample. Ion images were generated from raw files (obtained from oritrap tune software) and coordinate files (obtained from MALDI Injector Software) by imageinsight software from Spectroglyph LLC. MS/MS collision-induced-dissociation (CID) fragments were collected by isolating each m/z of interest and manually adjusting the collision energy for each compound. The “metabolites of interest” were identified by searching the accurate mass obtained with the Orbitrap instruments and the MS/MS data using MetFrag.

Results:
Metabolites in flax roots of susceptible and resistant cultivars inoculated with F. oxysporum or under control conditions were identified. For evaluation of metabolic responses to the fungal infection, comparative analysis was performed, and metabolites that were induced in resistant and susceptible flax genotypes after inoculation with F. oxysporum were identified. Resistance-related metabolites were determined. In the present study, we used susceptible and resistant flax cultivars, for which transcriptome analysis under the same conditions (inoculation with F. oxysporumisolate #39 and control) was performed by us previously, and diverse patterns of differentially expressed genes were revealed.

Conclusions
Complex analysis of transcriptome and metabolome data in flax plants with distinct resistance to fusarium wilt can be a promising tool for determination the mechanisms of resistance to F. oxysporum.

Novel Aspect:
Metabolite profiling was performed for inoculated with Fusarium oxysporum flax roots of susceptible and resistant to the pathogen cultivars.

References


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Introduction
Malignant Mesothelioma (MM) is an aggressive cancer of the mesothelium associated with exposure to specific Mineral Fibres (MF) [1]. There is an urgent need to develop methods to clearly identify and quantify the MF within biological samples [2]. The current project aim was to develop a biologically relevant MM model and use LA-ICP-MSI to determine the element distribution of MF within samples. Development of this technique would untimely lead to early MM diagnosis.

Methods
MM models were developed using immortalised cells (NCIH28, MSTO211H, NCIH1975, Met5A) cultured in both 2D monolayers and 3D spheroids. Cells were exposed to various MF (concentration 0-25µg/mL). Viability of the cultures was measured using Alamar Blue® and Hoescht/Propidium Iodide staining. MALDI and LA-ICP-MS images were obtained at post-exposure following cell cytospin and matrix coating. The spheroids were cryosectioned and fixed onto slides prior to the imaging.

Results
The MM models were successfully developed in both 2D monolayers spiked with MF and 3D spheroids with MF embedded within the cells. The Hoescht/Propidium Iodide staining showed an apoptotic core to the spheroids which translates to the in vivo tumour environments. The cell viability results after MF treatment declined with exposure period, MF dose and most importantly MF type, which is in line with published literature [3]. Cell viability data of 2D models were also supported by Hoescht/Propidium Iodide images.

Some preliminary data has been acquired using LA-ICP-MS imaging, proving that this analytical method has high potential in identifying the MF within the cell cytospins and can become a useful tool for clinical diagnosis of MM. Initial data has also shown that sections of the 3D MM model can be analysed by LA-ICP-MS and MALDI MSI to determine spatial distribution of lipids and proteins surrounding MF.

Conclusions
LA-ICP-MS has recently emerged as one of the most versatile imaging tools in biomedical research and the current study has showed great potential for its future integration in clinical settings. Method optimisation is needed before moving on to more complex 3D structures and histopathological lung tissue biopsies. MALDI MSI and flow cytometry will also be employed in future analysis of protein expression within MM samples and to support current data.

Novel Aspect
This project is the first attempt to develop a diagnosis method that uses LA-ICP-MSI to identify the full arrangement of MF at the cellular level within MM samples.

References
Introduction:
Non-alcoholic steatohepatitis (NASH) is an advanced form of Non-alcoholic fatty liver disease (NAFLD), and is recognized to often progress to cirrhosis and hepatocellular carcinoma[1]. A liver excised from an animal model raised to forcibly express NASH is quite useful to study the disease, because pathological findings of the organ are similar to the one from human patients. Whereas some metabolomics methods using LC- or GC-MS have been applied to the analysis of the organ, visualization of metabolites on pathological section is necessary to discuss disease states with relevance to various staining. We will present a metabolic profile of biochemical compounds in some pathological states, which is possibly related to a dynamics of the fibrosis in liver, by using MALDI imaging.

Methods:
Liver tissues were removed from 4w-, 8w-, and 16w-old male of NASH-model rat induced by high fat cholesterol diet. Dried liver sections were subject to MS imaging and some pathological staining (hematoxylin-Eosin, SMA, sirius red, azan). Three sections with three corresponding controls were mounted onto an ITO-coated slide glass. 9-aminoacridine was sublimated onto the tissues at 0.5 μm thickness with a commercial instrument (iMLayer, Shimadzu Corp., Japan). After sublimation, a recrystallization of the coated matrix was conducted under vapor of a solvent consisted with methanol and water. MS imaging at 20 μm spatial resolution were performed with a MALDI-TOFMS (MALDI-7090, Shimadzu Corp., Japan) in negative mode at 2 kHz laser repetition, after a diameter of ablation by laser was validated. Newly developed in-house softwares were applied to visualization and analysis of all data.

Results: (Limit 900 characters)
Three liver tissues, 4w, 8w, 16w-treated, and each corresponding control tissues were subject to the high throughput MS imaging simultaneously, the advantage of which is that distributions of biochemical compounds can be recognized undoubtedly under the same matrix coating and laser irradiation. Targeted m/z of biochemical compounds obtained from LC- or GC-MS was visualized with in-house software. As typical case, since an increase of PS(18:0,18:1) and PI(16:0,18:2) in 8w- and 16w-treated liver was indicated with LC-/GCMS, m/z of both lipids were visualized to be compared with stained sections. Whereas the PS was extremely localized at fibrosis area, which was stained with azan and sirius red deeply, the PI was found at nodules in the liver tissues. bile acids and their derivatives were also chosen as target molecules and visualized. Interestingly, whereas taurocholate and taurochenodeoxycholate distributed at nodule areas widely in 8w- and 16w-treated liver, there were significant narrow areas in the nodule, where only the taurocholate or the taurochenodeoxycholate were accumulated specifically. On the other hand, glycocholate were localized in fibrosis areas that were increasing along with the treated week. Two-dimensional operation included in the newly developed software was applied to find more precise relevance between a progress of fibrosis and a distribution of bile acids. Calculated images of NASH liver tissues obtained by dividing m/z 405 (glycocholate) by summation of m/z 498 and 514 (taurochenodeoxycholate and taurocholate respectively) indicated that a distribution of glycocholate was correlated with fibrosis areas stained with azan in detail. In general, fibrosis in liver is attributable to excessive production of collagen and extracellular matrix in hepatic stellate cell after liver injury by stress. Specific 2D profiles of bile acids and lipids shown here could
be caused with a change of global metabolism by the stress. A production of actual NASH liver in lab animals is still matter due to long necessary months. Raising the animals with high fat cholesterol diet is useful countermeasure to obtain almost the same pathology as the one in actual disease, however, pathological findings have been only the way to examine both tissues[2]. The 2D profiles obtained here is applicable to validate NASH-model at molecular level.

Conclusions:
Differently treated NASH-model livers were studied using MALDI imaging to discuss a relevance with a pathological progress. Not only a phospholipid but also both primary and secondary bile acids were mapped in fibrosis area that increases with the HFC-treated week.
2D profiles of those molecules targeted with metabolomics analysis indicate a validation of NASH-model tissue could be possible with MALDI imaging, which might contribute to investigation and development of novel medicine for NASH.

Novel Aspect:
MALDI imaging was applied to obtain metabolic profiles of bile acids and lipids in fibrosis of NASH-model liver.

References:
Introduction: The endogenous Jasmonic acid (JA) and abscisic acid (ABA) act as important roles in plant responding to wound stress [1]. Previous quantitative MS analysis has revealed JA and ABA levels in different damaged self-recognition stages, but changes of their spatial localizations on leaf are still unclear [2]. Aim to study JA and ABA biosynthesis and transportation in-situ, MALDI-MS Imaging was performed on leaf samples.

Methods: leaf samples treated by mechanical damages were harvested in different healing time. Samples were cryosectioned and mounted on ITO slides, and then applied DHB matrix. MALDI-MS imaging was performed using MALDI SYNAPT G2 MS System (Water) with the mass range from m/z 50 to 1000. Further phytohormones ion intensity map creation and bioinformatics analysis were processed using in-house R scripts.

Results: 1. Intact masses of JA, ABA and other phytohormones were detected in the MALDI spectra. 2. Spatial distributions of phytohormones on leaf samples were highlighted in their ion intensity maps. 3. MALDI-MS imaging results showed unique hormonomics patterns in different wound healing stages. 4. PCA analysis and hypothesis tests suggested levels of JA and ABA are correlated with other phytohormones.

Conclusions: MALDI-MS imaging demonstrates its applicability on phytohormones and their metabolites detection and imaging on plant tissues. Changes of JA, ABA and other phytohormones in the different wound healing stage are able to be visualized in-situ. Identified hormonomics patterns provide a better understanding on plant wound stress and damaged self-recognition.

Novel Aspect: It is the first time that the change of JA and ABA localizations in leaf wound healing are visualized in-situ using MALDI-MS imaging.

References:

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B.01 INSTRUMENTATION AND METHODS - HYPHENATED TECHNIQUES

24 - GC-MS WITH COLD EI - EXTENDING THE RANGE OF COMPOUNDS AND APPLICATIONS AMENABLE FOR GC-MS ANALYSIS

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GC-MS with Cold EI - Extending the Range of Compounds and Applications Amenable for GC-MS Analysis
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Introduction
The major shortcoming and Achilles Heel of GC-MS is its inability to analyze relatively non-volatile and thermally labile compounds. This problem is further exacerbated via the often weakness or absence of molecular ions in standard EI-MS.

GC-MS with Cold EI is based on GC and MS interface with a supersonic molecular beam (SMB) and electron ionization of the SMB vibrationally cold sample compounds in a fly-through ion source.

Method
We found that GC elution temperatures can be significantly lowered upon the reduction of column length and increase of carrier gas flow rate. The increase of column flow rate also lowers the elution temperatures from the injector liner into the column while ion source degradation is inherently eliminated in the fly-through ion source. Thus, GC-MS with Cold EI provides significant extension of the range of compounds and applications amenable for analysis.

Preliminary data
The transition from simple to incompatible for GC-MS analysis. As the sample compound size and polarity is increased its analysis by standard EI gradually becomes more difficult in all the performance aspects: TIC signal is reduced, molecular ion abundance is reduced, baseline noise is increased, peak tailing is increased, ion source degradation is increased, the response becomes non-linear and analysis time is increased until the analysis becomes non-practical. In contrast, with Cold EI these performance aspects decline are much weaker.

Large and labile compounds analysis. The analysis of all organic explosives including TATP, PETN, Tetryl and HMX will be demonstrated plus thermally labile carbamate pesticides, underivatized steroids and large triglycerides in oil and blood plus large synthetic organic compounds.

Isomer distribution analysis for fuels and oils characterization. The availability of molecular ions to all hydrocarbons uniquely enables isomer distribution analysis for improved fuels and oils characterization.

Novel Aspect
How GC-MS with Cold EI extend the range of compounds amenable for GC-MS analysis with new demonstrated applications

Keywords GC-MS with Cold EI
Introduction:
Identification and quantitation of steroid metabolites in biological samples are essential for screening for various hormonal disorders. Many of these metabolites are closely related isomers and cannot be easily separated by LC or one dimensional GC due to their chemical and structural similarity. Comprehensive GCxGC coupled to a high resolution TOFMS provides enhanced chromatographic separation, better detection and identification of the analytes of interest.

Methods:
All data was obtained using the high resolution multiple reflecting geometry time-of-flight (TOF) mass spectrometer equipped with GCxGC with quad-jet, liquid nitrogen cooled thermal modulator and secondary column oven. We have tested various GC columns sets and oven temperature programs during GC method development. Steroids standards were obtained from Steraloids (USA) and Fisher Scientific (USA). Samples were derivatized using MSTFA method.

Results:
The steroid standards were analyzed individually and from that runs deconvoluted and curated mass spectra were obtained and included in the custom accurate mass library to assist in analytes identification in the complex mixtures. We present a workflow and analysis method that enables detection of 33 steroids from different classes in urine. Enhanced peak capacity provided by GCxGC allows separation of all analytes of interest, including structural isomers, which cannot be resolved or identified by other techniques. The high mass accuracy data along with automatic deconvolution algorithms provide reliable mass spectral information allowing accurate peak assignment. The method was transferred on to another analytical setup – the low resolution (LR) TOFMS coupled to GCxGC. Use of GCxGC-LRTOFMS represents a more practical approach of implementing the method for steroid analysis, while the HRTOFMS results can by applied for reliable analyte assignment by using HRTOFMS RI data and mass spectral match.

Conclusions:
The method is developed for comprehensive analysis of steroids in urine by GCxGC-HRTOFMS as well as GCxGC-LRTOFMS. The method allows taking full advantage of increased separation capacity of GCxGC and mass accuracy and sensitivity of the TOFMS. The reproducible sample preparation (derivatization, cleanup, etc) was the main challenge, and the future work will be focused on automation of the sample preparation process.

Novel Aspect:
33 steroids were simultaneously analyzed in complex biological matrix using GCxGC separation coupled with low and high resolution TOFMS with automatic data processing.
Introduction:
Capillary electrophoresis (CE) is an orthogonal technique to LC separating compounds based on their charge. The properties of CE enable the reduction and often elimination of carryover and wall absorption which effects peak resolution and sensitivity of LC. In this work we will describe how a CESI-MS method has been developed to detect intact proteins between 15 -25,000 amu. CESI [the integration of capillary electrophoresis (CE) and electrospray ionization (ESI) into a single process in a single device] is now enabling the easy connection of CE to mass spectrometers.

Methods:
Protein standards were prepared in a variety of different solvents and injected by either pressure or electrokinetically onto a neutrally coated capillary under various conditions. The CE separation used MS amenable background electrolyte (BGE) consisting of a mixture of Acetic acid, Acetonitrile and Water. Proteins were detected by a MS system in MRM mode (using a capillary 30 μm ID, 91 cm long) which was fitted with a NanoSpray® III source which was used in either full scan or single ion monitoring mode with an ionspray voltage of 1600 - 1800 V which was optimized to the BGE being used.

Results:
The CESI-MS method was developed with the capability of detecting intact protein standards at <10 ng/mL. This study showed that electro kinetic injection was >40 fold more sensitive that an isotachophoresis (ITP) injection technique and a also more sensitive than a traditional LCMS approach and followed a similar trend as shown previously for neuropeptides. The response obtained was linear over the 2-3 orders tests with very low levels of carryover observed. Sensitivity depended on how well the protein ionized and one of the major factors shown to affect sensitivity on the electro kinetic injection was the levels of acetonitrile in the sample.

Novel Aspect: (Limit of 150 characters)
Increasing sensitivity for bioanalysis by using electrokinetik injection techniques
MULTIVARIATE STUDY OF MATRIX EFFECT IN THE ANALYSIS OF PHYTOESTROGENS IN SOY-FOOD BY LC-ESI-MS/MS

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Keywords: liquid chromatography-tandem mass spectrometry, matrix effect, experimental design, QuEChERS, phytoestrogens

Introduction
Matrix effect is an important aspect to consider when analyzing extracts from complex food matrices by LC-ESI-MS [1]. In fact, if no labeled standards are available, accurate quantitation is a difficult task. The work deals with the evaluation of matrix effect involved in the analysis of phytoestrogens in soy-based food. QuEChERS [2] were used as extraction/purification technique and the multivariate approach of experimental design was used to optimize the procedure.

Methods
The phytoestrogens daidzein (DAID), genistein (GEN), coumestrol (COUM), formononetin (FORM) and biochanin A (BIOCH) were extracted from soy-based burgers using the QuEChERS methodology: the steps involved are extraction by acetonitrile-water, phase separation by salts addition and clean-up. Two subsequent experimental designs were employed to study the factors influencing the procedure. LC-MS/MS in multiple reaction monitoring mode was used for the analysis.

Results
Matrix effect (ME) was assessed by comparing the analytes’ signals in neat standards and in soy-burger extracts. A screening design (Plackett-Burman) [3] was employed to select the important variables involved in the QuEChERS procedure and affecting ME (response). No factors were significant for DAID and GEN; in fact, they were determined in diluted extracts, where negligible ME was observed. As for COUM, FORM and BIOCH, the factors considered statistically significant (p<0.05) were: sample amount, PSA (Primary Secondary Ammine) sorbent and FLORISIL sorbent (used in the clean-up stage). A subsequent Box-Behnken design [4] allowed to obtain the response surfaces (models of the response as a function of the variables). The models for COUM and FORM were subjected to ANOVA and showed good R2 coefficients (0.85 and 0.78 respectively). Conversely, the model for BIOCH was not significant. The optimal conditions to minimize ME (ion suppression < 20%) were: 200 mg of sample (for 10 mL of solvent) and clean-up of 2 mL of extract with 40-50 mg of PSA and 80-90 mg of FLORISIL.

Conclusions
Thanks to the approach of experimental design and response surface methodology, it was possible to study the problem of matrix effect in LC-ESI-MS analysis of complex matrices (soy-based burgers). The QuEChERS procedure, including a clean-up step, was optimized with a limited number of experiments, despite the several factors involved. The method is cheap, rapid, does not require specific equipment and provides a valid alternative to the use of labeled internal standards.

Novel Aspect
Application of the multivariate approach to optimize matrix effect in the analysis of a complex and novel matrix (soy-based burger) without the use of internal standards.

References
Application of SPME GC-MS to explore possible differences in free-lactose milks from the market

Keywords: lactose-hydrolyzed milk, SPME GC-MS, volatile organic compounds

Introduction
Positive effects of milk on the human health are well-known. However, 70% of the global population is lactose intolerant [1]. For this reason lactose-free products, obtained adding lactase, have been released on the market [2]. According to the step in which lactase is added, downside effects can arise over time, due to its proteolytic side activity [3]. The present study aim to explore the potential of SMPE GC-MS to catch VOCs profiling of commercial lactose-free milk.

Methods
UHT lactose-free milks from the local market, three freshly produced (FM1, FM2, FM3) and two after 180 days of storage (SM1 and SM2) were analyzed by SPME GC-MS according to Bergamaschi et al. (2015) [4]. Compounds were identified using the NIST-98/Wiley library and calculated retention indices (RI). Samples were compared by one-way ANOVA and Tukey post hoc test, when necessary, using the STATISTICA software (Dell Software Inc., Palo Alto, CA).

Results
Fifty-nine volatiles were identified and 64% of them were present in all the samples, suggesting differences in the VOCs profile. Many of those were already reported in other studies focused on lactose-free milk [3, 5, 6]. Fresh samples (FM1, FM2 and FM3) differed significantly for 7 compounds and 6 of them were higher in FM3. At the moment, we cannot speculate on the observed differences but the results suggested that the SPME GC-MS can catch the differences among milk batches. Fresh and stored milk differed significantly for 6 compounds, 5 of them having higher intensity after 180 days of storage (SM1 and SM2). Several of the VOCs that differed among the samples were methyl-ketones, well-known for their contribution to the off-flavor in UHT milk [7]. Some, such as 2-undecanone and 2-tridecanone, have long carbon chain and can be responsible for the heated flavor of milk [7]. The formation of methyl-ketones can also be addressed to the Maillard reaction, whose proceeding can be referred to the higher levels of these compounds found in SM1 and SM2.

Conclusions
The study indicated that the SPME GC-MS approach was able to catch differences in volatiles profiling of the milk tested. Conservation had a relevant impact and most of the changes were related to compound intensity. As the proteolytic side activities of lactase may play a role, it will be verified further. A shelf-life study is envisaged to verify the evolution of the VOCs profile in the samples and its contribution to the products sensory profile.

Novel Aspect
This study is an attempt to verify the suitability of the SPME GC-MS for tracking the evolution of the off-flavours formation in lactose-hydrolysed milk during storage.

References


POST-COLUMN IN-SOURCE DERIVATIZATION IN LCMS: A TOOL FOR NATURAL PRODUCTS CHARACTERIZATION AND METABOLOMICS

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Keywords: Post-column derivatization, LC-MS, in-source derivatization

Introduction:
Metabolite characterization is crucial for many aspects of basic research, such as de-replication of crude extracts in natural products chemistry or tentative identification in untargeted metabolomics. This information makes the subsequent decision-making process easier and more efficient. Derivatization with hydrazines is a known method in this respect, which allows the characterization of carbonyl functional groups [1-2].

Methods:
Liquid Chromatography-Mass Spectrometry was performed using Electrospray or Atmospheric Pressure Chemical Ionization as ionization techniques. The post-column addition of the reactants (hydrazines) was done by means of a syringe pump via a T-junction before entrance into the ion source. Experimental conditions were optimized. LCMS runs without addition were also performed for comparison.

Results:
Different compounds with at least one carbonyl group in its structure were analysed by this method. Triterpenoids like cycloartanes, steroids, cardenolides and other terpenoids were included in this study. All the analysed samples showed a similar behavior. The spectra of compounds with aldehyde groups exhibited as main signals those corresponding to the hydrazones, which are the expected products for the reaction between aldehydes and hydrazines. On the contrary, compounds with conjugated ketones did not react with hydrazines and did not show additional signals, and compounds having non-conjugated ketones presented in their spectra mainly those signals corresponding to the product of the nucleophilic addition adduct of hydrazine to the carbonyl group.

Conclusions
The in situ derivatization of carbonyl compounds in the ionization source was achieved, and applied in high-performance liquid chromatographic analysis. The typical derivatives of carbonyl compounds with hydrazines were obtained. Other reactions are being evaluated to further extend this method to other classes of derivatives.

Novel Aspect:
The use of post-column in-source derivatization in LCMS allows the characterization of compounds with carbonyl groups in their structures in a quick and easy way.

References
CONJUGATION AMONG NUCLEOTIDES AND CYANOCOBALAMIN FUNCTIONALIZED WITH CISPLATIN: A STUDY BY LIQUID CHROMATOGRAPHY COUPLED WITH ESI AND MULTISTAGE MS

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Keywords: LC-MS, cisplatin, cyanocobalamin, RNA, prodrugs

Introduction:
Cisplatin has been largely used in antineoplastic therapy [1]; diverse drug delivery systems have been studied in order to reduce its serious side effects [2]. DNA is generally accepted as the critical target for platinum-based compounds, nonetheless adducts between cisplatin and proteins [3], lipids [4] and RNA [5] might contribute to their effectiveness. In this study, interaction between cyanocobalamin-cisplatin (CNCbl-CP) prodrug and RNA bases has been studied via LC-MS.

Methods:
After the reaction between CNCbl and CP, a complex with empirical formula \([\text{C}_{63}\text{H}_{94}\text{ClCoN}_{16}\text{O}_{14}\text{Pt}]^+\) is generated [6]; its reactivity with in vitro incubation of individual adenosine monophosphate (AMP), guanosine monophosphate (GMP), cytosine monophosphate (CMP) and uridine monophosphate (UMP) in aqueous solution at 37 °C was studied by comparing results obtained using two different HPLC columns coupled to electrospray ionization and a linear ion trap (ESI-LIT) mass spectrometer.

Results:
The use of innovative drug delivery systems able to increase selectivity against cancer cells is a common strategy to reduce cisplatin toxicity; CNCbl functionalized with cisplatin is an example of prodrug. The formation of adducts between CNCbl-CP and RNA purine and/or pyrimidine bases was studied following two approaches: reaction was performed first on individual NMPs and later with competitive reactions in simultaneous presence of four NMPs. An appropriate gradient elution program based on methanol was developed to attain a separation both on a conventional and an amide-embedded ODS stationary phase; those two columns may provide complementary selectivity towards platinum adducts. All the NMPs react with CNCbl-CP by substitution of the last chlorine ligand on the cisplatin. The comparison between obtained and simulated isotopic patterns together with MS/MS analyses confirmed the presence of platinum in the reaction products.

Conclusions
From the obtained data it was clear that AMP and GMP are the most reactive nucleotides due to the presence of purine rings; three isobaric chemical species have been identified at m/z 965.8 for AMP, while a single protonated adduct at m/z 973.8 was generated for GMP. Also, adducts with CMP and UMP have been identified at m/z 953.8 and m/z 954.3, respectively. Plausible structures have been proposed by using tandem (MS/MS) and MS3 spectra.

Novel Aspect:
The interaction between CNCbl-CP and RNA bases has been proved by using HPLC-MS; the examination of fragmentation patterns was found very useful to gain insight into detailed molecular information.

References
Analytical Method for Quantification of Bixin in Extract of Annatto Using V-EASI(-)-LC-MS a Method Softer than ESI-LC-MS

Keywords:
Analytical Methods, mass spectrometry, V-EASI, LC-MS, Bixin

Introduction
Ventury Easy Ambient Sonic-Spray ionization (V-EASI) is a ionization source softer than electrospray (ESI) derived from Sonic Spray (SSI)[1]. Until now it has been little explored, used only for qualitative analyses[2-4]. This study is a proof of concept of an LC-MS system using the V-EASI source applied to develop a quantitative method of of bixin, a natural dye from annatto sensitive to high voltage[5].

Methods
The quantification was performed in LC-MS system using a Waters 510 chromatographic pump coupled with V-EASI source, and the quadrupole-mass spectrometer (LCMS shimadzu 2010), where was used the column C18 in the measurements. Methanol, bixin standard, lauric acid (internal standard), acetic acid (modifier) were used. For the validation, the guidance of validation from FDA was employed[6].

Results
The method was developed and optimized using the SIM mode for the ion of m/z 199 (lauric acid) and ion of m/z 393 (bixin) by V-EASI(-). We have found with the optimization the best analysis condition, as follow: 6 Bar of source, MS analyzer and block at the temperature of 250 °C, detector voltage of 1.50 kV, chromatographic run with 100% of methanol (0.05% acetic acid, v/v) where internal standard eluting at 3.8 min and bixin in 4.4 min. The validation data showed a linear range of 0.5 to 10 ppm, limit of detection and quantification of 0.02 and 0.07 ppm, respectively. The imprecision interday and intraday were 4.6% and 8.4%, respectively. The method also was considered accurate in an agreement with the official method by HPLC-DAD[7], it provides statistically equal results and has been shown to be a selective method with no matrix effect.

Conclusions
We were able to do the validation of a new V-EASI(-)-LC-MS method for bixin quantification, which presented similar results in comparison with the official method[6]. The method opens the perspective of a new system aiming the quantification of sensitive compounds and may be a new possibility for omics sciences.

Novel Aspect:
A powerful coupling among mass spectrometer, liquid chromatography and V-EASI source for the bixin quantification.
References

Introduction:
Pulses represent one of the most important traditionally dietary component worldwide, supplying proteins, dietary fibers, minerals and vitamins. The Food and Agriculture Organization declared 2016 the International Year of Pulses [1]. The potential health benefits of legumes are attributed to the presence of a wide range of phytochemicals including polyphenols that, according to the literature, are directly associated with antioxidant activity [2].

Methods:
Different extraction procedures such as acidic, basic and neutral extraction have been tested at different pH, by combining different temperatures and extraction times and the first one was the best. The separation of the different analytes was performed by using a KinetexPFP column and the mobile phase was composed by water and methanol both containing 0.1% of formic acid. The analysis has been carried out by using HPLC-MS/MS Dynamic MRM triple quadrupole.

Results:
The polyphenols of interest are: shikimic acid, gallic acid, delphinidin, catechin, chlorogenic acid, epicatechin, vanillic acid, caffeic acid, siringic acid, cumaric acid, ferulic acid, 3,5 di-CQA, rutin, kaempferol, cyanidin, quercetin. Chromatographic run is very fast as 16 polyphenols elute within about 10 minutes. The approach used implied Dynamic MRM acquisition mode in which analytes are only monitored while they are eluting from the LC and valuable MS duty cycle is not wasted by monitoring them when they are not expected. The sensitivity of the Dynamic MRM method was higher with respect to the MRM one. The LODs achieved with Dynamic MRM method range from 0.0015 mg/l to 0.015 mg/l and LOQs ranged from 0.001 mg/l to 0.05 mg/l. Considering linearity, the obtained coefficients of correlation were in the range 0.9907-0.9998 for the MRM method and 0.9934-1 for the Dynamic MRM method.

Conclusions:
The application to real samples indicated that black beans (458.64 mg/kg) and ruviotto beans (189.02 mg/kg) displayed the highest amount of total polyphenols. This study highlights that the content of polyphenols is related with the dark color of coat pulses and that certain polyphenols are characteristic in specific pulses variety. These aspects may be useful for increasing the health knowledge of legumes and for proposing new functional foods.

Novel Aspect:
A new analytical method that uses HPLC-MS/MS and triple quadrupole in Dynamic MRM mode was developed for the analysis of sixteen polyphenols in pulses.

References:
THIN LAYER CHROMATOGRAPHY COMBINED WITH FLAME-INDUCED ATMOSPHERIC PRESSURE CHEMICAL IONIZATION MASS SPECTROMETRY (FAPCI/MS) FOR VOLATILE AND SEMI-VOLATILE COMPOUND ANALYSIS

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Keywords: thin layer chromatography, desorption flame-induced atmospheric pressure chemical ionization

Introduction:
The combustion of hydrocarbons in a flame produces reactive ionspecies. In addition, the high temperature generated in a flame allows thermal desorption of small compounds on surfaces. A flame is therefore directed toward the sample to characterize analytes on surfaces, so called desorption flame-induced atmospheric pressure chemical ionization (DFAPCI). In this study, thin layer chromatography (TLC) was combined with DFAPCI/MS to analyze a mixture.

Methods:
The developed TLC plates were set on a XY stage and moved by a robotic platform for DFAPCI/MS analysis. A combustion head fabricated by arranging two concentric stainless steel tubes was utilized to generate a micro-flame. The combustion head was set at an angle of 45° with respect to the TLC plate surface. Volatile and semi-volatile compounds such as caffeine, drug molecules, and plant extracts were analyzed by TLC-DFAPCI/MS.

Results:
A light-brown line with 1-1.5 mm width was produced on the gel bed after analysis. This line clearly revealed the surface bearing a steam of heated jet from an oxyacetylene flame. The surface temperature of a TLC plate was measured to be 300 ± 10 °C as the plate passing through the desorption/ionization (DI) region. A mixture containing nicotinamide, 2-phenylacetamide, and dibenzylamine was separated by a silica gel TLC plate for the subsequent DFAPCI/MS detection. Positive ions of nicotinamide (m/z 123, [M+H]+), 2-phenylacetamide (m/z 136, [M+H]+), and dibenzylamine (m/z 196, [M-H]+) were detected. The limit of detections (LODs) of TLC-DFAPCI/MS for the analysis of three standards were tested and concluded to be 50 ng/spot for nicotinamide, 25 ng/spot for 2-phenylacetamide, and 5 ng/spot for dibenzylamine. Samples such as amine and amide standards, drug molecules, and aromatherapy oil were successfully characterized by TLC-DFAPCI/MS.

Conclusions:
Compared with other TLC-AMS approaches, TLC-DFAPCI/MS shows advantages including cheap, simple instrumental setup, and easy to be fabricated. Since the analytes were vaporized by a heat, TLC-DFAPCI/MS was suitable to characterize volatile, semi-volatile, and thermally stable compounds. We are combing laser desorption (LD) with FAPCI/MS, which will be capable of detecting non-volatile substances.

Novel Aspect:
Thin layer chromatography (TLC) has combined with desorption flame-induced atmospheric pressure chemical ionization (DFAPCI) to analyze a mixture.

References
EVALUATION OF GAS CHROMATOGRAPHY COUPLED TO ELECTROSPRAY-ATMOSPHERIC PRESSURE PHOTON IONIZATION ORBITRAP MASS SPECTROMETRY AS AN ADVANCED STRATEGY FOR POLYAROMATIC HYDROCARBON ANALYSIS

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Keywords: Orbitrap, Gas chromatography, Mass spectrometry, High resolution, polyaromatic hydrocarbons

Introduction: Heteroatom containing PAHs (HPAHs) play an important role in petroleomics due to their toxicity, stability in links of the catalyst development during oil refining process. However, general GC-EI/MS is not suitable for polar PAHs due to ionization process.[1] Also, no single ionization technique has capability to ionizedifferent variety of compounds.[2] In this study, GCESI-APPI MS provides a technique with high sensitivity and accuratem/z values ofthe HPAHs.

Methods: About thirty PAHs including nitrogen, sulfur and oxygen containing compounds were used for the establishment of GC ESI-APPI orbitrap MS conditions. GC was performed using an Agilent 7890A equipped with in-house-heated column transfer line that was heated by a programmable temperature controller with a temperaturesensor. [3, 4] Mass spectrometer was orbitrap Q-Exactive (ThermoFisher Scientific Inc.) equipped with an ESI source and APPI lamp.

Results: Depending on the analytes, GC has been coupled to various detectors, e.g. MS, FID and NPD. GC-MS is the most commonly employed in EI. But EI may be lost the parent ion of the analytes because of extensive fragmentation. FID is the presence of heteroatoms in molecular decreases the detector’s response. To solve this problem, element-selective detectors, like NPD, have been used. But NPD has limited signal stability due to mechanism of detection that is based on the bead. [1] To overcome such concerns, orbitrap MS with high sensitivity and resolution were applied to the HPAHs combining ESI which is soft ionization causing minimal fragmentations and APPI that it has been used to study PAHs in oils [5] hyphenated GC. The several ESI solvents were compared [6] and acetone showed the best ionization efficiency among the tested solvents. Also it is observed that the results using GC ESI-APPI MS for HPAHs is higher sensitivity than using GC-MS with single ESI source. Therefore, the GCESI-APPI MSIs suited for simultaneous analysis of HPAHs as an advanced ionization.

Conclusions: The aim of this research was to evaluate an instrument to couple GC with high-resolution orbitrap MS using an ESI source and APPI lamp. The studies demonstrated that the GC ESI-APPI MS has more elements-selectivity, sensitivity and accuracy even better than obtained with traditional GCEI/MS for HPAHs. Therefore, we expect that this method should be useful in the selective analysis of complex mixtures as nitrogen-rich oil, spilled oil, metabolite and humic substances.

Novel Aspect: GC coupled to high-resolution orbitrap MS with ESI-APPI was used to evaluate heteroatom containing PAHs analysis at the molecular level compared with GC ESI MS and GC EI MS.

References


ONLINE COUPLING OF NON-DENATURING CHROMATOGRAPHY TO NATIVE MASS SPECTROMETRY IS NO MORE AN IMPOSSIBLE MARRIAGE

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Keywords: native mass spectrometry, ion mobility, LC-MS, therapeutic protein

Introduction:
Mass spectrometry performed in non-denaturing conditions (native MS or IM-MS with ion mobility) has gained interest for the qualitative and quantitative characterization of intact proteins. However, online hyphenation of non-denaturing chromatographic (ndLC) techniques to native MS/IM-MS is still not straightforward, which hampers its routine use in high throughput automated environments.

Methods:
The 1DLC-MS system consists of an Acquity H-Class coupled to a Synapt G2 HDMS (Waters). Several Acquity BEH SEC (Waters) columns were used for 1D SEC-native MS. For 2D LC-MS, an Acquity-I-Class was added to the 1D setup. For the 1st dimension, the column was a MAbPac HIC-10 (Thermo Scientific) or a Yarra SEC-X300 (Phenomenex); the 2nd SEC dimension was an AdvanceBio SEC (Agilent Technologies).

Results:
We present here 1D and 2D LC-MS setups for online coupling of ndLC to native MS for the intact protein analysis. Native MS is mostly performed in ammonium acetate (AcONH4) [1] while ndLC techniques (e.g. size exclusion SEC or hydrophobic interaction HIC chromatography) require large amounts of non-volatile salts [2]. We present here the benefits of 1D SEC-native MS in AcONH4 for automated online buffer exchange and protein aggregation studies [3]. As AcONH4 is not optimal for HIC/SEC, a multidimensional approach combining HIC/SEC in the 1st dimension and SEC in the 2nd one (HICxSEC or SECxSEC) was developed. The 1st ndLC dimension (HIC/SEC) aims at achieving optimal protein separation, while the 2nd ndLC (SEC) serves for fast desalting. Benefits of HIC/SECxSEC-native MS/IM-MS are shown for different types of proteins, including monoclonal antibody-based (mAb) products and amyloid proteins.

Conclusions
Our results demonstrate the possibility to online couple ndLC to native MS/IM-MS. Benefits of ndLCxLC-native MS/IM-MS setups provide comprehensive and streamlined characterization of different types of proteins [4]. The synergic online coupling HIC/SEC to native MS/IM-MS is envisioned to definitely push native MS approaches at the forefront of intact protein characterization.

Novel Aspect:
Online hyphenation of non-denaturing LC to native IM-MS
1D SEC-native MS/IM-MS
2D HIC/SECxSEC-native MS/IM-MS

References
Introduction: (Limit of 400 characters)
GCxGC-TOFMS is a powerful tool for measuring complex samples. Using EI with this method allows for NIST searches, but these searches may result in mis-assignments without additional exact mass information for the molecular ion as generated through soft ionization techniques like FI or PI. In this study, we measured perfume oil by using GCxGC-HRTOFMS with EI, FI and PI.

Methods: (384, Limit of 400 characters)
All samples were measured by using a high resolution TOFMS and GCxGC system. A pure perfume oil was qualitatively measured using EI, FI and PI. For the time studies, 0.1 uL of pure oil for the same perfume was placed in a 22 mL vial and heated at 35°C for 0 hour, 0.5 hour, 1 hour and 3 hours. Afterwards, the headspace was sampled by using a SPME fiber and then measured with GCxGC FI.

Results: (788, Limit 900 characters)
The 2D chromatograms for EI, FI and PI showed the same retention time (R.T.) patterns. Additionally, the FI and PI data showed stronger molecular ion signals than the EI data. As an example, the peak detected at R.T. 25.70 minutes (1st column) and 1.55 seconds (2nd column) showed relatively high fragment ion intensities for the EI measurements. The NIST search for this mass spectrum showed several compounds with a match factor over 900 but with different chemical compositions. In order to narrow down the possibilities, it was necessary to measure the molecular ion using FI and PI. The results for these techniques showed an accurate m/z value within 1 mDa of the calculated mass for C_{12}H_{20}O_{2}. This estimated composition narrowed the EI library search candidate to linalyl acetate.

Conclusions (337, Limit of 400 characters)
The combination of EI fragmentation information with the accurate mass molecular ion information from FI and PI provided a means for narrowing down the NIST library search to help identify the compounds measured. We will present the qualitative results for the other compounds in the sample along with the perfume time variation studies.

Novel Aspect: (104, Limit of 150 characters)
Qualitative and quantitative analysis of perfume by GCxGC–HRTOFMS with EI, FI and PI ionization Methods:
1116 - SECONDS-PER-SAMPLE: NOVEL ACOUSTIC HYphenated APPROACHES TO ENABLE TRULY LABEL-FREE ULTRA HIGH-THROUGHPUT AMBIENT MASS SPECTROMETRY ADVANCING DRUG DISCOVERY

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Keywords: Acoustic droplet ejection (ADE), Acoustic mist ionization (AMI), Open port probe (OPP)

Introduction:
A truly label-free ultra-high throughput screening technology is the “holy grail” platform for drug discovery. We have integrated acoustic droplet ejection (ADE) technology that couples ambient mass spectrometry with two novel approaches enabling low sample consumption (nanoliters), low cost and ultra-high-throughput.

Methods:
We first highlight the principles of ADE and key technologies that enable robust acoustic liquid handling operations. The first modality utilizes direct electro spray ionization (ESI) whereby ADE delivers up to 1,500 spray events per second from an assay well in a microplate into the MS via a heated transfer optic [1]. The second modality explores an exciting method of sample injection to the MS by integrating ADE with 2.5 nL droplets into an open-port probe (OPP) sampling interface [2].

Results:
The first modality, acoustic mist ionization (AMI), integrates ADE with a Waters Xevo G2-XS QToF mass spectrometer. The utility of this integrated system as an ultra-high throughput platform in this format was demonstrated on a biochemical screen of more than 250,000 compounds identifying new and novel inhibitors of a relevant drug target. The sample assay was able to tolerate the changes to buffer conditions without impacting enzyme activity or kinetics. A data processing algorithm was designed to deal with large sample batches. In the second modality the ADE-OPP-ESI-MS system operates with a continuous-flow of carrier solvent (MeOH) for direct sample dilution that also actively cleans the entire flow system, mitigating carry over. “Classic” ESI ion source used in this setup enables detection of a broad range of analytes including small drug molecules, antibody standards, peptides (MW>1500) and proteins. High sensitivity (attomol loading for small molecules, or sub-femtomol for intact antibody) and reproducibility (<8% CV) was demonstrated without internal standard normalization.

Conclusions
We highlight ultra-high throughput ambient mass spectrometry hyphenated approaches with an acoustic sample introduction interface — a MS based plate reader that enables broad applications in drug discovery. Next steps on the commercialization perspectives are addressed with these two modalities and the need to integrate the MS spectra “big data” collection to support label-free ultra-high throughput ambient MS and advance the state of the art for drug discovery.

Novel Aspect:
Novel acoustic hyphenated sampling approach with mass spectrometry for ultra-high throughput label free screening and other applications in drug discovery.

References

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Introduction:
The electric field radius is a fundamental character of an ion trap, it measures the basic properties of ion trap. For a quadrupole ion trap with idea hyperbolic electrode, its electric field radius can be simply obtained by its geometrical structure, but it is very difficult to calculate the electric field radius if it was built with non-hyperbolic electrodes. In this work, the electric field radii of different size rectilinear ion traps were measured by experiments.

Methods:
The quadrupole linear ion traps with 5.0mm×5.0mm, 5.50mm×5.00mm, 6.00mm×5.00mm, 6.50mm×5.00mm, and 7.00mm×5.00mm respectively were used for radius testing. All experiments were performed using an electrospray ionization (ESI) rectilinear ion trap mass spectrometry. The digital power supply was used for both ion trapping and dipolar excitation of mass selected ion during CID experiments. And the electric field radius of each ion trap was deduced by the results.

Results:
For a quadrupole linear ion trap (LIT) with hyperbolic electrodes, the ion motion inside ion trap can be described by Mathieu equation.

\[ q = \frac{4eV}{mr^2\Omega^2} \]  
\[ (1) \]

And we can get following (2) from (1):

\[ \frac{m}{e} = \frac{V}{4r^2\pi^2}T^2 \]  
\[ (2) \]

Whether an ion of m/e can be trapped depends on the radial size r, or it is so called the electric field radius, the RF angular frequency \( \Omega = 2\pi f \), the voltage (V), and q. The property of an ion trap, such as the ion trapping capability, ion mass range, and so on are mainly dependent on the ion trap parameters, And particularly, the electric field radius. For a LIT with non-hyperbolic electrode such as rectilinear ion trap, its r cannot be directly deduced from equation (1). But it can be measured by CID experiment when a ion of m/e was performed at the optimal RF period of \( T \) according to (2) when V is fixed.

For the rectilinear ion traps with 5.0mm×5.0mm, 5.50mm×5.00mm, 6.00mm×5.00mm, 6.50mm×5.00mm, and 7.00mm×5.00mm, their measured electric field radii were measured, and they are 4.89mm, 5.15mm, 5.30mm, 5.79mm and 6.10mm respectively.

Conclusions
The electric field radii of rectilinear ion traps can be measured by experiments. For proving the reliability of this method, a hyperbolic electrode ion trap with r0=4.00mm was built and its measured electrode field radius was 4.07mm.

Novel Aspect:
The electric field radii of different size rectilinear ion traps were measured by experiments.
Keywords: quadrupole mass filter, universal hyperbolic electrode

Introduction

It is offered to create quadrupole mass-analyzers as short identical units - monoblocks with the relevant electrodes concatenated mechanically and electrically to form the unified system of electrodes [1, 2]. Each unit - monoblock consists of all-purpose hyperbolic electrodes which are concatenated with the help of ceramic isolators.

Methods

To create an all-purpose hyperbolic electrode the electrolytic molding technology [3] which allows making thin-walled copper electrodes (1 – 2 mm) with high accuracy was applied.

Results

The design principle described is worked in practice. The all-purpose hyperbolic electrode is made of copper by the electrolytic molding method, its length is 75 mm, its wall thickness is 1 – 1.5 mm. The electrode is clothed in a coating of 5 – 10 micron thickness by means of electrical deposit. The peripheral zone houses manufacturing holes which allow fastening electrodes together with a screw through ceramic isolators. The webbing along the electrodes’ ends in addition to its intended purpose is for the fastening of separate electrodes’ units together during the assembling of extended systems as well as for the setting of the whole electrodes’ system in vacuum chamber. The quadrupole mass filter was created with the using of the all-purpose hyperbolic electrodes.

Conclusions

The all-purpose hyperbolic electrodes allow creating quadrupole electrodes’ systems as separate blocks which can be connected with each other to get transit-time mass-analyzers of practically any length. The longitudinal distortion of electrical field doesn’t occur as the accuracy depends on the performance quality of a separate monoblock.

Novel Aspect

The application of the all-purpose hyperbolic electrode created by the electrolytic molding method with one precision form usage for quadrupole mass-analyzers creation.

References


For information please contact: scientific@imsc2018.it
DEGRADATION STUDIES OF THE ANTIBIOTIC SULFAMETHOXAZOLE UTILIZING HPLC/MS.

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Keywords: Sulfamethoxazole, HPLC, ICP-MS, Hyphenation, Environmental

Introduction:
The removal of micropollutants from surface waters is a topic of growing awareness. Not all xenobiotics are sufficiently removed by wastewater treatment plants, so additional disinfection steps such as ozonation or direct photolysis utilizing UV light are investigated. Many of these procedures are already well elucidated regarding degradation efficiency or energy costs but only few studies deal with the identification of formed products and their toxicity.

Methods:
Sulfamethoxazole (SMX) solutions adjusted to pH 3 and pH 8 respectively were degraded using direct photolysis (220-500 nm). The resulting transformation product mixture was characterized using HPLC/ESI-HRMS and fragmentation experiments. Sulfur-containing compounds were quantified using HPLC/ICP-TQ-MS.

Results:
In the present study, the degradation of the frequently used antibiotic sulfamethoxazole (SMX) was investigated using photolysis as degradation process focusing on the identification and characterization of formed transformation products by means of high performance liquid chromatography coupled to electrospray-mass spectrometry (HPLC/ESI-MS). The SMX solutions were pH-adjusted using a phosphate buffer to study the pH dependency of the product generation. In a further step, formed transformation products containing sulfur were quantified using HPLC/ICP-TQ-MS. From the quantitative data, rate constants of the photolytic degradation could be determined.

Conclusions:
More than 30 degradation products were found, with some being already described in literature. It was shown that the pH value has a great impact on the SMX degradation behavior and on the selection of formed transformation products.

Novel Aspect:
An HPLC/ICP-TQ-MS hyphenation was developed for the quantification of sulfur-containing organic compounds and the method was optimized to an LOD of about 100 ng/mL.
Introduction
Fast analysis requires speed optimization of the whole analysis including sample collection, introduction, separation, detection, identification and quantitation. We developed instruments and methods that each facilitates faster GC-MS analysis:
Fast GC, Open Probe Fast GC and GC-MS with Cold EI.

Method
The fast GC is based on a short capillary column inside thin walled metal tube that is resistively heated to provide sub one minute separation. Open Probe is based on a heated oven that is mounted on a low thermal mass fast GC which is open to room air with helium purge flow protection to prevent air penetration. Open Probe fast GC-MS operation is simple: touch the sample with a holder, insert it into the Open Probe and start running with 30 s separation and 50 s ready for next sample.

Preliminary data
We evaluated our low thermal mass Fast GC with Agilent 5975 GC-MS with standard EI and with GC-MS with Cold EI and demonstrated sub-one-minute full analysis cycle time. With the concept of low pressure chromatography we found that a mere factor of four loss of GC separation could bring a factor of 64 faster GC separations. Open Probe Fast GC-MS provides sub-one-minute full analysis times, including sample preparation. Thus, Open Probe Fast GC-MS acts like the range of ambient desorption ionization techniques yet with separation, library identification at the isomer level, superior sensitivity and the use of low cost single quad MS of GC-MS.

Novel Aspect
New instruments and methods for low thermal mass fast GC and Open Probe Fast GC-MS for sub one minute fast GC-MS and real time analysis with separation

Keywords Real time analysis with separation
The Importance of Enhanced Molecular Ions in GC-MS
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Introduction
A major limitation of GC-MS is the weakness or absence of molecular ions in its standard EI mass spectra. Enhanced molecular ions by the use of EI of cold molecules (named "Cold EI") has many benefits.

Method
We used GC-MS with Cold EI based on the combination of an Agilent 5977 MSD with the Aviv Analytical supersonic molecular beam (SMB) interface and fly-through ion source with electron ionization of internally cold molecules in the SMB (hence the name Cold EI) and the following are its benefits:

Preliminary data
Cold EI provides both enhanced molecular and fragment ions thus it is the only "soft" ionization method that is compatible with library identification. Unexpectedly, enhanced molecular ion actually improves NIST library identification probabilities. Molecular ions strongly decline with mass in standard EI (20% for added carbon in hydrocarbons) while in Cold EI it is about size independent. Cold EI eliminates vacuum background and reduces column bleed thus make it easier to visually identify the molecular ions than standard EI and/or ESI-LC-MS. Enhanced molecular ions and high mass fragments provide extended structural and isomer information. Molecular ions enables the use of TAMI software with unit resolution quadrupole MS for the conversion of isotopomeric abundances into elemental formula. Matrix interference is reduced with mass thus it is reduced on molecular ions no less than with MS-MS on fragment ions. The molecular ions in large compounds provide superior sensitivity in SIM or RSIM.

Novel Aspect
How enhanced molecular ions in GC-MS with Cold EI improve GC-MS based sample identification and several other GC-MS performance aspects

Keywords: Benefits of enhanced molecular ions
208 - STUDY OF ENDOCRINE DISRUPTING COMPOUND RELEASE FROM MEDICAL DEVICES IN PLASMA SAMPLES OF PREMATURE NEWBORNS THROUGH AN ON-LINE SPE UHPLC-MS/MS METHOD

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Keywords: endocrine disrupting compound release; migration; medical devices; mass spectrometry; perfluorochemicals

Introduction: Endocrine disrupting compounds (EDCs) are exogenous substances that mimic/antagonize the endogenous hormone effect. Everyday people are exposed to many products containing EDCs such as plastic containers, cladding films in aluminum food cans, medical devices (MDs), and personal care products. The underdeveloped reproductive apparatus and immune system of newborns can be exposed to unusually high concentrations of EDCs difficult to metabolize or excrete [1].

Methods: The study deals with the development of an online UHPLC-MS/MS method for the determination of 25 EDCs belonging to different chemical classes (perfluorochemicals PFCs, bisphenols, alkylphenols, phthalates, and parabens). To evaluate the possible EDC migration from different MDs of diverse materials used for blood sampling or for parenteral nutrition therapies, the devices were put in contact with physiological and parenteral solutions at prefixed time.

Results: The first step of the study was to identify a possible release of EDCs from the MDs and successively the EDC accumulation in plasma of the hospitalized newborns during the therapies to which infants have been subjected. The results demonstrated the released of some ECDs (such as parabens at concentration of few pg mL⁻¹, diethylphthalate and dibutylphthalate at ng mL⁻¹ level), when using physiological solution as the leaching agent. In addition, the presence of a PFOS structural isomer was found at 25.0 ng mL⁻¹: this compound was released in part from the infusion tubes and mostly from the venous catheters. The releases from the infusion tubes of the same materials (PE) from different manufacturers are very comparable, whereas the catheter of silicone releases greater amount of EDCs than that of polyurethane. As concerns plasma samples, butylparaben has greater concentrations in healthy newborns than in premature ones, while PFOA and PFHpA show greater concentrations in newborns hospitalized in neonatal intensive care unit.

Conclusions: The developed and validated method is attractive for routine analysis as it is characterized by high sensitivity, selectivity, robustness and reduced runtime analysis. The release from MDs of different plastic materials were investigates and diverse amount of EDCs ranging from a few pg mL⁻¹ to about twenty ug mL⁻¹ were found. EDCs were present also in the plasma of newborns and their monitoring have to be taken into consideration to avoid pathologies.

Novel Aspect: A new method has been developed and validated to quantify the migration of 25 EDCs belonging to different chemical classes (including PFCs) from MDs in plasma.

References
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Keywords: microchannel plates, triode structure, low vacuum operation, miniature mass spectrometer

Introduction:
Recent interest in portable mass spectrometry applications has spurred a great deal of research into the development of miniature mass spectrometers (MS) such as desktop type explosives trace detectors (ETDs). In the near future, there is a possibility that ETDs used at checkpoint screening will change from the current ones based on ion mobility spectrometers to ones based on next-generation MS.
In order to retain the major advantage of portability, miniature MS systems typically use small vacuum pumps with relatively low pumping speeds, meaning the miniature MS has to be operated at relatively low vacuum levels (>1e-2 Pa). Also in terms of sensitivity and vacuum condition, almost all miniature MS systems under development have adopted the ion trap analyzer. In these system configurations, ion detectors are required to have both low vacuum operation (>1e-2 Pa) and high gain (>1e+6). Currently, channel electron multipliers (CEM) are mainly used in miniature MS, but it is difficult for CEM to satisfy the required specifications.

Conventional electron multiplier based detectors including CEM and microchannel plates (MCP) cannot be adapted to operate at low vacuum (>1e-2 Pa) because of ion feedback (IFB), which causes discharges and decreases in S/N. However, the reduced dimensions of a typical MCP detector, which can be less than the mean free path in conventional electron multiplier based detectors, make it suitable for operation at low vacuum. The major source of IFB, which is a peculiar phenomenon of secondary electron amplification, was found to be the creation of ions from residual gas molecules.
We concluded that controlling the residual gas ions is more important than suppressing the ion generation. In order to realize this idea, we introduced a triode structure into the conventional MCP detector to enable it to operate at low vacuum condition.

Methods:
The mechanism of IFB in an MCP detector was investigated, and it was found that 1) IFB is caused by EI-generated residual gas ions, which are generated between the MCP-out and an anode by the reaction of residual gas and electrons from the MCP, 2) after traveling in reverse through the MCP channel, the residual gas ion generates secondary electrons by impinging on the MCP channel surface, and 3) IFB occurs repeatedly like an avalanche phenomenon and it becomes a huge noise signal. We concluded that controlling residual gas ions is more important than suppressing ion generation.
Almost all MCP-based ion detectors have a bi-planar structure which consists of a chevron stack MCPs and an anode. The potential is MCP-in < MCP-out < anode, so the residual gas ions (+) return to the MCP and cause an IFB. The triode structure has a mesh electrode placed between MCP-out and a dynode as the third electrode. The aim of adopting the triode structure is to prevent positive ions returning to the MCP. Therefore, the novel potential is MCP-in, dynode < MCP-out < mesh, so that the dynode can function as an ion capture electrode and the mesh as an electron capture electrode.
It is confirmed that the triode structure prevents ion feedbacks at low vacuum condition by evaluating various characteristics. Since the usual electron gun could not be operated at low vacuum condition, ultraviolet rays were used as input signals instead of electrons. The vacuum pressure of the chamber which contained the triode MCP detector was controlled by opening and closing the manual gate valve and monitored with a vacuum gauge. It was
difficult to keep the vacuum pressure constant in the high pressure region, and it slowly deteriorated during the measurement.

Results:
The prototype triode MCP detector was examined using standard MCP detector characterization equipment in high vacuum levels (1e-4 Pa or less), which is the recommended environment for the standard MCP detector. The gain characteristic of the triode MCP detector was 5e+6.

The triode detector operated in two potential modes: a) bi-planar mode [MCP-in < MCP-out < mesh = dynode (connected)], b) triode mode [MCP-in = dynode < MCP-out < mesh]. When the gain of MCPs was lower (1e+3 or less), IFB did not occur in both modes. When the gain was higher (1e+5 or more), IFB occurred at 1e-3 Pa in bi-planar mode without input signals, but did not occur in triode mode up to 1 Pa. It was found that the MCP detector was capable of high pressure operation by adapting the triode structure with a novel potential mode.

Since the charge of the input signal could not be measured by using ultraviolet rays, the peak channel from PHD characteristics analysis using a multichannel analyzer system, which measured the amount of charge from the MCP output signal, indicates the gain at each degree of vacuum level. Despite more than 1e-2 Pa, the dark noise which derived from the IFB could not be confirmed in the PHD characteristics. When the vacuum pressure was 1 Pa, the calculated gain of 5e+6 was maintained.

In order to confirm the stability of the detector operation, the outputs of the detector were monitored for about 90 minutes while input signal was turned on and off at 15-minute intervals. The output of the triode detector was stable from the long-term examination results irrespective of the degree of vacuum.

Conclusions
The MCP detector for low vacuum operation was developed by combining the triode structure with a novel potential mode. A compact detector for miniature MS could be realized by simplifying the structure in terms of supply voltage conditions and achieve the target gain of 1e+6 at 0.1 Pa. We will confirm the MCP detector operation in a lower vacuum region by improving the current evaluation system, and optimize the MCP detector for a commercialized MS.

Novel Aspect:
The MCP detector for low vacuum operation was developed by combining the triode structure with a novel potential mode. A compact detector for miniature MS could be achieved with the target gain of 1e+6 at 1 Pa.

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419 - THE DYNAMIC BEHAVIOR OF NANO LIQUID BRIDGE DURING AMBIENT SAMPLING IONIZATION IN SCANNING PROBE ELECTROSPRAY IONIZATION MASS SPECTROMETRY

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Keywords: Tapping-mode scanning probe electrospray ionization (t-SPESI), Nano liquid bridge, Mass spectrometry imaging, Atmospheric pressure ionization, Ambient sampling ionization (ASI)

Introduction:
Tapping-mode scanning probe electrospray ionization (t-SPESI) have been used for mass spectrometry imaging. In t-SPESI, the formation of a liquid bridge and the generation of an electrospray by a vibrating capillary probe realizes the ambient sampling ionization (ASI) [1-4]. However, the dynamic behavior of the charged liquid bridge at the probe apex is unclear. In the present study, we studied a probe motion during ASI with the optical detection method.

Methods:
We developed the measurement system of the probe displacement. The optical lever method and the sample stage controller was incorporated in the experimental setup of t-SPESI. Mass spectrometry was conducted at the same time to capture the time changes of ion intensity. The commercial available silica emitter and the Rhodamine B thin film on glass was used as a probe and the specimen. The physical properties of the probe were simulated with finite element method.

Results:
The spring constant of the probe was estimated to be about 37 N/m (18 mm long probe) and 5.0 N/m (35 mm long probe). We found that the probe with lower spring constant is suitable for the measurement of the probe displacement during ASI with high sensitivity. The probe apex was drawn toward the sample just before the contact, indicating the timing of liquid bridge formation. During the retraction of probe from the sample, the probe was released from a liquid bridge. Considering the retraction distance and the simulated spring constant, the force applied to the apex of the probe was about 25 µN just before the breakage of liquid bridge. At that time, the ion intensity became stronger and it gently became weak within 1 to 2 second. From these result, it was found that the sampled molecules were ionized over several seconds after the breakage of liquid bridge. In addition, the result of experiment with a nanopipette (a nanoscale diameter opening glass pipette) will also be presented.

Conclusions
New optical lever method was developed for SPESI. The formation and the breakage of the liquid bridge was captured by the displacement of probe during ASI. The sampled molecules are ionized over several seconds after the breakage of liquid bridge. The pulling force by the probe to break the liquid bridge was estimated to be larger than the expected surface tension of liquid bridge.

Novel Aspect:
This report is the first result to capture the dynamic probe motion during the sampling and ionization process using liquid bridge and electrospray in SPESI.

References
Y. Otsuka et. al., Analyst 139, 2336 (2014).
Introduction:
A major bottleneck to directed evolution workflows is the throughput required to screen a large number of genomic variants for the most efficient biocatalysts [1]. Current screening methods often rely upon well-plate fluorescent readouts which become problematic when the product of interest is not inherently fluorescent; hence, ‘label-free’ detection is highly desirable.

Methods:
Mass Spectrometry coupled with a droplet microfluidic chip system offers a ‘label-free’ screening platform in which each genomic variant of the combinatorial library is encapsulated in a ~500 picolitre droplet suspended within an immiscible fluorous oil phase. MS analysis of the cell filled droplets allows for the identification and capture of the highest product producing genomic variants.

Results:
Droplet generation occurs at a rate of 5 Hz within a custom designed microfluidic chip followed by immediate flow of the droplets into a mass spectrometry source for ionization and then subsequent analysis. Loss of droplet order and droplet merging is avoided through ‘plugs’ of the oil phase and hence, each droplet arises in the TIC as a distinct peak at the same rate as generation. This allows for a mass spectrum for each peak to be obtained such that information regarding products or metabolome fingerprint in 1 discreet droplet can be extracted. This has been performed for a range of standards (including droplet mixtures [2]) and for bacterial cells cultured within droplets as proof of concept. Other on-chip processes such as droplet splitting have also been successfully trialed independently, with the aim to incorporate droplet capture of those identified by the MS as ‘hits’.

Conclusions:
We demonstrate direct coupling between chip and two different Ion-Mobility enabled Mass Spectrometers to analyse discreet droplets without the prior removal of the contents (both protein and small molecule standards and cells) from the oil phase. This has been achieved with a throughput of ~5 Hz, which already surpasses the rate of many current sample introduction systems.

Novel Aspect:
Novel aspects include ultra-high throughput capabilities (~5 Hz) and miniaturization of sample introduction platforms for life science applications.

References:
PORTABLE GAS CHROMATOGRAPHY MASS SPECTROMETER WITH A RAPID HEATING CARBON NANOTUBE PASTE

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Keywords: portable GCMS, cold electron, nanovalve, carbon nanotube, VOC

Introduction
Recently, a miniaturized ion trap mass spectrometer with cold electron ionization was developed by our group as an ultra-portable mass spectrometer [1]. To extend the applicable field of the portableMS to a complex gas mixture analysis, we integrated a gas chromatography (GC) system to the miniaturized ion trap mass spectrometer using a nano-litervalveinterface andevaluated the analytical performances of the system using typical volatile organic compounds.

Methods
A new portable GC was developed using a new carbon nanotube (CNT) heating paste. The GC column was heated by a bobbin type heater made of anodized aluminum and CNT heating paste applied on the inner surface of the heater. The miniature cylindrical ion trap MS was developed using a cold electron source to ionize analyte gas. An accurate nanovalvewas developed and used as an interface between GC and the MS to avoid the failure of ion pump by abrupt increase of chamber pressure.

Results
The analytical characteristics of the portable GCMS were evaluated as individual componentsand as complete GCMS system. The performances of gas chromatography were evaluated with an adsorption concentrator using standard VOC samples while changing the column temperature gradient up to 100 degree/sec for rapid analysis. The performances of cylindrical ion trap were tested with nanoliter injection valve using the standard gas. With built in 2 L/sec ion pump and nanoliter injection valve, maximum 10 times/min sample injection was the limiting factor of analysis frequency while maintaining the operatable pressure of the ion trap. The electron ionization was using electrons from electron multiplier induced by UVphotons from a diode. The optimal transmission condition of the electrons to the ion generation region was derived from SIMION simulation and used for the analytical performance test of ion trap. The developed GC/MS system was less than 10 kg and showed better than unit MS resolution and sub-ppm detection limits for typical VOCs.

Conclusions
Using a portable gas chromatography and a miniaturized cylindrical ion trap mass spectrometer, a new portable GCMSwas introduced with CNT heating paste showed a high temperature-gradient for rapid analysis, and with a cold electron ionization source optimized for higher electron transmission to the ion generation region showed improved detection efficiencies. The integrated system showed detection limit of down to sub-ppm levels for the standard VOCs.

Novel Aspect
A novel hand carriable GCMS was developed using a carbon nanotube heating paste for GCand cold electron ionization for portable ion trap MS to detect VOCs realtime in the field.

References

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1129 - ANALYTICAL PROCEDURE FOR DETERMINATION OF PERFLUOROALKYL ACIDS, PARABENS AND COTININE FROM A LOW VOLUME HUMAN SERUM SAMPLE BY LC-MS/MS

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Keywords: biomonitoring, cotinine, epidemiological study, paraben, perfluorinated substances

Introduction:
In studies on the health effects of environmental chemicals it is desirable that multiple compound groups can be analyzed from the same low volume serum aliquot. Thus, we have developed and validated a single analytical procedure for determination of three different compound groups, perfluorinated substances (PFAS), parabens and cotinine (as a metabolite of nicotine), which are associated with a variety of adverse health outcomes [1-7].

Methods:
Serum sample of 0.1 mL was pre-treated with two step extractions, and all the analytes were separated and detected with LC-MS/MS. The limit of quantitation (LOQs) for PFAS, parabens and cotinine were 0.1 - 0.3 ng/mL, 0.20 – 1.0 ng/mL and 0.10 ng/mL, respectively. These limits are highly sufficient for studies where the exposure effects of PFASs, parabens and tobacco smoke is evaluated.

Results:
Validation data shows that the method has excellent accuracies and repeatabilities. For PFAS the accuracies ranged from 94 to 106% and the repeatabilities (as RSD%) from 2.2 to 5.6 %. The accuracies and RSDs for parabens were 73 – 120 % and 2.2 - 9.7 %, respectively, and for cotinine 102 – 106 % and 2.9 - 3.5 %. The main PFAS found in population based serum were perfluorooctanesulfonate (PFOS, range 1.3 – 23 ng/mL), perfluorooctanoate (PFOA, range 0.54 – 6.6 ng/mL), perfluorononanoate (PFNA, range 0.19 – 4.1 ng/mL) and perfluorohexanesulfonate (PFHxS<0.15 – 5.5 ng/mL). For parabens the methyl (range <1.0 – 49 ng/mL) and propyl (range <0.2 – 1100 ng/mL) conjugates were the abundant compounds found in this study. Cotinine level in serum of tobacco smokers was over 50 ng/mL, whereas in serum of non-smokers the level was usually below 0.1 ng/mL.

Conclusions:
A simple, sensitive and high throughput method for analysis of 21 PFAS, 8 parabens and cotinine from 0.1 mL of serum was developed and validated. Analysis throughput time for 150 serum samples is one week. Besides the high throughput performance, the method is suitable for studies where the sample volume is limited since only a low sample volume is needed.

Novel Aspect:
This is the first method to gather these 3 classes of environmental pollutants (perfluorinated substances, parabens, and cotinine) in a single procedure.

References:
783 - COLD VAPORIZATION OF TISSUES BY PICOSECOND INFRARED LASER ABLATION (PIRL) - UNIQUE ACCESS TO THE PROTEOFORM COMPOSITION.

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Keywords:
Picosecond infrared laser ablation (PIRL)
Protein extraction
Tissue proteomics
Proteoform
Clinical proteomics

Introduction:
The proteoform composition of tissues has a high diagnostic value. Proteoforms regulate biological processes and an aberrant composition may cause diseases. Intact proteoforms are exposed to enzymatic degradation reactions during sample preparation and methods are needed that provide access to the original proteoform composition of tissues. PIRL ablation minimizes degradation reactions and provides access to the original proteoform composition of tissues.

Methods:
PIRL-ablated proteins were analyzed by LC-ESI-MS/M and MALDI-MS to investigate if their chemical composition and enzymatic activity remained unaltered. Tissues were homogenized either by conventional mechanical methods or by PIRL ablation. The homogenates were separated by 2-DE and SDS-PAGE, followed by quantitative LC-MS/MS analysis. Enzymatic degradation reactions were analyzed by proteoform SDS-PAGE migration profiles.

Results:
Comprehensive MS analysis showed that the exact chemical composition of proteins was not altered during the PIRL ablation process and even posttranslational modifications and enzymatic activities were reserved (1). 2-DE analysis indicated that PIRL yielded a higher number of intact proteoforms compared to mechanical homogenization. These results were confirmed by SDS-PAGE migration profiles. Relative degrees of proteolysis were significantly lower in case of PIRL (2%) compared to mechanical homogenization (23%) (2). These results were further confirmed by spiking experiments. Total yield of intact alpha-casein proteoforms and number of identified phosphopeptides were significantly higher using PIRL, whereas mechanical homogenization showed significantly higher amounts of enzymatically degraded proteoforms. PIRL-derived extracts are almost free of particles minimizing sample, thus providing a higher reproducibility and significantly higher protein identification rates compared to conventional tissue homogenization.

Conclusions
PIRL releases intact proteoforms from tissues without changing their exact chemical composition including posttranslational modifications. The ultrafast release and transfer of intact proteoforms into frozen condensates minimizes exposure to enzymatic degradation reactions and provides an unique access to the original proteoform composition of tissues.

Novel Aspect:
PIRL provides an unique access to the original proteoform composition of tissues and facilitates molecular diagnostics of tissues at the proteoform level.

References
From niche application to essential research tool; why SFC-MS is the must have technique

Keywords
Supercritical fluid chromatography - mass spectrometry

Introduction:
SFC now delivers the chromatographic promises with robust and reliable instrument. The solvation power, selectivity and peak capacity of modern Ultrahigh Performance Supercritical Fluid Chromatography (UHPSFC) coupled with mass spectrometry affords the ideal analytical platform to address classes of compounds that were previously a challenge for GC-MS and RP-UHPLC-MS approaches.

UHPSFC-MS sits between LC-MS and GC-MS affording extended capability and complementarity and delivers unique solutions across a broad range of application areas, e.g. synthetic organics, pharmaceuticals, petrochemistry, lipids, nucleotides and many other areas.

Methods
Ultra-high performance supercritical fluid chromatography (UHPSFC) methods have been developed using a simple column screening protocol. A Waters Acquity UPC2is coupled to a TQD mass spectrometer and a range of cosolvents and additives are utilised to deliver generic and bespoke methods. Further tuning of the organic make-up solvent has been used to deliver specific assays.

Results
UHPSFC, in the form of Ultra Performance Convergence Chromatography (UPC2) has been incorporated into the open access (walk-up and use) chromatography mass spectrometry facility within Chemistry at the University of Southampton in 2013. This technology complements existing liquid chromatography and gas chromatography options (HPLC-MS (ESI/APCI), GC-MS (El/CI) and affords high throughput reaction monitoring for new chemistries and highly specific research solutions, e.g. lipids, isocyanate polymers, petrochemistry, molecular wires and supramolecular chemistry.

Novel Aspect
New chromatography solutions, extension of open access mass spectrometry.
Keywords: Conventional-flow LC-MS, exploratory proteomics.

Introduction:
Although conventional-flow LC-MS platforms have penetrated targeted proteomics successfully, their possibilities in discovery-oriented proteomics have not yet been thoroughly explored. Our objective was to determine what optimization and adjustments to a conventional-flow LC-MS system must be undertaken to identify a comparable number of proteins as can be identified on a nanoLC-MS system.

Methods:
A trypsin digest of HeLa cells lysate was used to compare proteomic identifications between nanoLC and conventional-flow LC-MS setup. Following parameters of the conventional-flow LC-MS configuration were optimized in order to improve identifications: DDA settings, column diameter, extra-column peak dispersion, temperature, flow rate, column length, mobile phase acidifiers and additives.

Results:
We demonstrate that the amount of a complex tryptic digest needed for comparable proteome coverage can be roughly 5-fold greater, providing the column dimensions are properly chosen, extra-column peak dispersion is minimized, column temperature and flow rate are set to levels appropriate for peptide separation, and the composition of mobile phases is fine-tuned. Indeed, we identified 2,835 proteins from 2 μg of HeLa cells tryptic digest separated during a 60 min gradient at 68 μL/min on a 1 mm × 250 mm column held at 55 °C and using an aqua-acetonitrile mobile phases containing 0.1% formic acid, 0.4% acetic acid, and 3% dimethyl sulfoxide.

Conclusions:
Although we have not exploited all options for improving LC-MS performance, we conclusively show that conventional-flow exploratory proteomic analyses are feasible. Providing the instrumentation and method are adjusted appropriately, the extra sample amount need not correspond to the theoretical 178-fold greater burden [1].

Novel Aspect:
Our results document that nanospray-free, conventional-flow LC-MS is an attractive alternative for bottom-up exploratory proteomics.

References:
ON-LINE COUPLING OF SURFACE PLASMON RESONANCE TO AMBIENT MASS SPECTROMETRY

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Keywords: Ambient mass spectrometry, surface plasmon resonance, hyphenation method, molecular interaction

Introduction:
Surface plasmon resonance (SPR) is a powerful technique widely utilized in the studies based on interaction processes such as cellular analysis [1] and clinical researches [2]. Despite its versatility, there still exist some intrinsic deficiencies due to the principle of SPR, among which the insufficiency in qualitative analysis remains a significant one. Generally, the information acquired by conventional SPR instruments is inadequate for the identification of the samples, constraining the application of SPR in the researches dealing with unknown species such as ligand fishing [3] or active ingredient identification [4]. Consequently, the combination of SPR with other analytical approaches that can provide molecular information has been highly desired [5-9].

In this work, a newly developed interface to couple SPR with MS using spray tip and DART was reported. The SPR flow solution was led into a sprayer, whose tip was put in middle of the DART outlet and the MS inlet, thus the solution could be sprayed and ionized. Proof-of-concept experiments using 4 chemicals dissolved in 3 types of salt-containing solvents were conducted, showing the practicability of this interface by observing the consistent change of the signals of MS and SPR. The performance of this interface and its salt tolerance suggested its high potential in the screening of the unknown interaction under physiological conditions using SPR-MS.

Methods:
SPR sensor chip modification. The sensor chip was modified with HSA using common EDC/NHS coupling method. The sensor chip was first cleaned by immerging it in a solution blended by ammonia solution, hydrogen peroxide and water with the volume ratio of 1:1:5 and being kept at 90 oC for 10 min. After installing the cleaned chip into the SPR analyzer, the following modification process was carried out by successively injecting and flowing: a. 3-MPA with TCEP, for 60 min; b. EDC and NHS for 20 min; c. HSA for 12 h; d. ethanolamine for 10 min. The change of SPR angle before and after the HSA modification proved that the protein had been successfully immobilized onto the sensor chip.

SPR-AMS experiments. Acetaminophen, metronidazole, quinine and hippuric acid were selected to be the model analytes, with PBS, TBS and EBSS chosen as the representative solvents to simulate the physiological conditions that might be used in usual SPR experiments. There were all together 12 kinds of sample solutions. A single run was started by commencing SPR and MS data acquisition simultaneously. For the first 2 minutes, pure solvent was delivered. At the beginning of the 3rd minute, the sample solution started to flow, and lasted for 4 minutes. During these 4 minutes, the analyte might bind to the HSA immobilized on the sensor surface, leading to a change in the SPR signal. From the 6th minute, again the pure solvent was delivered, and the analyte would be washed away. After the SPR signal fell back to its baseline and the MS signal became stable, the data acquisition was stopped. The raw SPR data was denoised by fast Fourier transformation smoothing before further analysis.

The solution flow rate of all of the runs was 0.20 mL/min. The nebulizing gas was nitrogen at a pressure of 15 psi. The SPR analyzer was set at constant angle mode recording the reflected light intensity at the SPR angle. For the DART-MS ion source, the temperature was set at 450 oC with helium as working gas. The MS parameters were: capillary voltage, off; dry gas temperature, 325 °C; dry gas flowrate, 5 L/min. The range of m/z between 100 and 400 was scanned with a maximum ion accumulation time of 200 ms. MS was operated at positive mode when analyzing solutions containing acetaminophen, metronidazole or quinine, and negative mode for solutions of hippuric acid.

Results:
Optimization of the DART conditions. As preliminary experiments, the following parameters that might affect the DART ionization and the MS analysis were optimized: DART source temperature, the vertical distance between the spray tip and the DART-outlet-MS-inlet plane (d3), the total horizontal distance between the DART outlet and the MS inlet (d1), the flow rate of the solution and the nebulizing gas pressure for the spray. As these parameters were
A novel interface relying on DART ionization and spray tip for direct and on-line coupling of SPR with MS was designed and developed. Experimental results using different model compounds and buffers exhibited the feasibility of the online combination of SPR and MS. This system showed a high tolerance of the non-volatile salts during the test, suggesting its potential utilizations in SPR-MS experiments, especially when physiological conditions should be considered. The droplets in the spray would get smaller and better distributed as they flew away from the spray tip, so a larger d3 would be beneficial. Also, as the analytes were dissolved in the solutions and the sprayed droplets, the ionization would consist of the evaporation and desolvation process, thus a higher source temperature would be more productive. For the nebulizing gas pressure, if it was too high, the droplets would be blown away too fast from the DART ionizing stream, so a lower pressure would be better. The flow rate meant the amount of sample, and a higher value led to a stronger signal. The value of d1 showed least importance, thus it was selected considering other parameters.

The horizontal distance between the spray tip and the MS inlet (d2) could also affect the results. However, as there was a restrictive requirement that d2 must be less than d1, the value of d2 could not be included in the orthogonal experiments and it was optimized separately. Generally, a smaller d2 could allow more ions to go into the MS inlet, but it might also lead to the sample residue at the MS inlet. Also, a larger d3 would result in more residues. With consideration to the signal intensity and the sample residue contamination, the following parameters were selected: DART source temperature, 450 oC; d1, 2.0 cm; d2, 10 mm, d3, 6 mm; flow rate, 0.20 mL/min; nebulizing gas pressure, 15 psi. Additionally, helium was used as working gas for all of the sample solutions.

Performance of the SPR-DART-MS experiments.

As mentioned in the experimental section, there were 12 sample solutions. It was undoubtedly proven that the as-designed spray and DART-based interface could be used successfully and practically for on-line coupling of SPR with MS, as the MS signal changed consistently with the SPR signal. For the first 2 minutes during which the pure solvent flowed through the SPR analyzer, SPR signal stayed at its baseline, so did the MS signal. As the solution containing analytes started to flow at the 3rd minute, the SPR signal started to increase as the analyte bound with the HSA immobilized on the sensor chip. The MS signal also increased, as the excess analyte in the solution got sprayed and ionized. From the 6th minute, pure solvent flowed again, and the SPR signal changed to its original values because of the dissociation of the bound analytes, and the MS signal also decreased.

There is an observable lag of approximately 1 min between the increase of the MS signal and the injection of the sample solution. This was mainly because of the volume of the SPR flow cell and the connecting tubes. All of these volumes between the pump and the MS would act like the dead volume similar in a chromatography process, delaying the change of the MS signal. Besides the dead volume, there were another two reasons explaining the delay of this decrease. First, there remained analytes bound on the sensor chip, the elution of these analytes would last for some time, thus the MS signal would remain high during all this elution process. Second, as the aqueous solutions containing non-volatile salts were used, the evaporation of the sprayed droplets would often be incomplete, resulting in the residue of the analytes in the vicinity of the spray tip and the MS inlet, keeping the MS signal from decreasing. Notwithstanding this residue, it demonstrated the necessity and advantage of the DART-based interface, that it could prevent the residue from contaminating conventional ESI source.

Performance of the SPR-DART-MS experiments.

For most analytes and solvents, the interface showed a high tolerance of the non-volatile salts. For the 3 analytes in positive mode, both of PBS and EBSS showed even higher signal intensity than water, indicating that the low-molecular-weight salts might help the proton transfer to form positive species. TBS showed lower signal intensity, which could be explained by the competitive ionization between the analyte and tris contained in TBS, which was supported by the strong tris peak (m/z = 122, [M+H]+) observed in most of the MS spectra using TBS. For the analyte in negative mode, all of the salt-containing solvents showed a depression in signal intensity, suggesting the salts’ negative effects in negative mode. These results proved the as-designed interface had an adequate tolerance of the non-volatile salts, and should suffice the applications for direct on-line coupling of SPR with MS using salt-containing solutions.

Conclusions

A novel interface relying on DART ionization and spray tip for direct and on-line coupling of SPR with MS was designed and developed. Experimental results using different model compounds and buffers exhibited the feasibility of the online combination of SPR and MS. This system showed a high tolerance of the non-volatile salts during the test, suggesting its potential utilizations in SPR-MS experiments, especially when physiological conditions should be...
needed. This interface provided a new approach for online coupling of SPR-MS, and would contribute to the fast detection of the interaction between substrate and the ligand and simultaneous identification of the ligand [10].

Novel Aspect:
A novel interface for direct and on-line coupling of SPR to AMS was developed. A spray tip connected with the outlet of the SPR flow solution was conducted as the sampling part of the DART-MS, with which the online coupling interface of SPR-MS was realized. Four model samples, acetaminophen, metronidazole, quinine and hippuric acid dissolved in three kinds of common buffers were used in the SPR-DART-MS experiments for performance evaluation of the interface and the optimization of DART conditions. The results showed consistent signal changes and high tolerance of non-volatile salts of this SPR-MS system, demonstrating the feasibility of the interface for on-line coupling of SPR with MS and the potential application in the characterization of interaction under physiological conditions.

References
Introduction:
The fast development and exhaustive search for effective VOCs analysis with mobile MS has become an important research field, due to the advantages of miniaturized systems for on-site applications over laboratory-scale instrumentation. Nevertheless, it is important to rely on valid analytical platforms capable of achieving high sensitivity, reducing process times, and, specifically for on-site analysis [1], enabling multi-component determinations.

Methods:
An extensive evaluation of three commercially available mobile GCMS, the Hapsite ER (Inficon, Germany) and the E2M (Bruker, Germany) with quadrupole mass analyzers, and the Torion T-9 (PerkinElmer, Germany) with a toroidal ion trap mass analyzer, was performed using a complex mixture of 24 compounds introduced by different devices, and compared to a state-of-the-art bench-top GC-2010 Plus coupled to a TQ-8040 triple quadrupole mass spectrometer (Shimadzu, Japan).

Results:
First, the performance was assessed in terms of sensitivity, approximately only 50% of the analytes were detected in the mobile mass spectrometers independent of the introduction devices (thermal desorption TD, direct probe and SPME) and compared to the TD stationary system. In contrast, after average normalization, the reproducibility of consecutive measurements (n=3) seemed to be strongly influenced by the pre-concentration system. While ~30%RSD was determined for ab/adsorption-desorption methods (SPME and TD), even higher variance and unstable results were obtained using the direct probe system; all of them were considerably higher than the 15%RSD found on laboratory scale. In addition, spectra reproducibility was probed for its impact on spectral similarities with common reference spectra libraries, but still some of the corresponding softwares showed several limitations for data transformation, data transfer, and even selection of quantitation and confirming ions for proper determinations and statistical analysis.

Conclusions
Mobile devices for on-site applications of unknown samples still need to overcome several analytical limitations during qualitative and quantitative analysis, specifically for volatile profiling with multi-component determinations.

Novel Aspect:
Mobile GCMS was assessed for appropriate method development and validation for analysis of complex standard mixtures and compared to state-of-the-art bench-top GCMS.

References
Introduction

Antibiotics are used as human and veterinary medicines to treat diseases. Studies have found various antibiotics in surface water, which has raised concern on the human health effects. To investigate the occurrence and fate of these low level contaminants and measure them in a variety of water matrices; sensitive, selective and reliable methods are needed. In this study, a selective solid phase extraction (SPE) method was evaluated for sample clean-up and analyte concentration for analysis on an UHPLC-MS/MS system.

Methods

Water samples were subjected to solid-phase extractions. Analytes were determined by coupling the UHPLC system to a highly sensitive PerkinElmer QSight 220 triple quadrupole mass spectrometer with dual ESI/APCI sources. Under the optimized conditions, the method was validated in terms of its specificity, sensitivity, linearity, accuracy, and precision. Recoveries from sample matrix were evaluated by fortifications of analytes at 3 different concentrations.

Results and Conclusion

In this study, to effectively remove interfering components and enriched analyte of interest from complex environmental water sample matrices, several SPE extraction phases with different functionalities were evaluated, such as reversed phase, ion exchange and mixed mode cartridges. The extraction and elution conditions were also optimized for SPE procedures. Calibration curves were built with matrix-matched standards to compensate for any matrix effects. The regression coefficients (R2) for the calibration curves obtained for all antibiotics studied were all greater than 0.990, and the limits of quantification (LOQs) ranged from 0.1 to 5µg/L for the targeted compounds. The mean recoveries from the samples spiked at three different concentrations, respectively, were between 70% and 120%. The intra-day and inter-day variations, expressed as RSD, were less than 5%, respectively. The results demonstrated that the accuracy and precision of the present method were acceptable for routine monitoring purposes. The analyte confirmation in sample was achieved by comparing their respective retention times and peak area ratios of two MRM transitions with those obtained from the corresponding reference standards. The method developed in this study provides an effective tool for determination of multi-antibiotics in surface water and a large number of actual water samples were analyzed using this method to evaluate the occurrence of the analytes in local water systems in Chengdu, China.

Novel Aspect

Highly selective SPE extraction method coupled to highly sensitive UHPLC-MS/MS for low level antibiotics analysis in surface water.
Introduction:
Cyclic lipopeptides (CLPs) are produced by bacteria in a ribosome independent manner and consist in a hydrophilic peptide chain linked to a β-hydroxy fatty acid chain. CLPs are a very large and diverse group of molecules, that has recently gained lots of attention for their potential applications in agricultural or pharmaceutical industries [1, 2]. In that aim, the development of reliable analytical methods for new CLPs isolation and characterization is essential.

Methods:
Capillary Electrophoresis-Mass Spectrometry was performed on a CESI 8000 instrument coupled to a tandem linear ion trap and high resolution mass spectrometry FT-ICR. A bare fused silica capillary of 90cm x 375µm (OD) x 50µm (ID) was used for the separation. In addition, CLPs were analyzed by ion mobility mass spectrometry on a timsTOF instrument.

Results:
The obtained separation of up to four isobaric CLPs by capillary electrophoresis mass spectrometry will be presented. The fragmentation spectra enabled the identification of each compound detected. The mobility of the CLPs in solution will then be compared to the measured collision cross section in the gas phase by ion mobility mass spectrometry.

Conclusions:
Capillary electrophoresis appears to be a reliable technique for cyclic lipopeptides separation and shows promising applications for new CLPs identification. Capillary electrophoresis and ion mobility mass spectrometry are complementary techniques for CLPs structural analysis.

Novel Aspect:
To our knowledge, this is the first report on cyclic lipopeptides separation by capillary zone electrophoresis method.

References:
Introduction:
Electron ionisation (EI) at 70 eV is standard practice for GC-MS, due to the high degree of ionisation (and thus good sensitivity) and consistent mass spectra generated.

Nevertheless, the identification of individual compounds in complex samples remains challenging when multiple compounds in a chemical class have similar spectra, or weak molecular ions at 70 eV EI. This problem can be addressed by the use of soft ionisation to reduce the degree of ion fragmentation, but this approach has been cumbersome to implement until now.

Methods:
The mass spectrometer used in this study incorporates a unique ion source design enabling both hard and soft electron ionisation spectra to be acquired simultaneously – in a technique deemed Tandem Ionisation. Multiplexing between ionisation energies allows conventional 70 eV spectra to be obtained - for routine identification against commercial libraries - as well as simplified soft EI spectra for enhanced confidence in identification of compounds which may exhibit weak molecular ions and/or similar spectra at 70 eV.

Results:
The soft EI spectra consist of only structurally-significant fragments for reduced demand on dynamic range and an increase in peak capacity. In addition, greater orthogonality between the mass spectra of isomeric compounds is evident, thus simplifying compound identification and reducing reliance on retention indices.

Conclusions
Fast multiplexing during acquisition greatly improves the performance of a TOFMS, enabling two complementary MS datasets to be acquired simultaneously.

Novel Aspect:
The use of a novel, patented ion source design to provide both hard and complementary soft electron ionisation in a single run.
EVALUATING THE CAPABILITY OF DIFFERENT NON-TARGETED MS-BASED APPROACHES FOR THE COMPREHENSIVE CHARACTERIZATION OF OLIVE TREE-DERIVED MATRICES

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Keywords: non-targeted approaches; food metabolomics; LC-MS; GC-MS; complementarity platforms

Introduction:
Virgin olive oil consumption has been associated to a lower incidence of some chronic diseases, largely due to its non-glyceridic minor compounds, which are also found in other vegetal tissues derived from the olive tree. Thus, the deep characterization of the main parts of the olive fruit and tree might be a key step when trying to find natural sources of bioactive compounds. To do it so, different MS-platforms were used (LC-ESI/APCI-MS and GC-APCI-MS).

Methods:
Picudocv. samples were prepared using an unselective liquid/solid-liquid extraction protocol. The extracts were analyzed by LC and GC coupled to a QTOF detector by means of ESI and APCI interfaces in the case of LC and an APCI source in GC. In LC, the analytes were eluted in a C18 column (2.1x 100 mm, 1.8 μm), with acidified water and acetonitrile at 40 ºC. The silylated extracts were also analyzed in GC (BR-5 column) with a T gradient from 150 to 320 ºC (ramped at 4 ºC/min).

Results:
The extracts (from lyophilized olive pulp, olive seed, fruit skin, leaves and wood from the branches, as well as virgin olive oil, olive oil obtained from pitted and dehydrated fruits and oil obtained from the seed contained inside the pit) were prepared and analyzed. Data acquired were processed with MetaboScape® 3.0 (Bruker), which automatically extracts and combines isotopes, adducts and fragments belonging to the same compound into one feature. Information obtained from MetaboScape was studied to point out the adequacy of each methodology for the determination of each family of compounds, as well as to describe the distribution of the identified plant metabolites within the selected olive oils and tissues (their presence, absence, relative area in each matrix and relative response in each platform were checked). The identification of more than 130 compounds from the extracts (phenolic compounds, triterpenic acids and dialcohols, tocopherols, sterols and free fatty acids) was achieved.

Conclusions:
- The potential of different non-targeted MS-based approaches to cover the metabolome of 8 Oleaeuropaeaderived matrices was assessed.
- The coupling to a high resolution MS through different ionization sources (ESI and APCI in LC and APCI in GC) allowed the detection of compounds from different chemical classes.
- The use of a great number of standards and the annotation strategies (MetaboScape) lead to the identification of more than 130 compounds in the extracts.

Novel Aspects:
Study of biosynthesis and distribution of minor bioactive compounds in olive oils and tissues applying innovative non-targeted LC-ESI/APCI-MS and GC-APCI-MS approaches.
Identification of isomeric organic molecules would become a big challenge for mass spectrometry with ambient in situ setup where no chromatography is available. We explore a viable generic strategy for differentiation of small isomeric metabolites in such situations; the potentials and limitations of widely available low-energy CID MS/MS and ion mobility analysis have been evaluated by using a set of isomeric C4 amino acids as a model.

Methods:
Analyte ions were generated by electrospray ionization on directly injected standard solutions. Quadrupole/time-of-flight (Q-TOF) hybrid and triple quadrupole tandem mass spectrometers were used for energy-resolved (ER) CID MS/MS experiments. A traveling wave ion mobility analyzer built in one of the Q-TOF systems was used for ion mobility analysis in N2. For the CID experiment, He, Ne, Ar, or N2 was used as the collision gas.

Results:
Six C4 amino acids, i.e. 2-aminobutyric acid (2-ABA), 3-aminobutyric acid, 4-aminobutyric acid, 2-aminoisobutyric acid (2-AIBA), 3-aminoisobutyric acid, and N,N-dimethylglycine (DMG) are one of the simplest examples of small isomers. Among them, 2-ABA, 2-AIBA, and DMG gave similar CID spectra that were attributable to common major fragmentation pathway, loss of 46 Da (CO and H2O) from protonated molecules (m/z 104) to form m/z 58. Obviously, the spectral patterns were condition dependent; however, the difference in energy requirement to give m/z 58 from each isomer could be clearly visualized in breakdown diagrams obtained by ER MS/MS experiments. On the other hand, ion mobility analysis was shown to have limited discrimination power on these isomers. The predicted collision cross section (CCS, calculated by IMoS software [1]) of 2-AIBA protonated molecule was roughly 2% larger than those of the other isomers whereas the predicted CCS of m/z 58 from 2-ABA was 3% larger than the CCS of m/z 58 from others.

Conclusions:
An ER MS/MS approach by using commonly available instruments was shown to be useful for differentiation of small isomeric organic molecules. As the lab-frame collision energy values depend on geometry of the CID device, collision gas species, and gas pressure, method for standardize collision energy axis should be established to facilitate platform independent differentiation and identification of isomers.

Novel Aspect:
Potentials and limitations of an ER-MS/MS-based integrated approach for differentiation and identification of small isomeric organic molecules have been evaluated.

References:
HIGHLY EFFICIENT ON-LINE PROTEIN DIGESTION AND PEPTIDE ANALYSIS: EXPLOITING NEW SHORT-BED TRYPsin-REACTORS

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Keywords: Trypsin; Immobilized enzyme reactor; Monoliths; Liquid chromatography-mass spectrometry; Proteomics

Introduction:
Trypsin is the gold standard enzyme used in shotgunproteomics for the conversion of proteins into MS-friendly peptides[1]. However, long incubation times and possible covalent modifications induced by digestion conditions are common drawbacks of classic in solution protocols. To achieve high digestion efficiency, automation and a significant decrease in analysis timewe consideredthe development of trypsin-based immobilized enzyme reactors (tryp-IMERs).

Methods:
Trypsin-based monolithic tryp-IMERs were obtained by covalent immobilization on short-bedCIMac™ columns(5 mm x 5.2 mm I.D.) exploiting different chemistries and immobilization protocols.IMERswere inserted in an integratedplatform featuring analytical columns for peptide analysis and an ESI-Q-TOF mass spectrometer.Optimized procedure was validated in terms of intra- and interday and IMER to IMER reproducibility,ruggedness, stability and sensitivity.

Results:
The IMER-based analytical platform allowed the on-linedigestion and identification of protein substrates, the separation of peptides and acquisition of MS/MS spectra in an automated manner.In the optimized working conditions no memory effects, nor significant nonspecific interactions were detected. Very short digestion time(90 s), good intra–day and inter-day analysis reproducibility (%RSD in terms of %SQ=≤ 1.2) and good sensitivitydown to picomol level were achieved. The high digestion efficiency was confirmed by the analysis of high-molecular weight (HMW) proteins such as a pool of human immunoglobulins G and the HMW fraction of plasma proteins, as challenging real sample.Indeed, efficient protein digestion could be achieved albeitsample complexity, allowing the identification of 281 plasma proteins. Furthermore, the excellent sequence coverages achieved with best performing tryp-IMERs were generally higher than those obtained by in solution digestion (overnight) or with the commercially available tryp-IMER Poroszyme™.

Conclusions:
CIMac columns were suitable supports for the development of highly efficient IMERs.Tryp-IMER performance in terms of digestion of single proteins, protein mixtures and real plasma samples were comparable with that of some capillary and nano-IMERs, without requiring hyphenation to micro- or nano-analytical instrumentation. Automation and high digestion efficiency make the short-bed tryp-IMER appealing new tools for shotgun proteomics and protein analysis.

Novel Aspect
Short-bed CIMac columns were for the first time exploited for the preparation of tryp-IMERs which can be inserted in classic LC-MS systems enhancing automation and throughput.

A COMPARISON OF THE RESULTS OBTAINED FOR THE ANALYSIS OF SOUTH AFRICAN PINOTAGE WINES USING GCxGC-TOFMS WITH HYDROGEN AND HELIUM AS CARRIER GASES

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Keywords: Pinotage wine, carrier gas comparison, GCxGC-TOFMS, principal component analysis, taste profile

Introduction
The relatively low price and availability of helium have made it the default carrier gas for the majority of GC and GC-MS users over the last 40 years. Since the helium shortage of 2012 a number of GC methods have been revalidated for use with hydrogen. This has been driven not only by availability, but also by price, as the cost of helium has doubled in the last 24 months. We compare the analysis of pinotage, a uniquely South African wine, using hydrogen and helium as carrier gas.

Methods
We have analysed seven samples of South African pinotage wines using comprehensive gas chromatography – time of flight mass spectrometry (GCxGC-TOFMS) and both hydrogen and helium as carrier gases. The results were then subjected to principal component analysis (PCA) to investigate the comparability of the components identified using the different carrier gases.

Results
Within the carrier gas groups (i.e. helium or hydrogen) results were comparable, but some differences were observed between carrier gas groups. These differences relate primarily to detectability and signal to noise variations for many components, and an attempt has been made to clarify and explain the discrepancies. There are no obvious differences in the mass spectra obtained for the same compounds detected with different carriers, and the mass spectra using both carriers give good quality library matches with spectra contained in commercial mass spectral libraries. By and large the same compounds were found in the same samples analysed with different carriers. In addition the wines were compared by a tasting panel, and an attempt has been made to correlate the wines scoring most highly with their chemical composition and price.

Conclusions
It is likely that helium will be required for GC applications for a long time to come, but as its price continues to rise and it becomes harder to source, laboratories will face continued pressure to pursue alternatives. Hydrogen is the most suitable alternative to helium for many analyses and it is likely to be adopted by GC labs because of its cost effectiveness, availability, and the existence of programmes permitting easy adaption of existing helium based Methods:

Novel Aspect
This is the first time that the compounds providing the organoleptic properties of pinotage wine have been compared using different carrier gases, and GCxGC-TOFMS. The study provides information on analysis of wine using hydrogen as carrier gas, and also highlights several caveats when using this approach.
564 - ANALYSIS OF AN ANTIPARASITIC FRACTION AGAINST GASTROINTESTINAL NEMATOSES OF SHEEP FROM LEAVES OF MAYTENUS ILCIFOLIA BY LC-ESI-IT-MSN.

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Keywords: Maytenus ilicifolia Leaves extract, antiparasitic fraction, gastrointestinal nematodes of sheep, LC-ESI-IT-MSn.

Introduction:
Maytenus ilicifolia is an important folk medicinal plant, largely found in Paraguay, Uruguay, Argentina, and Brazil. Contributions to human health have been reported [1-3]. A purified fraction from the ethanol extract of Maytenus ilicifolia leaves exhibits antiparasitic activity against gastrointestinal nematodes of sheep. The aim of the present study was to perform a preliminary structural characterization of this bioactive fraction by LC-ESI-IT-MSn.

Methods:
The ethanolic extract of M. ilicifolia was subject to a bioguided fractionation on a VLC column and subsequent separation on C18 SPE. The antiparasitic activity was determined against parasites in naturally infected sheep in Uruguay, through egg hatching and inhibition of larval migration in vitro assays. The bioactive fraction was investigated by liquid chromatography/electrospray ionization ion trap multiple-stage mass spectrometry in negative ion mode.

Results:
The bioactive fraction was adequately separated by chromatography on a C18 reverse phase UHPLC column using a mobile phase gradient of acetonitrile in water at acidic conditions. The mass spectrometer was set to monitor, select and fragment the most abundant ions [M-H]− in the range m/z 200–1500. According to their [M-H]- ions, characteristic product ions and retention times, several constituents were identified. Catechin and epicatechin were found. Flavonoid glycosides ranging from 1 to 4 monosaccharide units, having mainly quercetin and kaempferol as aglycones moities were identified. Tannins were also found, the main ones being composed of catechin/epicatechin and afzelechin/epiafzelechin. The results of the egg hatching and the larval development assays will be presented at the Conference.

Conclusions
The LC-ESI-IT-MSn is a method quick and easy that can be extended or transferred to other purified bioactive fractions from other plant extracts, allowing a preliminary characterization of its main compounds. In this way the structure of the compounds could be related to the biological activity.

Novel Aspect:
This work shows that a purified fraction of ethanolic extract of Maytenus ilicifolia rich in flavonoids and condensed tannins present a good antiparasitic activity against gastrointestinal nematode of sheep.

References
563 - FIRST REPORT ABOUT THE STRUCTURE OF AN IMMUNOADJUVANT SAPONIN FRACTION FROM QUILLAJA BRASILIENSIS LEAVES BY LC-ESI-IT-MSN

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Keywords: Triterpenic saponins, Quillaja Leaves extract, Immunoadjuvant saponin fraction, ESI-MSn, UHPLC.

Introduction:
Quillaja brasiliensis is an endemic tree species in Brazil and Uruguay. It has been shown that the aqueous extract and some of the purified saponin fractions obtained from its leaves have immunoadjuvant activity comparable with Quil-A®, the main commercial adjuvant product based on Q. saponaria saponins [1, 2]. The aim of this study was to perform a preliminary structural characterization of the immunoadjuvant fraction of saponins designated as Fraction B (FB).

Methods:
The aqueous extract of Q. brasiliensis was subjected to a separation on an SPE column to remove non-saponin components and the fractions containing saponins were reunited. This fraction (FB) was investigated by direct infusion and liquid chromatography/electrospray ionization ion trap multiple-stage mass spectrometry in negative ion mode (DI-ESI-IT-MSn and LC-ESI-IT-MSn). The characterization was performed based on MS2 and MS3 experiments of the [M-H]− ions.

Results:
The saponins contained in FB were adequately separated by chromatography on a C18 reverse phase UHPLC column using a mobile phase gradient of acetonitrile in water at acidic conditions. The mass spectrometer was set to monitor, select and fragment the most abundant ions [M-H]− in the range m/z 1300–2000, using different collision energies. One of them allowed the production of the precursor ion [M-H]− in good yield, while the second energy was set to a higher value in order to produce more product ions that provided more structural information. According to their [M-H]- ions, characteristic product ions and retention times, twenty seven saponins, were tentatively identified [3]. These saponins feature four different triterpenic aglycones, namely quillaic acid, gypsogenin, phytolaccinic acid and its O-23 acetate. All the saponins are bidemosidic, bearing oligosaccharides chains linked to the aglycone at C-3 and C-28, the last one containing or not a branched dicarboxylic acid linked or not to a terminal Ara residue.

Conclusions
This is the first detailed report about the structure of Q. brasiliensis saponins. Further work will be necessary to fully characterize all the immune stimulating saponins present in the Q. brasiliensis species.

Novel Aspect:
Q. brasiliensis is an alternative source of immunoadjuvant saponins. The production of Fraction B could scale and become a commercial product comparable with Quil-A®.

References

Introduction:
Identification of the chemical structure of impurities and characterization of their biological activities in the early stage of product development as well as in final product is very important in industrial biotechnology. This is a crucial aspect of “process understanding” and “product specification”. Furthermore, regulatory authorities (ICH, FDA, EMA) have established clear guidelines demanding close monitoring, in-depth investigation and unambiguous confirmation of the chemical structure of impurities in drug products.

Methods:
Various approaches can be considered to identify compounds but most of them require relatively pure compounds. While MS has a very high sensitivity, NMR identification in the past required more material. Nowadays NMR instruments have higher sensitivity and approximately 50 μg of pure compound is sufficient for the identification of unknowns, meaning that the composition (identity and relative quantities of elements) and constitution (nature and sequence of bonds), can be determined. Such small amounts can be obtained easily from a semi preparative HPLC platform using 10 to 20 mm i.d. columns.

Results:
This study presents a workflow to effectively isolate impurities by using semi preparative HPLC followed by high resolution mass spectrometry and high field NMR analyses for structure elucidation. Furthermore, the workflow can be applied as untargeted approach to characterize complex matrices where the whole chromatogram is fractionated and each fraction separately tested on e.g. biological activity and further characterized by HR-MS and NMR. The workflow includes checks on purity and stability of collected fractions and concentration and desalting steps before HR-MS and NMR analysis.

Conclusions
A practical workflow has been developed for the identification of the chemical structure of impurities. The workflow is based on isolation of impurities using a semi preparative HPLC and followed by high resolution mass spectrometry and high field NMR analyses for structure elucidation. The workflow has been applied successfully for structure elucidation of a number of unknown impurities in various biotech products. Furthermore, it has been also demonstrated that this workflow can be used to characterize samples consisting of complex mixtures.

Novel Aspect:
Fast and robust protocol for semi-prep chromatography, HR-MS and NMR workflow for targeted and untargeted fractionation in combination with intermediate checks on purity of collected fractions.
Introduction: Since first introduction in 1990, Solid phase microextraction techniques have continually evolved in terms of architecture in order to further push down detection limits while maintaining a clean extraction, portable and easy to handle design. With this portability in mind, the SPME fiber has been appropriately coupled with emerging miniaturized MS technologies for on-site analytical applications. In this way various complex, including living systems can be monitored. The most challenging are biomedical applications. Patient monitoring, metabolomics and biomarkers discovery are an integral part of medical diagnosis and biomedical research. However, tissue analysis remains the bottleneck of such studies due to the invasiveness of presently used sample collection based on biopsy, as well as the laborious and time-consuming sample preparation protocols. In the proposed technology, we have integrated in vivo sampling, sample preparation including global extraction of metabolites together with convenient coupling to analytical instrument resulting in medical biopsy tool.

Methods: This approach based on a small sorbent coated flexible metal fiber format of solid phase microextraction was developed and evaluated during organ treatment and transplantation in rodent or pig model as well as applied to human organs. Other formats will be discussed as well. In this approach, only molecules of interests are extracted onto biocompatible sorbent from the investigated system, however no tissue is not removed from the organ. By using extraction phase with appropriate chemical property and morphology, the analytes are enriched onto the sorbent with minimum of interferences collected. Therefore, the direct coupling of the chemical biopsy probe to mass spectrometry is feasible. Such hyphenation facilitates rapid quantification of the compounds of interests resulting in possibility of close to real time monitoring. This approach has potential in biological and clinical on-site investigations including bedside medical diagnosis.

Results: In the presentation we will discuss diverse SPME-based devices recently developed in our laboratory for the extraction/enrichment of analytes of interest from tissue and small volumes of complex sample matrices, which can be directly coupled with mass spectrometry instruments for rapid analysis. In these applications we use SPME devices containing coatings with matrix compatible morphologies. The approaches tested include Coated Blade Spray (CBS), SPME-transmission mode-direct-analysis-in-real-time (SPME-TM-DART-MS), SPME- microfluidic open interface (SPME-MOI). Total analysis time was typically 5 minutes, but did not exceed 15 minutes and sample volumes ranging between 1-100 µL to the whole organs were used. Sampling/sample-preparation is performed either by spotting the sample onto the SPME-device, or by immersing the SPME-device in a vessel containing the sample or placed directly into tissue. Despite short extraction times, limits of detection in the sub-ng/mL range were obtained, while good accuracy, and linearity were attained for all the studied probes (e.g. therapeutic-drugs, drugs of abuse, and immunosuppressants) in the diverse matrices scrutinized (e.g. urine, plasma, blood, saliva, brain, muscle and other tissue). In the presentation we will describe exemplary cases in which the mere coupling of SPME to MS is not sufficient to answer relevant analytical questions and the use of strategies that allow for removal of co-extracted interferences or source artifacts, such Ion Mobility and Multiple Reaction Monitoring with Multistage Fragmentation (MRM3), will also be discussed.

Conclusions: Matrix compatible coatings provide clean extraction suitable for direct coupling to mass spectrometers.

Novel Aspect: SPME biopsy probe hyphenated to MS facilitates on site monitoring even of complex and living systems.

References

Introduction: Quercetin is one of the most discussed flavonoid compounds with beneficial effects to human health due to its antioxidant properties. In human body, it is metabolized to two groups of health affecting metabolites with different physico-chemical properties including acidity, molecular weight, and log P. Therefore, it is difficult to determine these compounds in a single analytical run [1][2].

Methods: Three chromatography methods with different selectivity using mass spectrometry (MS) detection in both electrospray positive and negative ion modes were chosen and optimized. These methods included reversed phase ultra-high performance liquid chromatography (RP-UHPLC), hydrophilic interaction chromatography (HILIC), and complementary supercritical fluid chromatography (UHPSFC).

Results: We carried out columns screening with emphasis on separation of isomeric pair of tamarixetin and isorhamnetin. Shield RP18 column using methanol and 0.1% formic acid as the mobile phase enabled the baseline separation of all analytes in RP-UHPLC. Pentahydroxyl, amide, silica, and zwitterion columns with acetonitrile and different aqueous solutions in wide pH range were used for separation in HILIC mode. However, the separation of flavonoids was less successful due to the peak broadening. Ten different columns were screened for SFC. The most promising results were obtained using the diol column and methanol with the addition of organic acids, buffers, ammonium hydroxide, and water. Finally, the MS parameters were tuned to get sensitive detection.

Conclusions: The optimized methods will be compared in the terms of selectivity, retention and sensitivity. The targeted analysis of quercetin and related compounds including small phenolic acids and large metabolites with preserved flavonoid structure in combination with high throughput sample preparation step will be used to explain their metabolism and the health effects.

Novel Aspect: The simultaneous analysis of quercetin and its metabolites with different physico-chemical properties using various separation mechanisms with MS.

References:

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MULTIDIMENSIONAL GAS CHROMATOGRAPHY COUPLED TO ISOTOPE RATIO MASS SPECTROMETRY IN SPORTS DRUG TESTING

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Keywords: testosterone misuse, carbon isotope ratios, Deans switch, long term detectability

Introduction:
Testosterone (T) is frequently misused in sports and its administration is forbidden under the rules of the World Anti-Doping Agency [1]. Its detection remains challenging as T is an endogenous substance. Only by carbon isotope ratio determinations it is possible to distinguish endogenous from exogenous T. Sample preparation is commonly based on twofold high performance liquid chromatography clean-up, which is laborious and requires up to 2 working days [2].

Methods:
Multidimensional gas chromatography (MDGC) is suitable for the separation of complex matrices. Therefore, one GC equipped with a column of lower polarity is used for sample clean-up and hyphenated to a second GC that employs a more polar column for analyte separation by a pressure-controlled Deans switch device. The effluent from the second GC is partitioned between an isotope ratio and a single quadrupole mass spectrometer to enable simultaneous determinations of isotope ratios and peak purity.

Results:
The developed method was fully validated following the guidelines of the World Anti-Doping Agency [3] and employing linear mixing models and reference population derived thresholds [2]. The method demonstrated comparable results to the current screening procedure and sample preparation was shortened to 1 day. Additionally, it was possible to implement measurements of a recently detected new long-term metabolite of testosterone administrations. This epiandrosterone prolongs the detection time by a factor of 2 to 3 [4]. Only for testosterone itself a bias between the screening and the new method was found, which can be partly compensated by population-derived threshold levels.

Conclusions:
The developed method employing multi-dimensional gas chromatography instead of high performance liquid chromatography was found to be fit-for-purpose and has been applied to sports drug testing complementing the current routine procedure. Epiandrosterone was found to be a suitable marker for steroid administration and was successfully implemented into doping controls.

Novel Aspect:
Multi-dimensional gas chromatography hyphenated to isotope ratio mass spectrometry was successfully employed for doping control analysis substantially reducing the workload for sample preparation.

References:
Introduction: Mass Spectrometry detection on-site
Fast and precise in situ gas sensing and identification is of utmost importance in the case of an industrial chemical accident, of atmospheric pollution mapping, for homeland security... The systems used for such kind of purpose need to be of small overall dimensions and weight in addition of their top level performance. Towards this goal, a few research groups [1,2] have developed miniaturized mass spectrometer systems based either on standard or on microfabricated techniques.

Methods: Micro Mass spectrometer on chip
This microsystem is fabricated using Micro Electro Mechanical Systems (MEMS) technology. This technic is more promising for size reduction, but also more challenging than standard manufacturing techniques. The MEMS developed in this work have a size of only 1 cm × 2 cm for a micro-mass spectrometer based on Time-of-Flight (TOF) separation. The micro-TOF ion source is studied and optimized subsystem by subsystem [3,4].

Results: Mass analysis by a linear Micro-TOF
Using electron impact ionization (EI 70 eV), ions are generated in an ionization chamber, then extracted and focalized by electrostatic lenses constituted by 6 electrodes. Ion beam is collected in linear mode in a Micro Channel Plate (MCP) detector and mass spectra are recorded. Using ultrafast pulsing of these ions, the micro ion source is used as a linear TOF spectrometer that is able to separate simple gas mixtures efficiently. Ionization of neutrals atoms is shown to be efficient by electron impact. Using comparison with ion trajectories simulations, the extraction and focalization are optimized to be able to extract the highest ionic current from the ionization chamber. Pure electrostatic simulation can be used as guideline and limits of the simulation values are discussed. The electrode voltage setting of ionization and focusing areas were studied and influences on signal intensity and on mass resolution are followed. Mixes of noble gas, alkane or BTEX were tested in linear micro-TOF.

Conclusions
Miniaturization on chip of mass spectrometer Time-of-Flight is operational and provides repeatable and reproducing results. These devices allow studies of extraction and ion focusing on chip. Electrostatic simulation could be used as global guideline and is a tool for the understanding of ion beam behaviour. Resolution will be improved using orthogonal injection.

Novel Aspect
Gas analyses by linear micro-TOF are available and this work shows encouraging results and pulls the μ-TOF one step closer towards a fully integrated portable analytical system.

References

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Online high pressure proteolytic biosensor- mass spectrometry (PROTEX-SPRMS): A new powerful tool for protein epitope and affinity identification and antibody characterization

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Keywords
Online-Biosensor-MS combination; T-SPR biosensor; continuous flow microfluidics; antibody epitope identification; epitope peptide affinity determination.

Introduction
Analysis of biomolecular interaction epitopes is a key step in the development of therapeutic antibodies, biomedical peptide and protein biomarkers and molecular vaccines. Bioaffinity analysis using biosensors has become an established technique for detection and quantification of biomolecular interactions. However, a principal limitation of biosensors is their lack of providing chemical structure information of affinity-bound ligands. Proteolytic excision/extraction (PROTEX-MS) is a major techniques for mass spectrometry based elucidation of protein-ligand interactions, but affinity-MS analysis does not provide quantitative affinity data. A new online proteolytic excision SPRMS combination (PROTEX-MS) enables simultaneous structure identification and affinity quantification of antibody epitopes from a protein-ligand complex immobilized on a gold chip.

Methods
Key tools of the online proteolytic excision SPRMS Epitope Analyzer are (i), a new SPR biosensor that enables the application of microfluidic systems suitable for direct ESI-MS that is not sensitive to buffer change normally required for SPR (T-SPR); (ii), an integrated, automated interface that provides elution and sample concentration for direct MS analysis of the eluted epitope peptides [1]; and (iii), a new high pressure proteolytic system, high pressure barocycler digestion. The high pressure digestion enables substantially enhanced speed, efficiency and automation of protease digestion and proteolytic affinity-extraction, compared to conventional digestion.
Results
Initial validation of the new PROTEX-To validate SPR-MS system, analysis on a well-defined affinity pair has been studied. Results from the tests on anti-Beta Amyloid antibody interacting with Abeta 1-40 and 1-16 protein provided us with a KD values of 0.628 μM and 0.796 μM and with the protein/peptide structural information. The broad application potential of the PROTEX-SPR-MS Epitope Analyzer has been ascertained by identification of protein epitope structures from therapeutic and pathophysiological antibodies, such as an unusual mixed-disulfide antibody epitope of the rheumatoic target protein, HLA-B27; and the interaction site identification of chaperone complexes of lysosomal enzymes [2, 3]. Interaction epitopes as diverse as antigen-antibody and lectin-carbohydrate complexes [4, 5], with affinity binding constants (KD) from milli- to nanomolar ranges can be directly analyzed. Applications amenable with the PROTEX-SPR-MS Epitope Analyzer include affinity-based biomarker identification; identification of protein and peptide epitopes; precise antibody affinity determinations; direct label-free antigen quantification; and clinical epitope analyses of pathophysiological antibodies, suitable for clinical apheresis and hyposensitization of patients after immunogenic protein therapy [4-6].

Epitopes from antisera of Fabry disease (FD; α-Galactosidase deficiency) patients were identified using the PROTEX-SPR-MS combination. Tryptic peptide mixtures were loaded onto the SPR-MS; after washing out nonbinding peptides, epitope peptides were eluted with 0.01% TFA into the affinity interface, ESIMS was performed with a Waters QuattroUltima MS. The epitope peptide, α-Gal(309-332) was identified from 2 FD patients and revealed high affinity (KD, 39 nM) comparable to the full length enzyme (KD, 16 nM). Linear, stabilized epitope peptides were prepared by SPPS.

Conclusions
A number of applications are amenable with the new online PROTEX-SPR-MS epitope analyzer, including affinity-based biomarker identification, identification of protein and peptide epitopes, precise antibody affinity determinations, and direct label-free antigen quantification. In addition, the clinical application potential was shown in epitope elucidation from therapeutic and pathophysiological antibodies of lysosomal enzymes, with affinity constants (KD) amenable from milli- to nanomolar ranges.

Novel Aspect
A new powerful tool for epitope and affinity analysis of biomolecular interactions was developed. The PROTEX-SPRMS Epitope Analyzer provides a breakthrough (i) for proteolytic digestion of proteins; and (ii), direct affinity determination in combination with MS.

710 - ATMOSPHERIC PRESSURE CHEMICAL IONIZATION – CONSTRUCTING IONIZATION EFFICIENCY SCALE AND DERIVATIZATION REAGENT SUITABILITY.

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Keywords: ionization efficiency; atmospheric pressure chemical ionization (APCI); derivatization reagents.

Introduction:
Recent evidence has shown that the atmospheric pressure chemical ionization (APCI) mechanism can be more complex than generally assumed. In order to better understand the factors affecting the APCI, an ionization efficiency scale for an APCI source has been created for the first time. Moreover, based on the scale, five different derivatization reagents for amino acid analysis were tested for APCI suitability (eluents, sensitivity, matrix effects etc).

Methods:
The MS responses were recorded in a flow injection mode as previously done by Kruve et al. [1] For every compound, the response versus concentration slope was measured and the ionization efficiencies were expressed in relative terms, using pyrene as the reference compound. Comparison of derivatization reagents was carried out with optimized LC-APCI-MS methods on a reversed phase column.[2]

Results:
For the first time an ionization efficiency scale for 40 compounds has been created for APCI source. All logIE values are presented relative to pyrene, chosen as the reference compound with the assigned logIE value 3.41, and range from −0.62 up to 4.32, i.e., spanning almost 5 orders of magnitude. [3]
Suitability of five derivatization reagents for amino acids analysis (diethyl ethoxymethylenemalonate (DEEMM), benzyl ethoxymethylene malonate (DBEMM), dansyl chloride (DNS), 9-fluorenylmethyl chloroformate (FMOC-Cl) and 2,5-dioxopyrrolidin-1-yl N-tri(pyrrolidino)phosphoranylideneamino carbamate (FOSF)) were tested for APCI. Acetonitrile proved to be more suitable for the analysis and sample dilution approach in green tea showed that APCI is, in general, less prone to matrix effect than electrospray ionization. FMOC-Cl had poor sensitivity and also high matrix effects (ME). DEEMM and DNS-derivatives were less influenced by the ME than DBEMM and FMOC-derivatives. [2]

Conclusions:
The results show that the best ionized compounds in the APCI are not small volatile molecules. Overall the ionization efficiency vs molecular structure trends in APCI are very similar to ESI. Results imply that in APCI several ionization mechanisms operate in parallel and mechanism(s) not relying on evaporation of neutral molecules from droplets has significantly higher influence than commonly assumed. Moreover, derivatization reagents for APCI were DEEMM and DNS.

Novel Aspect:
For the first time an ionization efficiency scale spanning over 5 logIE units and including 40 diverse compounds has been created for APCI source.

References
Introduction: After birth several assays are typically performed to determine if an infant suffers from conditions that could have long-term health effects. The compounds measured range from small molecules to larger proteins. Few techniques are capable of effectively analyzing both small and large molecules rapidly and simply. In this work we present a microfluidic CE-MS system for analyzing both small molecules and intact proteins related to newborn screening assays.

Methods: Microfluidic chips with a 10 cm separation channel were used for small molecule assays and chips with a 22 cm separation channel were used to analyze protein biomarkers (908 Devices Inc.). Samples for small molecule assays a liquid extraction was performed to elute the compounds from dried blood spots. Protein biomarkers were measured from whole blood or isolated erythrocytes. MS analysis was performed using a Thermo Orbitrap MS.

Results: Results will be presented demonstrating microfluidic CE-MS analysis of several common biomarkers regularly monitored during new born screening. Using the same method, amino acids and acyl carnitines can be monitored to assess whether samples are indicative of Phenylketonuria (PKU) or fatty acid metabolic disorders, such as Carnitine acylcarnitine translocase deficiency (CACT). The small molecule biomarkers are able to be quantified over the appropriate physiological ranges indicative of disease states. Hemoglobin subunits from whole blood or purified erythrocyte lysates were analyzed to detect hemoglobin variants indicative of hemoglobinopathies, such as Sickle Cell Anemia. The alpha, beta, gamma, and abnormal subunits were resolved in the microfluidic CE separation within 4 minutes and the identity of the subunits was confirmed by mass. In the samples analyzed, beta chain variants indicative of sickle-cell disease were characterized.

Conclusions The microfluidic CE-MS platform can be used to perform rapid small molecule and protein assays related to new born screening. Additionally, multiple small molecules can be assessed with a single method providing the ability to screen for different disease states in a single run. By analyzing hemoglobin at the subunit level, minor modifications can be identified and detected by both their mass and anticipated change in migration times due to modification.

Novel Aspect: In this work a single system is used to demonstrate new born screening assays for small molecule and protein biomarkers.
413 - CHEMICAL PROFILING OF THE HUMAN SKIN SURFACE FOR MALARIA VECTOR CONTROL USING A NON-INVASIVE SOLVENT MODIFIED SORPTIVE SAMPLER WITH GC×GC-TOFMS AND UPLC-IMS-HRMS

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Keywords: GC×GC-MS, UPLC-IMS-HRMS, human skin compounds, sorptive sampling, malaria vector control

Introduction:
Odour mosquito lures are currently being used as part of integrated vector control strategies in the fight against malaria. Variation in inter-human attractiveness to mosquitoes, as well as the preference of mosquitoes to bite certain regions on the human host are possible avenues for identifying lead compounds as potential attractants[1-4]. These may prove more effective in lure and kill strategies.

Methods:
Two different areas of the human skin, namely wrist and ankles, were compared following a 1 hour sampling period. Skin surface compounds were concentrated using a solvent modified polydimethylsiloxane (PDMS) sampler. Sampling was followed by analyses with GC×GC-TOFMS and UPLC-IMS-HRMS. The sampler was thermally desorbed directly in the inlet liner of a GC system, while for LC analyses the PDMS sampler was solvent back-extracted prior to analyses.

Results:
The skin profiles for the ankle and wrist groups were compared using principal component analyses. Compounds responsible for the differences in the chemical profiles were tentatively identified based on a comparison of sample mass spectra to that of the NIST14 library for the GC analyses. Accurate mass, isotope fit values and fragmentation patterns were compared to online ChemSpider databases for the LC analyses. Binary compare using an OPLS-DA Model highlighted the differences between the ankle and wrists groups for the LC-MS data sets. Viburtinal and Menaquinol, as well as Alloxycholic acid and N,N-Diethylbenzeneacetamide, were tentatively identified using the Human Metabolome Database. These form plausible lead markers for the wrist and ankle groups, respectively. A comprehensive list of collision cross section data of human skin chemicals was compiled for LC amenable markers.

Conclusions:
In this study, methods were developed to chemically profile the human skin surface with the aim of determining the compounds responsible for attracting the mosquito vector to its human host. A PDMS sorptive sampler was modified with solvent to give improved extraction of polar analytes from the skin surface. This multi-approach gave a comprehensive human skin chemical profile consisting of volatile, semi-volatile, and non-volatile compounds.

Novel Aspect:
Skin surface compounds were concentrated using a solvent modified, non-invasive, in-house developed PDMS sampler for GC-MS and LC-MS. CCS library for skin surface compounds.

References
B.01.1 INSTRUMENTATION AND METHODS - HYPERENATED TECHNIQUES

1311 - DEVELOPMENT OF RAPID SCREENING APPARATUS WITH DIP AND PI/MS FOR ADDITIVES IN POLYMER MATERIALS.

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Keywords: direct inlet probe, fragmentless ionization, screening, silent change, additive,

Introduction:
The globalization of the economy has made risks of quality control due to the “Silent Change” apparent in manufacturers, particularly in the electrical and electronic industries. The Silent Change refers to material specifications being modified without approval in the supply chain, and lead to serious accidents that cause product recall and the loss of the brand image.

For its quality control, rapid screening technique is required with all detection of additive species because of many specimens of lots, and with simple operation of apparatus.

Methods:
For all detection of additives, it is better to use DIP, direct inlet probe that extract additives thermally in vacuum. However low boiling point chemicals are vaporized and disappeared at measurement, so that technique to cover the specimen is used, named SET, Suppressing Evaporation Technique. Separation technique as GC and LC takes time, so that it is required a fragmentless ionization that realizes informational separation in a mass spectrum because one peak indicates one species.

Results:
JMS-Q1500GC by JEOL was accepted as a base instrument for the developed new apparatus. This instrument has both options of DIP and photoionization that is one of the fragmentless ionization techniques. However these options are exclusive. To be consistent with both, an alteration had been required. Further new design of DIP had been required. We manufactured a new design vacuum chamber and DIP by way of trial. At this stage of development, performance of the trial apparatus had been checked with some specimens. For example, good calibration curve has been obtained from VitaminE within 10 min., but not from Decabromodiphenyl because of the design of instrument.

In this design, DIP was inserted to an underside of the chamber, so that the operability was not good. The performance of coupling of DIP and Photoionization had already ascertained, consequently we moved to the next step to solve the operability problem. Newer designs of DIP and vacuum chamber were required for that DIP is possible to insert to a front of the chamber. And the new design has resolved the detection problem of Decabromodiphenyl.

Conclusions
The new apparatus with both of DIP and photoionization consistently has been successfully developed. The measurement is without property dependence from chemicals, e.g., a difference of boiling point, as all detection.

Novel Aspect:
The developed apparatus offers high level quality control to avoid the silent change with rapidity and easy operation. It can be a deterrent to the silent change.
Keywords: on-line-two-dimensional-liquid-chromatography-tandem-mass-spectrometry (2D-LC-MSMS); displacement chromatography; proteomics;

Introduction:
The extreme complexity of proteomes is a major challenge for LC-MSMS-based proteomics. Two-dimensional-(2D)-LC applying cation-exchange chromatography (CEX) in the first dimension has been successfully used for increasing the number of identified proteins. In this study it was investigated if CEX performed in the displacement mode (DM) within an online-2D-LC-MSMS system offers advantages in comparison to the gradient mode (GM) for the analysis of proteomes.

Methods:
Tryptic peptides of Hela cells (5 µg I total) were applied to an online-2D-LC-MSMS system (1st dimension: CEX column; 2nd dimension reversed-phase column). Tryptic peptides were eluted from the CEX either by pulsed injections of increasing amounts of ammonium acetate (GM) or by pulsed injections of spermine (DM). Resulting MS data were subjected to OpenMS, MaxQuant and mathematica scripts.

Results:
The total number of identified peptides and proteins as well as the sequence coverage was significantly higher by using DM compared to GM. A major reason for this phenomenon is, that DM was giving a significantly better separation via CEX of doubly charged tryptic peptides (charge state in the mobile phase of the LC), which represent the majority of tryptic peptides in mammalian proteomes. The results are in good agreement with former studies about the application of the displacement mode for the separation of complex mixtures in combination with mass spectrometry [1, 2]. However, in comparison to the former study [2] in the current study a big step forward was achieved regarding the operating time (reduction from 6 days to approximately 12 hours) of the 2D-LC-MSMS analysis. In addition the total sample amount was reduced down to 5 µg.

Conclusions
In comparison to gradient mode the displacement mode for cation-exchange chromatography within an online system comprising two-dimensional liquid-chromatography coupled to tandem mass spectrometry for identifying proteomes via tryptic peptides is significantly more efficient.

References

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Low-flow atmospheric pressure chemical ionization based on a gas dynamic virtual nozzle nebulizer

Keywords: low-flow APCI, GDVN,

Introduction:
Atmospheric pressure chemical ionization (APCI) is a useful tool for analyzing small and relatively stable molecules, e.g., lipids [1], at milliliter-per-minute sample flow rates. Despite the previous effort to develop low-flow APCI [2,3], no such an ion source has been commercialized yet. We are currently developing a nebulizer for an APCI source operable at sub-microliters per minute flow rates.

Methods:
The sprayer was manufactured from a borosilicate glass tube (I.D. 1.15 mm) and two fused-silica capillaries, which were fixed inside the tube: (i) tapered-tip capillary for the sample and (ii) capillary for gas delivery. The device generated liquid jet thanks to gas-dynamic forces exerted by a co-flowing nitrogen (gas dynamic virtual nozzle, GDVN [3]). It served as a nebulizer for low-flow APCI.

Results:
The spray created by the GDVN nebulizer operated at 120 ml/min nebulizing gas flow rate and 200 nl/min liquid sample flow rate had a conical shape with the diameter of 1.5 mm at the distance of 2.4 cm. To facilitate fast solvent evaporation, a short, resistively heated glass tube was placed between the sprayer orifice and corona discharge region. Test compounds including acridine in methanol and acetonitrile, cholesterol in toluene, cis-11-hexadecenol in acetonitrile (all in positive mode), and 5-nitrosalicylic acid in methanol (in negative mode) were successfully detected. Their spectra were similar to those recorded from the conventional APCI. The signal stability was rather moderate (RSDs of acridine signal recorded for ten minutes were about 30 – 40 %). High signal intensities of the analytes suggested the enhanced sensitivity of the GDVN APCI source.

Conclusions:
An APCI source with a GDVN nebulizer was found appropriate for nanoliter per minute sample flow rates. Various compounds were detected using a liquid flow rates down to 200 nl/min. No clogging of the sprayer was observed throughout the measurements, which, together with its high sensitivity, makes it a good candidate for a nano-HPLC and mass spectrometry hyphenation.

Novel Aspect:
For the first time, GDVN sprayer was successfully used as a nebulizer for liquid samples in an ion source.

References
Analysis of Enzymes in laundry detergent using SDS-PAGE and MALDI-TOF-MS
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Keywords: Enzyme, laundry detergent, SDS-PAGE, MALDI-TOF

Introduction:
There is a major challenge for the development of method in order to identify the enzymes in raw and manufactured materials in cosmetic industries[1, 2]. In this study we used SDS-PAGE and MALDI-TOF-MS instrument for identification of main types of enzymes used in laundry detergents: protease, lipase, cellulose, amylase, and mannanase.

Methods:
A certain weight of granular solid concentrates, was dissolved inTris-base, and the suspension was stirred for 30 min. After centrifugation, equal volume of TCA and supernatant were mixed, and enzyme precipitation was accomplished with TCA protocol. These mixtures were separated according to their molecular weights on 12% Bis–Tris gels and visualisation was performed using Coomassie brilliant blue. After cutting of gel and remove of Coomassie reagent, the mass values has obtained by using MALDI-TOF-MS.

Results:
Samples were load on the gel, and the separation was done based on the peptides' molecular weight. The main protein bonds were detected at approximately 26, 72& 40, 32, 55, 55, and 35 kDa for Everlase, Carezyme, Mannaway, Termamyl, Duramyle, and Lipolase respectively. In order to obtain precise mass values, the same solutions were analyzed by MALDI-TOF-MS. Finally, the precise mass values were obtained, 26.6, 72.5& 35.6, 33.7, 55.6, 55.6, and 31.7 kDa for Everlase, Carezyme, Mannaway, Termamyl, Duramyle, and Lipolase respectively and results have compared with reported MS results of these enzymes. Also, the protein bonds were detected successfully in ternary, quaternary and quinary mixtures of crude enzymes. As a final point the granular enzymes were analyzed with SDS-PAGE and MALDI-TOF. According to the obtained results, we can identify every kind of granular enzymes.

Conclusions:
In this study, determination of protease, lipase, cellulose, amylase, and mannanase was accomplished by SDS-PAGE and MALDI-TOF-MS. The MALDI-TOF-MS as a high-throughput screening method can be proposed for monitoring of enzymes used in laundry detergents, owing to its advantages such as speed, sensitivity, reproducibility and label-free readout.

Novel Aspect:
SDS-PAGE and MALDI-TOF-MS can be proposed as a versatile quality control approaches for detergent industry to identify used enzymes.

References

MECHANISTIC REACTION INSIGHTS BY IN-SITU MONITORING OF AN AACVD REACTION USING GC-MS: STUDYING THE ZNFE2O4 THIN FILM GROWTH PROCESS

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Keywords: AACVD, Gas Chromatography Mass Spectrometry, Reaction Monitoring, Volatile profile, Exhaust gas.

Introduction
A variant of the conventional chemical vapour deposition (CVD) process, the aerosol-assisted (AA) CVD method, uses aerosol droplets to transport the precursors, with the aid of inert or reactive carrier gases to the substrate for thin film coating. It is important to monitor the reaction that takes place in the AACVD system. A novel adaptation of GC-MS is developed which enables direct monitoring of AACVD exhaust gases for the first time providing valuable information.

Methods
The AACVD setup described by Tahir et al. (1) was directly interfaced via a heated transfer line and semi-automated port valve to GC-MS with DB-1 column. AACVD was conducted by systematically increasing the temperature from 300 to 550 °C in 50 °C intervals. Measurements were performed under programmed temperature conditions and the resulting films were characterised by X-ray diffraction and Raman spectroscopy to understand material properties and chemical nature.

Results
The exhaust gases were monitored for the solvent (ethanol) and the individual precursor components; zinc acetate and iron (III) chloride and [Fe2(acac)4(dmaeH)2][ZnCl4] the precursor. The GC-MS peaks were successfully observed for the entire experiment at the various temperatures. Key observation noted were: the exhaust gas profile changes depending on temperature; the ethanol changes into acetaldehyde if no precursor is present; when the precursor dissolves in ethanol, other species are observed which are from either the direct breakdown of the precursor, or reaction in the AACVD system, acetone, ethyl ether, ethyl acetate, butenal, 2-butanone and 3-penten-2-one. The optimal temperature required to produce ZnFe2O4 was 450 °C from precursor in ethanol. The exhaust gas profile obtained from the GC-MS was confirmed through (XRD) and Raman spectroscopy studies. This study shows that this method can be used to give a mechanistic insight into the processes that take place in the AACVD system for the first time, with potential for further research.

Conclusions
The volatile profile obtained from the exhaust of an AACVD system provides clear insight to the progression of the overall reaction. Observation of specific reaction products acetone, ethyl acetate, ethyl ether and butenal in exhaust gas phase can be used as an indication for the formation of ZnFe2O4 film from precursor [Fe2(acac)4(dmaeH)2][ZnCl4] in ethanol at 450 °C. The technique has the potential of being incorporated into simple sensor technology for a wider use.

Novel Aspect
A new application of GC-MS has been developed for in-situ monitoring of AACVD process, with mechanistic information from the volatile profile of the exhaust gas being so vital.

References
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Keywords: Sensitivity, Time of flight, Ion Extraction, Pulse

Introduction:
Better sensitivity is the long-lasting pursuit of an instrument designer. In most cases of the time of flight (TOF) mass spectrometer (MS), ions were extracted from the ion source continuously and path through the push area of the TOF-MS. Hereafter, most of the ions in the push area was repeatedly accelerated to the free field area, and will finally reach the detector in sequence, which is decided by their mass to charge ratio.

Methods:
A pulsed extracting field and an accelerating field was introduced into a TOF-MS between the ion source and the push area. Thus the ions could be extracted and spread along its path to the push area. Where an ion’s position among them depends on its mass to charge ratio. If the push pulse is synchronized to a certain time, it could seize the interested ions by their mass to charge ratio, leave other ions passing by.

Results:
Experiments were done on an off-axis TOF with thermal ionization ion source and Cs sample with contamination of Na and K. Working in continuously extracting mode, the acquired mass spectrum will show that the mass peak of Na is much higher than that of Cs. While in pulsed ion extraction mode and synchronized push, the mass peak of Cs is enhanced to several times higher if Cs was focused. The mass peak of Na could be suppressed to below 20% at the same time. If it is synchronized to Na, the mass peak of Na would be enhanced and Cs be suppressed. In this experiment, the difference of the synchronize time, or push pulse delay, between focused to Na and Cs is about 5 micro-seconds, which can be easily handled.

Conclusions
Pulsed ion extracting mode could enhance the interested mass peak by several times and greatly suppress other mass peaks at the same time. Comparing to the continuously extracting mode, it could greatly improve the sensitivity of the instrument without significant change for an off-axis TOF-MS.

Novel Aspect:
Pulsed ion extracting mode could enhance the interested mass peak by several times, which shows its great talent on improving the sensitivity of an off-axis TOF-MS, and other instruments.

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Introduction:
The proliferation of relatively cheap small-footprint MS instruments presents an opportunity for their application to on-line reaction monitoring, particularly when used in combination with other small footprint analytical instruments [1]. The opportunities and limitations of small footprint ESI-MS in reaction monitoring are explored here.

Methods:
A combined system consisting of a Waters QDa single quadrupole ESI-MS instrument and a Magritek Spinsolve 43 MHz NMR instrument were used to monitor a variety of reactions, with on-line dilution provided by a system of Teledyne-SSI LS class HPLC pumps and a Rheodyne Mass Rate Attenuator.

Results:
The versatility of the combined ESI-MS/NMR system are demonstrated, as well as the value of a combinatorial approach to reaction monitoring, with examples presented in which data from a single technique would be misleading, and in which the combined data can be used to overcome inherent issues with the techniques in question. The capabilities of the system with regard to extremes of temperature, pH and other reaction conditions are reported.

Conclusions:
System is shown to be robust to a variety of conditions, though the performance of rare-earth magnet based NMR to reactions with off-gassing or extremes of temperature is found to be poor. Challenges are found with the use of the single quadrupole MS instrument for the monitoring of reactions with particularly complex mixtures, with methods developed to correct for suppression in ESI-MS.

Novel Aspect:
Combined portable small footprint NMR/MS system utilized on a variety of reactions, capabilities of system explored and discussed.

References
Introduction:
The Laser-Resonance-Ionization Mass Spectrometer (LRI-MS) is a type of mass spectrometer with high elemental selectivity and high sensitivity, which is able to overcome the possible isobaric interferences [1]. Although frequently utilized in the reported LRI-MS facilities or instruments [2-5], time-of-flight (TOF) analyzer is not very appropriate for precise isotope ratio measurements due to its inherited shortcomings of the detection system, such as variation of signal detection efficiency [6]. In our laboratory, a LRI-MS system equipped with a magnetic analyzer (MA) has been used to measure the isotope ratios of plutonium [7] and tin [8] with peak-jumping method. Recently, a LRI-MS system equipped with an electrostatic analyzer (ESA), a magnetic analyzer (MA) and a fixed multi-collector system has been developed for precise isotope ratio measurement. In this instrument, the distance between the ion beams of different mass numbers at the focal plane is proportional to the relative mass dispersion. In order to achieve simultaneous detection of ions with different central mass by using the fixed multi-collector system, a zoom lens based on electrostatic quadrupoles has been developed to adjust the distances of the ion beams at the focal plane [9]. However, the zoom effect, that is, the horizontal magnification simulated by SIMION is quite different from that predicted by theoretical model.
In this work, the reason why the zoom effect in the simulation is so different from that in theoretical model is carefully analyzed. Based on the analysis, the zoom lens was improved to obtain a more accurate zoom effect. By using a home-made diagnostic tool capable of visualizing the shape of ion beam, the zoom effect of the improved zoom lens was evaluated experimentally.

Theory and Methods:
Quadrupole lenses are focusing in one plane and defocusing in another perpendicular one [10]. Thus, several quadrupole lenses could be combined for a useful lens system. A perfect quadrupole consists of four hyperbolic rods. Since hyperbolic rods are difficult to be manufactured and fabricated, round rods with a diameter of 1.148 times the desired diameter of the quadrupole field were used instead [11]. The shortcoming of such quadrupole lenses is that it occupies more space. In electrostatic field, the solution, which satisfies Laplace equation and given boundary conditions, is unique. Thus, a rectangular boundary field which saves more space could be used. Several segmented electrodes can be used to fit the rectangular boundary of the quadrupole field. However, high-order field components will be introduced. In order to reduce the high-order field components, the structural parameters of the electrodes must be carefully designed. In ideal quadrupole lenses, the electrostatic field strengths are assumed to start and end abruptly, which is impossible in real lenses. To eliminate the influence of the fringing field, an effective length should be evaluated accurately. One method to evaluate the effective length of the quadrupole with round rods is to use the empirical formula $l_e = l_0 + 1.1 a_0$, where, $l_e$, $l_0$ and $a_0$ mean the effective length, real length and aperture of the quadrupole, respectively[12]. But it is not verified in the segmented quadrupole with a rectangular boundary. Another method to evaluate the effective length is fringing field integration. It needs the accurate fringing field distribution, which can be determined by the finite element method (FEM) or finite difference method (FDM). To achieve point-to-point focusing in horizontal direction and make the lens system not too complicated, a better choice is to use two quadrupoles, the parameters of which can be calculated based on the design requirement and ion optics theory.
Experiment and Results:
In previous work[9], 24 electrodes were used to fit the rectangular(30 mm×30 mm) field boundary in which 11×2 electrodes were installed in vertical position while 1×2 electrode in horizontal position. It was found that high-order field components and a large constant field existed, the potential of which was 18.5% of the quadrupole field amplitude voltage. To eliminate the constant field and reduce the high-order field components, the rectangular was changed into 60 mm×30 mm, and the left and right electrodes were replaced with three electrodes each side, the potentials of which were equal and the positions of which were hyperbolic. This improvement was verified by the simulation results. After simulated the fringing field distribution by the finite element method, it was observed that the effective length of the field was roughly equal to the actual length of the electrode plus 25 mm. The improved zoom lens was manufactured and fabricated into the LRIMS. By visualizing the ion beams of europium and barium isotopes at the focal plane, respectively, using a home-made diagnostic instrument, the zoom effect was evaluated experimentally. The results showed that the horizontal magnification ratio is between 0.6 and 1.2 when the longitudinal magnification ratio varies from 0.7 to 1.1. The relative difference between the measured value of the magnification ratio and the predicted value is better than 1%. By fine adjustment of the real voltage of the electrodes, relative to the theoretical voltage, simultaneous detection of the isotopes of different elements has been realized by the fixed multi-collector system.

Conclusions and Novel Aspect:
Based on the simultaneous detection requirement of the LRI-MS equipped with an ESA, a MA and a fixed multi-collector system, an ion zoom lens based on the quadrupoles was developed and improved. The influence of the fringing field of the quadrupole is evaluated. The experimental results showed that the lens system achieved accurate zoom effect of the ion beams in the range of 0.6 to 1.2.

References
Introduction:
Many proteins in cerebrospinal fluid (CSF) exist in processed forms, many of which reflect corresponding species present in the brain. Previously an abundance of endogenous fragments from amyloid precursor protein, including variants of amyloid $\beta$ has been identified both in CSF and brain. Until recently, the other core biomarker for Alzheimer’s disease, tau, has been more difficult to assess with mass spectrometry (MS) due to its lower abundance in CSF.

Methods:
Tau was enriched from human CSF by immunoprecipitation (IP) using six different antibodies directed towards different tau regions. Top-down was the main approach, but bottom-up was also utilised for these samples. Analysis was performed by nanoflow liquid chromatography–Fourier transform MS. Quantitative data were obtained by addition of an isotope labeled protein standard before IP. Data processing was performed using Mascot Distiller and PEAKS Studio.

Results:
Starting with three commercially available antibodies endogenous tau fragments were identified using top-down MS of IP’d CSF samples. Based on these data two new neospecific antibodies (Tau123_C and Tau224_C) were developed. In total more than 100 endogenous tau proteoforms were identified by IP-MS using all six antibodies. These mainly covered the N-terminal half, but there were also species originating from the C-terminal quarter. The protein N-terminal was found to be acetylated and phosphorylation at Thr181 was observed. The bottom-up approach tryptic peptides covering slightly more than the N-terminal half were identified. From the quantitative measurements it could be observed that proteoforms covering the mid-region are most abundant, followed by the N-terminal region and a small contribution from the C-terminal part. Three major cleavage sites were identified; in the N-terminal quarter, in the mid-region, and in the C-terminal quarter. These results are in concordance with published data indicating that CSF tau appears in various processed forms, dominated by species originating from the N-terminal half of the protein [1].

Conclusions:
IP-MS is a powerful tool for proteoform mapping in biological samples. It is vital to understand tau processing and its relation to neurodegenerative diseases. These data can be used to guide development of new immunoassays targeting specific tau proteoforms.

Novel Aspect:
Characterisation of endogenous tau fragments in CSF using IP-MS methodology reveals the complexity of biomarker development and evaluation.

References:
Introduction:
Methylglyoxal, a non-enzymatic by-product of glycolysis, is the most relevant glycation agent in vivo. Protein modification by methylglyoxal is found in association to several human pathologies, most notably amyloid neurodegenerative diseases [1]. Characterization of glycated proteins is extremely challenging [2] calling for extreme resolving power and multiple ion-fragmentation Methods:

Methods:
Methylglyoxal modified proteins were analysed by direct infusion in a 7 Tesla Bruker Solarix XR mass spectrometer equipped with the dynamically harmonized FT-ICR ParaCell, in magnitude and absorption modes, at 4 to 8 MB acquisition sizes. Proteins were fragmented by using different ion-activation methods, including ECD.

Results:
Top down analysis of glycated proteins allows the identification of most modified species at MS, while MSMS analysis pinpoints location and chemical nature of modifications. While in vitro glycated proteins are highly heterogeneous, in vivo glycated ones show a higher degree of homogeneity. Most of the methylglyoxal derived glycation products can be identified and their location defined.

Conclusions:
Glycated proteins have often been analysed by bottom-up methods that do not allow any insight on the diversity and heterogeneity. Top-down analysis reveals the extreme complexity of glycated proteoforms, paving the way for interpreting the glycation effects on protein structure and function, essential for understanding its effects in living cells.

Novel Aspect:
Extreme resolution is a key requirement for analysing heterogeneously modified proteins, combined with a diversity of MSMS methods and in cell isolation of modified proteoforms.

References:
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IDENTIFICATION OF NEW LIGAND-PROTEIN COMPLEX FOR MYCOBACTERIUM TUBERCULOSIS USING FOURIER TRANSFORM MASS SPECTROMETRY

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Keywords: natural products, native mass spectrometry, target identification

Introduction:
Natural products are an infinite resource for drug discovery and development. Native mass spectrometry (MS) is an approach used to study proteins in the native folded state and useful to investigate non-covalent ligand protein complexes. We applied this method to screen our natural-product-based fragment library against Malaria proteins [1] and natural product fraction library against Tuberculosis (TB) proteins.

Methods:
37 frozen, shipped proteins are buffer-exchanged with ammonium acetate solution and then diluted to run the mass spectrometry assay. The concentration range used for protein is between 1-10 uM. Screening compounds or fractions are pooled together, dried and treated with 1uL methanol. The molar ratio of protein to single compound is 1:5. Screening is performed using Triversa Nanomate (Advion) coupled with a FT-ICR-MS SolariX (Bruker).

Results:
37 proteins were tested for visibility in native state using FT-ICR-MS. 31 proteins have been screened against 362 pooled active fractions. 26 proteins are able to bind a component with high (ligand-protein complex > 50%), medium (ligand-protein complex > 15%), or weak (ligand-protein complex < 15%) affinity. We identified components able to bind only one protein, components able to bind different proteins and proteins that show different ligand-protein complexes from the same pool fraction. Two proteins showed high ligandability (more than 7 binding complexes), 16 proteins showed medium ligandability (more than 3 binding complexes) and 7 proteins showed low ligandability (less than 3 binding complex). Fraction were confirmed and compound isolation is in progress.

Conclusions:
We used the native mass spectrometry approach for a high throughput screening with natural fractions active against TB. We investigate 37 potential targets and we have identified 26 proteins that bind to a total of 90 components from active tuberculosis fractions.

Novel Aspect:
Native mass spectrometry can be considered an alternative approach for High Throughput Screening of pure compounds as well as fractions libraries and target validation.

References

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COUPLING GAS-PHASE FRACTIONATION AND PROTON-TRANSFER REACTION FOR IMPROVED TOP-DOWN PROTEOMICS

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Keywords: proton-transfer reaction; top-down; proteomics; Orbitrap; gas-phase fractionation.

Introduction:
The major limitation in top-down proteomics is its reduced throughput. Here, we implement a series of gas-phase fractionation strategies on a Thermo Scientific Orbitrap Fusion Lumos Tribrid mass spectrometer to improve the number of detectable proteoforms. Specifically, we applied proton-transfer reaction (PTR) to both deepen the proteome coverage in the 0-30 kDa mass range and to facilitate the analysis of large proteoforms up to ~60 kDa.

Methods:
Classic data-dependent acquisition (DDA) methods (i.e., “top-N” strategies) based on either full MS1 or selected ion monitoring (SIM) scans for precursor identification were compared with gas-phase fractionation based on PTR. Quadrupole-isolated (width: 10-50 m/z units) multiply charged protein ions were subjected to PTR MS2 in the ion trap; precursors identified by PTR MS2 were isolated for HCD MS3 fragmentation using a MultiNotch method in the linear trap.

Results:
Orbitrap MS allows for the characterization of proteoforms from 0 to ~60 kDa.[1] However, even when applying extensive fractionation only the most abundant proteoforms are typically characterized. We performed gas-phase fractionation as previously described by the McLuckey and Hunt groups, by applying PTR on a selected portion (10-50 m/z units) of the spectrum at a time, to reduce spectral complexity. By reducing the charge state of protein cations, PTR also limits the problems in the narrow isolation of single proteoform precursors ahead of dissociation. We analyzed both bacterial (Pseudomonas aeruginosa) and human (HeLa cells) proteomes, in order to determine the different effects of PTR-based gas-phase fractionation on samples with different complexity. We also modified the synchronous precursor selection (SPS) method to adapt it to intact protein cations of different masses and charge states, making the selection of multiple precursors combined with HCD fragmentation efficient even for large proteoforms (>30 kDa).

Conclusions:
We implemented a novel data acquisition strategy based on PTR MS2 followed by isolation of multiple precursors for MS3 fragmentation that is aimed at improving the proteome coverage in top-down proteomics. The flexibility offered by the Tribrid Orbitrap platform offers the possibility of adjusting the degree of gas-phase fractionation in consideration of the complexity of the targeted proteome.

Novel Aspect:
Proton-transfer reaction is performed on a Tribrid Orbitrap mass spectrometer to detect and characterize more proteoforms in top-down proteomics.

References
Introduction:
In FT-ICR MS, many different m/z ions are simultaneously trapped and excited in the cell and the temporal variation of each m/z ion cloud distribution is complicated. In particular, at high ion density the Coulomb interactions are important. To analyze the Coulomb-interaction-induced effects by many-particle simulation, we have combined a special-purpose computer for many-body problems with a PC and the computer configuration has been accelerated[1].

Methods:
On the above accelerated configuration, the influence of Coulomb-interaction-induced degradation of excited coherent ion cyclotron motion on FT-ICR mass spectral peak shape was investigated by many-particle simulation. The simulation particles were equally assigned to each of m/z 130.0 and 130.1 ions. Ion excitation was performed by frequency sweep at 7 T and the mass spectra were simulated as a function of total ion population initially trapped in the cell.

Results:
From analysis for temporal variations of the ion cloud distributions during detection period it has been found that the coherent ion cyclotron motion of m/z 130.0 ion (lighter weight ion) cloud is out of phase earlier than that of m/z 130.1 ion cloud with increasing initial ion population. However, under certain conditions it has also been found that the spectral peak shape obtained from m/z 130.1 ion cloud appears to be distorted earlier than that from m/z 130.0 ion cloud. We have extracted respective ICR signal components resulting from m/z 130.0 and 130.1 ion clouds. In comparison between the two components, it has been found that the component from m/z 130.1 ion cloud is more significantly modulated than that from m/z 130.0 ion cloud. Further we have analyzed in more precise detail temporal variations of the ion cloud distributions and have presumed that the phase locking may occur for a part of remained (non-ejected) m/z 130.1 ion cloud with m/z 130.0 ion cloud.

Conclusions:
The simulation results suggest that the relationship between the Coulomb-interaction-induced degradation and the spectral peak shape is quite complicated.

Novel Aspect:
Temporal variations of the ion cloud distributions undergoing Coulomb interactions in the FT-ICR cell have been analyzed in more detail by many-particle simulation.

References:
Keywords: FT-ICR MS, transient, absorption mode FT, data acquisition, ion phase

Introduction:
Unlike the modern Orbitraps, many FT-ICR instruments worldwide offer magnitude-mode FT as a default, and sometimes the only, method to generate mass spectra. Previously, we introduced a data acquisition technology that enables recording FT-ICR mass spectra directly in absorption mode, and tested this with a 7T LTQFT Ultra. Here, we extend this technology to more FT-ICRs, and evaluate it in wide-range applications including petroleomics, imaging, and proteomics.

Methods:
Peptides, calibration mixtures, proteins, oils, and tissues were analyzed on commercial FT-ICR MS (9.4T solariX XR with ParaCell or custom NADEL cell, and two 7T LTQFT Ultra). Measurements were conducted in the w+ regime on the 7T and 9.4T instruments (original ICR cells), and in the wc and w+ regimes on the 9.4T instrument (NADEL cell). Transients were acquired in parallel with the instrument manufacturer’s data, using a custom data acquisition system (FTMS Booster).

Results:
A wide use of absorption-mode FT in FT-ICR MS applications is limited by high numerical costs of phase correction procedures, substantial phase distortions from standard data acquisition systems, and other complications. To avoid these, we developed a high-performance data acquisition system for recording FT-ICR data as transients with in-phase ions signals (or as mass spectra generated in absorption mode), compatible with both types of FT-ICRs from this work. The system operation was defined based on the textbook principles, namely: (i) the convolution property of FT and (ii) sampling of both detect and excite signals. The system was designed to sample and in-line process the two signals in order to produce a single transient for each measurement cycle. Our preliminary results demonstrate that with this technology FT-ICR mass spectra can be generated in absorption mode with no or a little tuning of the phase function. As a result, improved analyses of petroleum and proteomics samples, and increased throughput in tissue imaging are achieved.

Conclusions:
This work’s high-performance electronics and methods for acquiring FT-ICR mass spectra in absorption mode significantly simplify the use of absorption-mode FT processing in real-life high-resolution FT-ICR MS applications, reduce phasing artifacts in mass spectra, increase numbers of identified and quantified peptides, lipids and compound classes; and make for bringing the absorption mode processing to FT-ICR MS as a standard method to generate mass spectra.

Novel Aspect:
Method development, implementation in hardware, and evaluation in applications of absorption-mode data acquisition for FT-ICR MS, including both, Bruker and Thermo, makes.
Introduction:
Of the 1400 species of scorpions worldwide[1], the Chinese scorpion is used in Chinese medicines for cardiovascular problems[2], antimicrobial[3], and tumors[4]. The venom is a complex mixture of proteins containing bioactive components[5], some showing inhibition of enzyme factor Xa, which prevents blood coagulation[6]. These proteins are often modified and crosslinked with multiple disulphide bonds, with only partial genome sequences, therefore requiring de novo sequencing.

Methods:
Crude scorpion venom was fractionated using step elution on C18 cartridge, and in-vitro inhibition of factor Xa was tested. Nano-LC-FTICR MS/MS analysis of each fraction was acquired with collisionally activated dissociation (CAD) and electron capture dissociation (ECD) as fragmentation Methods: Chemometrics was used to obtain possible pharmaceutical targets. Spectra were collected on a 12 Tesla Bruker SolariX FTICR MS, using both top-down and bottom-up proteomics.

Results:
Using direct infusion FT-ICR MS, more than hundred unique molecular species (approximately 2-12 kDa) were observed in the crude venom, but with bottom-up proteomics, only a small number of proteins can be identified, even though many of these unidentified peptides have good fragmentation spectra. To accurately identify potential bioactive compounds, methodologies were developed to correlate the spectra with the bioactivity results. Quantification of compounds present in each fraction made it possible to use linear regression approaches such as partial least square (PLS) to establish a correlation, which allowed for the identification of the compounds that have stronger correlations with the bioactivity. Therefore, a list of compounds that strongly correlate to the bioactivity could be potential pharmaceutical targets. In addition, from the current results, most of these target compounds are proteins that range from 2k to 5k Da, with 30% of the proteins have sequences that are not in the genome database from Uniport.

Conclusions
The potential factor Xa inhibitors were de novo sequenced using both top-down and bottom-up approaches. The high mass accuracy and resolving power of the FTICR-MS allowed for the highest possible confidence during analysis, which aids the sequencing study of these novel scorpion venom proteins. The method is applicable to identifying potential pharmaceutical leads from other natural products.

Novel Aspect:
Ultra-high resolution FTICR MS for the analysis of scorpion venom reveals bioactive anticoagulant proteins.

References

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Native top-down Fourier transform mass spectrometry of proteins and protein complexes as a tool for structural biology and drug development

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Keywords: native mass spectrometry, FT-ICR, protein complexes, ligand binding, membrane proteins

Introduction:
Native mass spectrometry (MS) of protein assemblies reveals size and stoichiometry. But elucidating their structures to understand their function is more challenging. Membrane proteins are involved in many biological functions, but their characterization by a variety of techniques is challenging. We use top-down Fourier transform ion cyclotron resonance (FT-ICR) MS to derive ligand-binding sites and topological information for large proteins and complexes.

Methods:
Proteins were analyzed by nanoESI-MS under native buffer conditions (pH 6.8, 200 mM ammonium acetate) using a high resolution15-Tesla BrukersolariXFT-ICR system. The FT-ICR MS instrument is equipped with electron capture dissociation (ECD; 1-3 eV) and a CO2-laser operating at 10.6 um for activated ion-ECD and infrared multiphoton dissociation (IRMPD). Electron ionization dissociation (EID) was effected by using higher energy electrons (30-50 eV).

Results:
Native top-down MS generates information on the surface topology, ligand binding sites, and post-translational modifications of protein complexes [1]. We are using native MS/MS to investigate the molecular action of compounds that prevent amyloid fibril formation in neurodegenerative diseases such as Alzheimer’s and Parkinson’s disease [2]. To date, membrane proteins have not been addressed by native top-down MS. Optimal activation of membrane proteins in detergent micelles is required. For the homotetrameric 97 kDa water channel, Aquaporin-Z (AqpZ), increasing the collision energy yielded varying degrees of 4M liberation from the detergent micelles as well as fragmentation into the asymmetric dissociation 3M and M substituents. Activated ion ECD to release weakly-bound products generated a top-down spectrum rich in c-/z-products. Product ions originate from regions in the C-terminal portion of the structure, with most localized to the periplasmic domains.

Conclusions:
Electrospray ionization’s gift for transforming solution-phase macromolecules into gas-phase ionized counterparts without disrupting weak noncovalent interactions is key for applying MS to study protein complexes. For the first time, native top-down MS yields structural and sequence specificity for characterization of membrane proteins. The various activation methods available with FT-ICR MS allow protein complexes to be measured with high accuracy.

Novel Aspect:
Native top-down mass spectrometry yields higher order structural parameters and ligand binding sites for protein-ligand complexes and membrane proteins.

References:
Introduction: Analysis of high-mass protein and protein complexes poses unique challenges to mass spectrometry (MS), driving deep re-thinking of principles earlier validated on small molecules and peptides. In this work we examine limitations of standard MS for high-mass analysis and explore new ways for extending high-mass performance in various directions.

Methods: Experiments were performed using a modified Thermo Scientific™ Q Exactive™ Plus Orbitrap™ MS with a series of enhancements, including pulsed trapping of ions in the front end of the MS, reduction of RF frequencies on all ion guides and changes in Orbitrap injection. Separately, a new geometry of the Orbitrap analyzer with better vacuum within the trap was explored.

Results: Advances towards analysis of ions in the megadalton mass range and m/z detection in excess of 20,000 became possible only after tackling several major technical challenges, especially insufficient or poorly controllable desolvation and low transmission of high-mass ions. These challenges were addressed by instrument modifications listed above and guided by ion-optical modeling. Performance was evaluated in MS/MS and pseudo-MS3 experiments for several homomeric and heteromeric megadalton non-covalent protein complexes, including ribonucleoprotein ones. Separately, a new research-only Orbitrap design facilitated better mechanical balance and vacuum within the trap, enabling for the first time baseline isotopic resolution on intact 150 kDa antibodies both under denaturing and native conditions in infusion mode while employing conventional nitrogen supply for C-trap and HCD cell.

Conclusions: Significant improvements in sensitivity and resolving power were demonstrated for intact proteins, MS/MS and pseudo-MS3 for native top-down analysis of membrane proteins and megadalton non-covalent protein and ribonucleoprotein complexes. This progress yields new insights into the composition, dynamics and interactions of some of nature’s most elaborate and enigmatic molecular machines.

Novel Aspect: Theoretical and practical aspects of improving Orbitrap performance for high mass analysis.
Introduction:
Mass spec characterization of proteins often requires multiple “levels” of analysis for complete characterization. For LC-MS methods these analyses typically require completely different columns and mobile phases. With Microchip CE-ESI-MS a single set of experimental conditions can be used for all levels. This enables rapid multilevel characterization of proteins, with no down-time between samples.

Methods:
All work was performed using a commercially available microfluidic CE-ESI system (ZipChip, 908 Devices Inc.), attached to an orbitrap mass spectrometer (Thermo). Proteins were analyzed intact by diluting to an appropriate concentration before analysis. Middle-down and bottom-up analyses utilized standard reduction and enzymatic digestion Methods: All samples were analyzed using the same microfluidic device and the same background electrolyte.

Results:
To achieve successful analysis at all three structural levels, a BGE with a relatively low pH was chosen. In this denaturing BGE we observe just a single mAb peak, but the electrophoretic separation still functions to electrokinetically “desalt” the sample. This yields a clean mass spectrum with a simple dilute and shoot method. Middle-down analysis of mAbs yields separation of three main segments of the molecule: the free light chain (LC), and the Fc and Fd domains of the heavy chain. We also observe separation of variant forms of each of these segments. Top-down analysis of smaller proteins using this method yields similar separation of variants. The separation of sialic acid variants of alpha-1-acid glycoprotein will be presented as an example. Additionally, a high degree of sequence coverage in <20 minutes will be demonstrated for bottom-up analysis. Results will focus on the ability to rapidly characterize protein structure and determine biologically relevant information.

Conclusions
The microfluidic CE-MS system is a simple and rapid way to perform multiple levels of protein characterization. By choosing a BGE compatible with all types of analysis, there is no need to change the system or analysis conditions between types of characterization assays. Therefore, users can obtain intact, middle-up, and peptide mapping data without any downtime due to system turnover.

Novel Aspect:
Demonstration of a unique mass spec front end separation capability for rapid characterization of proteins.
635 - ION TRAPS WITH QUADRATIC POTENTIALS IN FOURIER TRANSFORM BASED MASS SPECTROMETRY. HARMONIZED MULTI-ELECTRODE KINGDON TRAPS AND FT ICR CELL.

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Keywords:
FT MS, ion traps, Harmonized Kingdon traps, dynamically harmonized FT ICR cell

Introduction:
The review of current stage of development of the ion trap devices, which are using electric fields with quadratic dependence on coordinate and FT MS approach to signal detection and processing, will be given with emphasize on multiple electrode harmonized Kingdon traps[1] and dynamically harmonized FT ICR cells [2]. The general approach both analytical and digital to electric field calculation for such devices will be presented as well.

Methods:
Trap electrode geometries were obtained as the result of solving Laplace and Hamilton-Jacobi equations. Regular high precision machine work and three different 3D printing methods: Selective Laser Sintering, Lost-wax Micromolding (Microcasting) and Inkjet Metal Printing as a methods of traps electrode making are discussed. Simulation of electric field distribution and ion motion dynamics was done by using combined SIMION and SIMAX software.

Results:
Ion motion in the electric field with quadratic potential is oscillation, which frequency depends only on m/z and the field curvature. By measuring this frequency and applying FT MS processing method, we can determine m/z of oscillating ions. For static electric field it is possible to create quadraticity in only one direction what follows from basic laws of electrodynamics. One dimensional quadratic electric fields have been offered for the first time by Knight [3]. Golikov [4] and Koester [5] have offered the options for harmonized traps with multiple internal electrodes. The general approach both analytical and digital to electric field calculation for such devices will be presented. Many variants of the four electrode harmonized Kingdon trap were designed and manufactured. Initial evaluations of different traps performances will be given. The idea of electric field quadraticity emerged in FT ICR mass spectrometry through dynamically harmonized FT ICR cells as a method of significant improvement of resolving power will be discussed as well.

Conclusions
Harmonized multi-electrode ion traps of Kingdon type with quadratic dependence of electric potential along one of the coordinates could be manufactured by different modern technologies both conventional and additive with electrode surface precision high enough to deliver resolving power comparable to Orbitrap [6] analyzer. Dynamically harmonized FT ICR cell and harmonized Kingdon traps could be described analytically using common mathematical approach.

Novel Aspect:
The new approaches to characterization of FT MS ion traps based on harmonized multi-electrode Kingdon traps and dynamically harmonized FT ICR traps was developed.

References
657 - THE NEXT DIMENSION IN PROTEOMICS

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Title: The Next Dimension in Proteomics
Authors: PuiYiu (Yuko) Lam, Chris Wootton, Federico Floris, Cookson K. C. Chiu, Tomos Morgan, Remy Gavard, Maria van Agthoven, Meng Li, Peter B. O’Connor*

Abstract:
2-dimensional mass spectrometry (2DMS) offers intriguing new advantages in proteomics analysis. In particular, 2DMS provides the ability to fragment all proteins/peptides simultaneously in a complex mixture and to accurately correlate which fragment derives from which precursor. The resulting mass spectra are somewhat unusual in appearance, resembling the 2D-NMR results from which the experiment is derived, but the spectra are relatively easy to understand in the context of modern MS/MS experiments. In this presentation, we will discuss the current results on how to best optimise 2DMS experiments for proteomics (in both Bottom-up and Top-down modes). In particular, we will explore the comparison between 2DMS-based proteomics and more traditional nLC-MS/MS results.

For this comparison, a series of standards including BSA and mixtures of standard proteins were used so that the proteomics comparison would focus on known proteins and peptides. In addition, yeast is compared between the two methods where the proteins are also largely known.

The current results clearly show that, in bottom-up proteomics mode, 2DMS can provide very similar levels of sequence and cleavage coverage on these standards, albeit at lower sensitivity at the moment. Additionally, 2DMS doesn’t suffer from the co-eluting peak problem in LC-MS/MS, or rather 2DMS is a method that is uniquely capable of handling many precursor and fragment species simultaneously by expanding the spectra into a second ‘modulation’ dimension which allows correlation of precursors with fragments.

For top-down proteomics, we can use MS/MS in the hexapole collision cell, followed by 2DMS in the ICR cell to effectively achieve MS3 data for all primary fragments for each and every precursor protein. Our current results show that this methodology can improve cleavage coverage for proteins in top-down proteomics experiments, but further research is ongoing. Latest results will be presented.
1107 - TWO-DIMENSIONAL (2D) FT-ICR MS AT HIGH RESOLUTION ON PRECURSOR IONS.

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Keywords: FT-ICR, 2 dimensional, high resolution on precursor ions

Introduction:
Most of modern mass spectrometers use quadrupole analyzer isolation with a selection window in the range of one m/z to the best as the transmission is decreasing fast beyond. We developed also non-uniform sampling (NUS) acquisition for 2D FT-ICR MS which consists in skipping points randomly on the first dimension which give access to a higher resolution in the same amount of time.

Methods:
Nevertheless, the maximum delay between the two encoding pulse increases as the resolution and reaches 1 second using for a 2Megapoints acquisition with a 0.5 microsecond dwell corresponding on a 9.4 Tesla instrument to a resolution of 380,000 at m/z 200. Obtaining sounded 2D FT-ICR implies that ions stays in place without drifting during this time with a precision better than the ECD or IRMPD beam.

Results:
We will describe here the optimization of the acquisition and of the treatment which allow us to obtain square 2D spectra. First of all we continue our analytical calculations of the ion movement to better understand the fundamental of 2D FT-ICR during the complete sequence. With this tool in hands the acquisition was optimized in two ways: (i) by modifying the Bruker IRMPD device to obtain a wider beam and a more precise control of the alignment; (ii) by a fine tune of the ICR cell for 2D acquisition (iii) and by using an optimized 2D pulse sequence. The data treatment was based on the iterative use of an improved version of the urQRd algorithm, which we developed previously for denoising, considering the missing points as noise.

Conclusions:
Currently we are recording 2D of size F1 (precursor) 256k and size F2 (fragments) 256k points which allows us to reach a resolution of 50,000 at m/z 200 for precursors and fragments which is five times higher than our previous best results. The acquisition is performed with a NUS ratio 1/16 which means a physical size of 16k which can be recorded overnight.

Novel Aspect:
We describe here for the first time the analysis of a complex mixture by MS/MS analysis without chromatography with high resolution on precursors.

References
722 - FLAVONOID ANNOTATION USING A PRODUCT ION-DEPENDENT MSn DATA ACQUISITION METHOD ON A TRIBRID ORBITRAP MASS SPECTROMETER

Reiko Kiyonami (1) - Iwao Sakane (2) - Seema Sharma (1) - Graeme McAlister (1) - Amanda Souza (1) - Caroline Ding (1) - Andreas Huhmer (1)

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Keywords: Flavonoid annotation; MSn; Tribrid Orbitrap; Mass Frontier 8.0; Compounder Discoverer 3.0

Introduction: (346/400 characters without spaces)
Flavonoid annotation from natural products remains challenging because of their structural diversity and lack of authentic standards. Simple MS2 based analyses are often not sufficient for complete structural annotation of flavonoids. We present a new flavonoid annotation workflow that uses comprehensive fragment ion information from HCD MS-MS and higher order FTMSn for rapid flavonoid annotation.

Methods: (329/400 characters without spaces)
Flavonoid extracts from different types of natural products were analyzed using LC-MS. A modified Orbitrap Fusion™ Tribrid™ mass spectrometer was used for collecting HRAM MS and MSn data. The data were processed using Compound Discoverer™ 3.0 software. A novel structure ranking algorithm was applied to the MS-MS and MSn data for confident structure elucidation of the unknown flavonoids.

Results: (672/900 characters without spaces)
The flavonoid extracts from multiple tea and fruit/vegetable juice samples were analyzed using the developed flavonoid annotation workflow. The MSn (up to 5) scans were only triggered when one of the sugar neutral loss product ions (162.0523, 180.0628, 146.0574, 164.0685, 176.0315, and 194.0421) was detected in the MS/MS scan. The collected data were processed using Mass Frontier 8.0 and Compound Discoverer 3.0 software. A novel structure ranking algorithm included in the Compound Discoverer 3.0 software was applied to the MS-MS and MSn data for confident structure characterization of the unknown flavonoids based on ChemSpider database and custom flavonoids database. The MSn data were critical, especially for the annotation of flavonoid glycoconjugates and flavonoid isomers.

Conclusions (323/400 characters without spaces)
The new LC-MSn workflow allows intelligent MS/MS fragment ion dependent MSn data collection. In combination with dedicated software for data processing, the new workflow enables improved throughput, identification coverage and confidence for flavonoids annotation from natural products by using comprehensive fragment ion information from HCD MS-MS and higher order FTMSn.

Novel Aspect: (150/150 characters without spaces)
The innovative LC-MSn workflow on a modified Tribrid instrument enables improved throughput, identification coverage and confidence for flavonoids profiling experiments.
Introduction:
Recently, we introduced and initially described FT-ICR MS at the cyclotron frequency instead of the reduced cyclotron frequency using the narrow aperture detection (NADEL) ICR cells and applicable for biomolecular applications [1-3]. Here, we will present an analytical model for signal generation at the cyclotron frequency based on our understanding of the underlying principles of this new concept of ion motion, excitation and detection in the NADEL ICR cells.

Methods: (Limit of 400 characters without spaces)
Transient waveforms at single and multiple cyclotron periods were measured experimentally and calculated theoretically by taking into account: (i) the trajectories of ion-oscillators with time-dependent instantaneous frequency (TDIF) [4], and (ii) different angular size of detection electrodes, including traditional wide (e.g., 90-120 degrees) and narrow (e.g., as in NADEL ICR cells) configurations. Ion motion simulations performed using SIMION [2].

Results:
Constant in time frequencies of ion motion, including cyclotron and magnetron components, have been widely described in the FTMS literature using solutions of a well-known algebraic ICR frequency equation. However, the latter is applicable only when ions are trapped in a quadratic potential with axially-symmetric trajectories and their Coulombic interaction is negligible. However, in a real-life FTMS the instantaneous frequency of ion motion is normally time-dependent. Contrary to the quadratic trapping potential of the modern ICR cells, the non-quadratic trapping potential of the NADEL ICR cells appears to be responsible for the signal generation at the cyclotron frequency. By using the TDIF theory, in a combination with the experimental and computational results, we establish a relationship between the asymmetric trajectories of ion oscillations, transient modulation, detection electrode dimensions, and composition of resulting Fourier spectra. That allows us to formulate the analytical model for cyclotron frequency approach.

Conclusions
FTMS performance is limited by the space charge. It is responsible for peak interferences in mass spectra which result in peak shifts and, ultimately, coalescence. Experimental and simulation data with FT-ICR MS at the cyclotron frequency indicates it as a possible candidate for the space charge-tolerant FTMS. Presented here considerations and analytical models provide further insights into the fundamentals of this novel approach to FTMS.

Novel Aspect: (Limit of 150 characters without spaces)
An analytical model is developed for signal generation at the reduced and true cyclotron frequency in FT-ICR MS, including for the narrow aperture detection electrode ICR cells

References
Introduction:
Previously, we implemented a high-performance data acquisition technology enabling users to access unprocessed data (transients) from any FTMS instrument (ICR or Orbitrap). Now, we extend this capability to enable acquisition of much longer, than typical, transients on their FTMS instruments. This instrumentation development, together with advanced data processing software, unlocks previously unseen ultra-high resolution (UHR) FTMS performance.

Methods:
Drugs, metabolites, complex mixtures, proteins, and tissues were analyzed using commercial ICR and Orbitrap FTMS instruments; including Q Exactive and Fusion Orbitrap FTMS series. Mass spectral and transient data were acquired in parallel using original manufacturer and in-house developed data acquisition hardware (FTMS Booster). Custom software was developed for acquisition and processing of very large datasets of transients and mass spectra.

Results:
Orbitrap FTMS applications are normally performed with transients (much) shorter than 1 s. Our preliminary results demonstrate that modern Orbitrap mass analyzers can sustain the high coherence level of periodic ion motion enabling routine acquisition of 3-10 s transients, even from benchtop Orbitraps. As a result, resolution levels exceeding 500000 at m/z 800 can be achieved. This ultra-high resolution (UHR) enables isotopic fine structure analysis, up to at least 1000 Da, providing increased molecular identification confidence for, e.g., drugs and metabolites (lipids). This UHR performance may also support quantitative analyses of lipids and peptides subjected to neutron-encoded isotopic labeling that yields nearly isobaric species. Interestingly, top-down protein analysis and imaging at these UHR levels is an uncharted territory, which is now ready for exploration. Among other examples, we will demonstrate increased sequence coverage and confidence in product ion assignment for top-down UHR FTMS using HCD MS/MS on a Q Exactive HF.

Conclusions
High manufacturing quality of Orbitrap mass analyzers together with high performance of modern data acquisition and processing technologies enable routine UHR FTMS for applications beyond petroleomics. We will show how the UHR FTMS may leverage life sciences applications, including metabolomics, top-down, and imaging. Interestingly, even the benchtop Orbitrap technology (for selected mass spectrometers) is capable of delivering the UHR FTMS performance.

Novel Aspect:
Implementation, performance evaluation, and application of ultra-high resolution capability on Orbitrap FTMS instruments, including on a benchtop series
Introduction
Sodium hypochlorite (NaOCl) is a widely used disinfectant1,2 and in dentistry, is routinely used as an irrigant in disinfecting infected root canals. In this study, we used selected ion flow tube mass spectrometry (SIFT-MS) to investigate the formation of toxic volatile organic compounds resulting from the reactions of NaOCl with a model system for infected root canals.

Methods
Samples representing a model tooth model system consisting of combinations of dentine powder (from ground freshly extracted molars), microbial suspensions and serum in a buffered suspension were incubated at 37oC under both aerobic and anaerobic conditions and then NaOCl was added. After incubation for 30 minutes, they were centrifuged. Volatile compounds in the headspace of the supernatants were analysed by selected ion flow tube mass spectrometry (SIFT-MS).

Results
During root canal chemomechanical preparation, NaOCl interacts with dentine, leading to denaturation of the organic matrix and changes in mineralised dentine3,4. However, the production of potentially toxic organic compounds has not been widely investigated. SIFT-MS analysis of samples using the precursor ions H3O+, NO+ and O2+ showed that various volatile compounds were produced in abundance when NaOCl was added to different model tooth system components. NH3 was produced when serum, dentine and bacteria were present, but there was much less in samples without serum. NaOCl also led to CH3CN formation, possibly due to NaOCl’s reaction with aldehydes and monochloramines, themselves formed through protein degradation by NaOCl. Furthermore, ions were detected at m/z 83, 85 and 87 using O2+ precursor. These ions are present with peak intensities in the ratio of 9:6:1, implying a 2 chlorine compound of which the only candidate is CHCl2+. This closed shell ion is known to be the product of the O2+ reaction with chloroform (CHCl3)5.

Conclusions
SIFT-MS was an effective technique at identifying the volatile compounds produced in a model infected root canal system with the addition of the irrigant, NaOCl. Toxic compounds were formed by the interaction of NaOCl with the components of the model system, including chloroform and acetonitrile. These have the potential to contaminate the environment and lead to dental health worker exposure or may enter the patient’s bloodstream.

Novel Aspect
The identification of toxic compounds produced during dental treatment is a novel application of SIFT-MS.

References
Keywords: Two-dimensional mass spectrometry, FT-ICR MS, ECD, ubiquitin, denoising

Introduction:
Two-dimensional Fourier transform ion cyclotron mass spectrometry (2D MS) is a tandem mass spectrometry technique that requires no isolation and that maps the fragmentation patterns of all ion species in complex samples in a single 2D mass spectrum [1]. Scintillation noise in 2D mass spectra is minimized using the uncoiled random QR denoising algorithm (urQRd) [2]. The effect of denoising on the structural information available in 2D mass spectra is studied.

Methods:
Seven 2D ECD mass spectra of ubiquitin were acquired on a 12 T solariX FT-ICR mass spectrometer. All spectra were processed using the SPIKE processing software with the urQRd denoising algorithm using different ranks and without the urQRd denoising [3]. All horizontal fragment ion scans were peak-picked and the resulting peaklists were analyzed using the Themis software to assess the reproducibility of the 2D mass spectra [4].

Results:
A 2D mass spectrum shows the fragmentation patterns of all the compounds in a sample. Each peak corresponds to a particular dissociation, where fragment ion scans, precursor ion scans and dissociation lines can be extracted from the 2D mass spectrum. The main source of noise is scintillation noise in the precursor ion dimension. The urQRd algorithm is routinely applied in the precursor ion dimension to lower the signal-to-noise ratio, but also removes peaks in the precursor ion dimension that are below the signal-to-noise ratio. In the fragment ion dimension, the signal-to-noise ratio is also drastically reduced, but many peaks are revealed by the orthogonal application of urQRd. We have extracted the peaklists from the fragment ion scans of seven 2D ECD mass spectra of ubiquitin processed with decreasing urQRd ranks, which corresponds to increasing levels of denoising. The peaklists have been compared using the Themis software to separate peaks corresponding to actual fragments from noise peaks.

Conclusions
This study enables a more confident assessment of signal-to-noise levels fragment ion scans in 2D mass spectra. With decreasing ranks of urQRd denoising, fragment ions are revealed in fragment ion scans. Below the optimal denoising level, fragment ions are removed alongside the noise and structural information is lost. This study improves the accuracy of the structural information that can be extracted from a 2D mass spectrum in terms of ubiquitin cleavage coverage.

Novel Aspect:
Use of Themis software on protein top-down 2D MS. Study of the effect of urQRd denoising algorithm in 2D MS in the dimension orthogonal to the one it is applied to.

References
Keywords: desorption electrospray ionization (DESI), mass tag, signal amplification, Wilms tumor gene

Introduction:
The use of mass spectrometry (MS) as a readout system provides several advantages of high sensitivity, high resolution, rich structural information, and absolute quantification capability. Fast qualitative and quantitative analysis of biological samples, such as whole blood drops[1], tissue sections[2], and single cells[3], has been realized by MS, especially after ambient mass spectrometry (AMS) emerged[4,5]. However, it has limitations for ultrasensitive direct detecting of large biomolecules by AMS[6]. Herein, a simple desorption electrospray ionization mass spectrometry (DESI-MS)-based bioassay utilizing signal amplification strategy was developed for ultrasensitive detection of large biomolecules, such as proteins and DNA.

Methods:
A glass chip was functionalized with thiol-modified capture oligonucleotides, while gold nanoparticles were functionalized with both DNA capture oligonucleotides and mass tags. Here, a new ionic small molecule was synthesized as mass tag. The target, WT1 DNA (67 base long ssDNA oligonucleotide, an important biomarker for minimal residual disease (MRD) in leukemia)[7], was captured by a sandwich assay between biochips and nanoparticles. After being dried, the active biochips were detected by DESI-MS. The dissociated mass tags, together with internal standards, were detected by MS for quantitative analysis of target oligonucleotides.

Results:
Interesting dissociation results were found that a large number of mass tags were dissociated from gold surface and produced abundant mass tag derivatives by DESI just in the open air without high-energy laser or high temperature. As hundreds of mass tags are carried by one gold nanoparticle, good amplification can be achieved for target molecule. Benefiting from the signal amplification, the detection of WT1 DNA could achieve ultrahigh sensitivity with a limit of detection of 0.5 amol (1 μL, 0.5 pmol/L) and a wide linearity range from 1 amol to 500 amol.

Conclusions:
A novel platform for large biomolecule detection was developed, which combined the signal amplification strategy and DESI-MS to achieve ultrahigh sensitivity and high efficiency detection of a disease biomarker (WT1 DNA). As a general method, this bioassay could be utilized for other DNA or protein biomarkers, which would be valuable for the early diagnosis in clinical diagnosis.

Novel Aspect:
Multistep signal amplification with mass tags was realized by DESI-MS in the open air without high-energy laser or high temperature, and high sensitive detection (lower than attomole) of WT1 DNA was achieved by the simple DESI-MS bioassay.

References
Introduction:

Monoclonal antibodies (mAbs) are an important class of innovative medicine for the treatment of cancer and other immune mediated diseases. Five of the top ten best-selling medicines in 2017 worldwide were mAbs. Traditionally, bioanalysis of mAbs has been done almost exclusively using ligand binding assays with some structural characterization provided by high resolution mass spectrometry (HRMS). More recently, direct intact level quantification based on HRMS has gained attraction, as it offers direct analysis, simpler sample preparation compared to surrogate peptide approach, and also provides some structural and isoform information associated with the mAb in study. In this presentation, the quantification of several mAbs in biological matrix is performed. Best practices for sample preparation, LC separation, MS detection, and data processing for achieving the high sensitivity are described. Challenges and the needs for next generation sample preparation and analysis of intact mAb analysis are discussed.

Methods:

Adalimumab, infliximab, or trastuzumab samples were prepared by spiking mAb solution into mouse plasma. Each mAb was immuno purified from plasma using goat anti-human Fc prepared either in streptavidin coated plate1 or magnetic beads. A Tecan Genesis liquid handling platform was employed in sample preparation. After elution under acidic conditions, extracted mAb was quantified by UPLC-HRMS. The LC-MS system consists of a binary pump, sample manager, column manager, and a Vion QTof HRMS. The mass range used for data acquisition was 500-4000 m/z, and the system was operated in ESI+ full scan sensitivity mode. A generic gradient was used with a flow rate of 0.2 mL/min and a total run time of 13 minutes. Data was acquired, analyzed and quantified using the UNIFI informatics system.

Results:

A standard calibration curve was prepared by spiking a serial dilution of monoclonal antibody into mouse plasma using BSA as a carrier protein. After immunoaffinity purification, the solutions were analyzed by LC-MS under generic reversed phase conditions using LC/MS friendly mobile phase consisted of acetonitrile, H2O, and formic acid. LC-MS conditions were optimized including chromatographic separation of the mAb from the carrier protein, glycan isoform spectral profile, and charge state summation and XIC tolerances for processing, yielding best bioanalytical performance. Excellent sensitivity (<50 ng/mL) using the optimized conditions was acheived. Comparing immunoaffinity purification from streptavidin plate vs magnetic bead, it was found that the plate offered lower background signal compared to magnetic bead. Preliminary results showed that LLOQ determined from streptavidin coated plate is <50 ng/mL compared to 250 ng/mL using streptavidin coated magnetic bead. Data processing parameters were also extensively evaluated and optimized. It was found that both XIC mass tolerance window and the number of charge states included in the peak area summation can have a large effect on data quality, especially at the LLOQ level. A flexible informatics workflow evaluating multiple parameters was used for a quickly viewing the data and determining the best analysis method.

Conclusions
HRMS quantification of intact mAbs offers analytical scientists many advantages over traditional approaches. In addition to simpler sample preparation (versus surrogate approaches), structural information at the whole molecule level can provide additional information in understanding the state of mAb in question in biological systems and interpreting quantitative outcomes. In this study, high sensitivity quantification of monoclonal antibody is presented. Results show that the combination of a sensitive mass spectrometer with the best practice in sample preparation and data processing parameters are critical in obtaining the highest sensitivity. Combination of these best practices brings us a step closer to routine intact level quantification of mAbs in plasma.

Novel Aspect:

The present study provides a complete workflow for intact level mAb quantification in plasma from automated sample preparation through automated data processing and data review. The LLOQ of <50 ng/mL represented the best sensitivity so far and further closing the gap between intact level and surrogate peptide approaches in the quantification of monoclonal antibody in plasma.

References

PROGRESS TOWARDS IMPLEMENTING ACCURATE MASS MS FOR ROUTINE BIOThERAPEUTIC ANALYSIS

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Keywords: fit-for-purpose time-of-flight MS, biotherapeutics, intact mass analysis, peptide mapping, glycan analysis

Introduction: The adoption of accurate mass MS for routine analysis has been hindered by instrument size, operation complexity, hardware robustness, and reproducibility. Yet, there is an need for this approach; for example, efficient data collection and analysis of quality-relevant information within the pharmaceutical industry. Here, we describe the performance evaluation of a prototype small-footprint time-of-flight MS that could overcome many of the above challenges.

Methods: To assess the performance and reproducibility across a range of biotherapeutics, trastuzumab, NIST mAb, infliximab, trastuzumab emtansine, brentuximab vedotin, and EPOs were analyzed using the prototype MS coupled with various chromatography separation modes for intact, subunit, peptide mapping and released glycan experiments. Data acquisition, processing, and reporting were all achieved within a single workflow-centric informatics platform.

Results: For intact mass analysis, a dilution series for both trastuzumab and NIST mAb established a limit of detection (LOD) and intra-system LOD reproducibility. Results were comparable to other high resolution MS systems. More structurally complex proteins (inflimab, trastuzumab emtansine, and EPOs), were analyzed and data demonstrated the greater heterogeneity of these molecules. The average drug-to-antibody ratio (DAR) for a cysteine-conjugated ADC agreed with values from commercial MS systems and hydrophobic interaction chromatography (HIC).

NIST mAb subunits showed good mass accuracy. Low oxidation levels were observed for both subunits after forced degradation experiments.

Peptide mapping of the NIST mAb was used to evaluate system robustness. A high sequence coverage was determined through precursor accurate masses and identification of fragment ions.

Glycan analysis was performed on the NIST mAb. All major glycans were correctly identified as well as many lower abundance glycans, including some potentially immunogenic glycans.

Conclusions: Several key biopharmaceutical experiments were performed on a small-footprint prototype TOF MS instrument. The data generated for all experiments (intact mAb mass, peptide mapping, glycan analysis) were very comparable to currently available commercial high resolution MS instrumentation. Thus, these data show great promise that this instrument could be an appropriate choice for routine MS-based biopharmaceutical analyses.

Novel Aspect: A prototype small-footprint oa-TOF MS provides fit-for-purpose data for multiple experiment types relevant to routine biotherapeutic analysis.
MULTIPLEXED ANALYSIS OF ENVIRONMENTAL SAMPLES BY GCxGC-HRMS WITH MULTI-IONIZATION CAPABILITIES

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Keywords: GCxGC, TOFMS, HRMS, Non-targeted, Environmental

Introduction:

The Gas Chromatography coupled to Mass Spectrometry provides powerful analytical tools, combining selectivity, sensitivity, reliability, and information capacity for targeted or non-targeted methods of environmental analysis. Yet, the complexity of the environmental samples and variety of the analytes of interest in them demands novel methods of analysis, taking advantage of modern advances in gas chromatography, mass spectrometry and data processing.

Methods:

The GCxGC equipped with thermal modulator and coupled to High Resolution TOFMS was used for untargeted screening of snow samples. The mass spectrometer was equipped with a novel ion source, capable of three modes of ionization - EI, PCI and ENCI. The snow samples were analyzed in different ionization modes using same instrument and at the same chromatographic conditions. The chromatograms were realigned for assisting in identification of the analytes.

Results:

The high resolution TOFMS is an ideal GC-MS detector for screening and quantifying unknowns as it collects high mass accuracy full mass range mass spectra at very high acquisition rates without any data loss. The technique provides highly reliable data suitable for automatic accurate spectral deconvolution of the coeluting analytes present in the samples in the wide concentration range. The GCxGC technique increases separation power, allows chromatographic separation of closely eluting constituents, making analyte identification more reliable and makes comprehensive analysis of environmental samples more realistic. Comparison of analytes detected in the same sample using EI, PCI and ENCI ionization methods shows that individual ionization method may miss certain types of the analytes, and combining data from three modes of ionization improves the quality of the results. The presentation will demonstrate the practical implementation of the technique and will report examples of false negative results if only one ionization method is used.

Conclusions:

Application of the highly efficient multiplexed technique of GCxGC-HRTOFMS with multi-ionization capabilities has proved to be very powerful for increasing coverage and reliability of the untargeted screening results of environmental samples. Combining data from all three ionization modes increased the number of detected analytes by at least 30% and improved reliability of the analyte assignments.

Novel Aspect:

GCxGC-HRMS analysis with EI, PCI and ENCI ionization of analytes was used for comprehensive analysis of snow samples collected at the same day in various parts of large city.
758 - GAS CHROMATOGRAPHY-ATMOSPHERIC PRESSURE PHOTOIONIZATION-HIGH RESOLUTION MASS SPECTROMETRY FOR THE DETERMINATION OF SEMI-VOLATILE FLUORINATED COMPOUNDS

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University of Barcelona, Department of Chemical Engineering and Analytical Chemistry, Barcelona (1)

Keywords: gas chromatography, atmospheric pressure photoionization, high resolution mass spectrometry, semi-volatile fluorinated compounds, water

Introduction:
Semi-volatile fluorinated compounds are currently determined by GC-MS using electron and chemical ionizations[1], but problems related to their low ionization efficiency have been reported. Considering the proved advantages of atmospheric pressure photoionization (APPI) for the analysis of non-polar compounds [2], in this work we explored the applicability of this technique for the determination of several families of fluorinated compounds by GC-MS.

Methods:
AGC-APPI-HMRS (Orbitrap) method combined with HS-SPME was developed for the determination of fluorotelomer olefins (FTOs) fluorotelomer alcohols (FTOHs) and fluorinated sulfonamides (FOSAs) and sulfonamide-ethanols (FOSEs) in river water samples at trace levels. The HS-SPME method was performed using a PDMS/DVB/CARB fiber and the GC-APPI-HRMS determination was carried out in the negative ion mode using fullscan acquisition at a resolution of 35,000 (FWHM).

Results:
Semi-volatile fluorinated compounds were ionized via dopant-assisted in negative ion mode, generating the [M+O2]- as base peak of mass spectra for most of FTOHs and FOSEs, while the [M-H]- and several in-source CID fragment ions were mainly observed for FOSAs and FTOs, respectively. Acetone was found to be the best dopant for achieving the highest responses for all the compounds. The GC-APPI-HRMS method provided low limits of detection, ranging from 0.05 to 10 pg injected, and non-significant matrix effects for the analysis of water samples with different pollutant loads. HS-SPME was used to extract analytes from water river samples and the method limits of detection achieved ranged from 24 pg L-1 to 15 ng L-1. The HS-SPME GC-APPI-HRMS method provided good linearity (R2>0.993) in the calibration working range. The precision and trueness of the developed method were determined using spiked blank river water samples, obtaining for all the compounds RSD% values lower than 11% and relative errors down to 12%, respectively.

Conclusions
GC-APPI-HRMS has shown to be an excellent alternative to the existing GC-MS methods using classical ionizations for the determination of semi-volatile fluorinated compounds, providing LODs up to 80 times lower than EI and CI techniques. Moreover, the response of the GC-APPI-HRMS is not significant affected by the matrix. On that way, the developed GC-APPI-HRMS method in combination with HS-SPME allowed the detection of the target compounds down to pg L-1 levels.

Novel Aspect:
This is the first time that a GC-APPI-HRMS method is proposed for the determination of semi-volatile fluorinated compounds in river water samples at very low concentrations.

References
Introduction:
A sample was found to contain two similar protein variants (“F” and “Y”), only differing by amino acid substitutions at a few positions. An attempt to estimate the ratio between the two variants had previously been carried out, using proteolytic digestion of the sample. Two peptides separating the variants were used for calculation of a chromatographic peak-area-ratio. However, this method suffered from poor precision mainly caused by variations in the nanoLC-MS system. Accordingly, the aim of the present work was to improve the precision of the ratio determination and to know the exact quantitative amount of the variants in the sample.

Methods:
Initially, protein concentration was estimated using a BCA assay, to control protein to enzyme ratio, and for the final quantitative calculations of the content of variants relative to the total protein amount.
One of the positions at which the variants were differing, was captured by digestion using endo-proteinase Asp-N, giving two good responding peptides. The Asp-N digested sample was spiked with corresponding AQUA peptides, purchased as synthesized variants with the C-terminal amino acid being heavy isotopically labelled (+4 Da). Accordingly, it was possible to distinguish the spiked peptides from the peptides originating from the sample. As preparation for nanoLC-MSMS analysis the peptides were purified by solid-phase extraction.

Results:
Adjusting the “simple area based ratio estimation” using heavy isotopically labelled peptide standards, eliminated most of the previous observed deviation. By spiking the heavy isotopic labelled peptides in known concentrations, it should be possible to quantify the amounts of variants in the sample, corrected for differing MS response and possible sample matrix effect. However, recovery of the spiked internal standard is not the only challenge of an absolute quantification, which also depend on recovery of the many steps in the sample preparation procedure (solubilization/extraction, BCA protein quantification, digestion and SPE purification).

Conclusions:
The precision, of a “simple chromatographic peak-area-ratio” describing the ratio between two protein variants in a sample, was remarkably improved by adjusting to internal standards in the form of heavy isotopic labelled peptides. Absolute quantification depends on many steps of the sample preparation procedure and each step require validation.
Introduction:
Croconaine dyes are appealing molecules based on the condensation of croconic acid and a reactive electron-donating aromatic or heterocyclic structures [1,2]. Fast investigation of these dyes was accomplished by matrix assisted laser desorption/ionization (MALDI) MS in positive ion mode after systematic matrix selection. Since few or no information on the gas-phase ion energetics of croconaines are available, an empirical approach was followed.

Methods:
All experiments were performed using a 5800 MALDI ToF/ToF analyzer in reflectron positive mode. Different matrices such as protonating 2,5-dihydroxybenzoic acid (DHB), and α-cyano-4-hydroxycinnamic acid (CHCA), electron-transfer (ET) secondary reaction matrices as trans-2-[3-(4-t-butyl-phenyl)-2-methyl-2-propenylidene]malononitrile (DCTB) and 1,5-diaminonaphthalene (DAN) and basic as 9-aminoacridine (9AA) were examined.

Results:
The investigated croconaines bring symmetrical substituents on the indolenine core including heteroatoms as fluorine and bromine or different aromatic structures. Typically, when dyes were analyzed by using proton transfer (PT) matrices as DHB and CHCA the base peak assigned to the protonated adduct [M+H]+ and the concomitant presence of an odd electron molecular ion, M+•, was observed. This outcome suggests that gas-phase PT and electron transfer ET secondary reactions are competing processes when acidic matrices are employed. The presence of many signal contributions represents a drawback in terms of sensitivity and trouble when running tandem MS. Among the ET matrices, DAN provided the highest ionization yield leading to the specific formation of odd-electron molecular ion M+• without other contributions. This allowed to select the precursor ion in a unique form for MS/MS analyses. Singular behavior was observed for bromine substituted croconaine when fragmented with DAN since bromine ion was detected as unusual Br+ cation.

Conclusions:
Protonating matrices generate a mix of odd molecular ions and protonated adducts alongside sodiated species. Among the ET matrices, DAN provided the highest ionization yield leading to the specific formation of odd-electron molecular ions M+• with a negligible fragmentation. Finally, MALDI MS/MS of the radical charged molecular species provide useful structural information, thus making identification very straightforward for all croconaines investigated.

Novel Aspect:
The analysis of croconaines by MALDI MS is presented for the first time. Tandem MS and DAN reveal common fragmentation patterns and the occurrence of uncommon positive ions.

References
Introduction:
Polyoxometalates (POMs) are discrete anionic metal-oxygen clusters which exhibit a great diversity of applications ranging from catalysis, medicine, etc.[1] POM-based inorganic/organic hybrids have drawn enormous attention in the past decades as exciting applications of these materials have been discovered in such diverse areas as light-emitting diodes, field-effect transistors, and solid-state lasers. Such hybrid materials may provide additional and/or enhanced functions and properties as a result of synergistic interactions between their inorganic and organic components.

Peng et al. reported a facile and efficient method to synthesize organoimido derivatives of POMs by using dicyclohexylcarbodiimide (DCC) as a catalyst, which was implemented by refluxing the reaction mixture of aromatic amines and TBA[Mo6O19] in acetonitrile for 12 hours.[2] Leidenfrost droplets have been used as microreactors for the preparation of metal nanoparticles and for accelerated organic synthesis.[3] However, there is no report on the preparation of inorganic-organic hybrids which show promise in material science and homogeneous catalysis. Herein, we report preliminary result for the functionalization of POM clusters with aromatic amines, a model example of this type of reaction and its first implementation using the Leidenfrost effect to perform accelerated chemical synthesis in droplets. We show that interfacial effects are involved in acceleration and prepared macroscopic amounts of synthetic products within minutes. Compared to Peng’s method, which was reported to be faster, more efficient, and the reaction conditions are much milder than earlier methods, Leidenfrost experiment is more competitive in which interfacial effects may play a pivotal role in the reaction acceleration.

Methods:
Efficient Preparation of Organoimido Derivatives of Lindqvist hexamolybdate in Leidenfrost Droplets.

Results:
Four reactions of Lindqvist hexamolybdate [Mo6O19]2- with aromatic amines to generate organoimido derivatives were used as model reactions to explore the use of the Leidenfrost effect for accelerated chemical synthesis of organo-modified POMs in droplets. It is clear to see that observable amounts of products were formed over the time course of the experiment (240 s) for the Leidenfrost droplet experiment while in the parallel bulk-phase reactions no detectable product formation is evident.

Conclusions:
We show that interfacial effects are involved in acceleration and prepared macroscopic amounts of synthetic products within minutes. Compared to Peng’s method, which was reported to be faster, more efficient, and the
reaction conditions are much milder than earlier methods, Leidenfrost experiment is more competitive in which interfacial effects may play a pivotal role in the reaction acceleration.

Novel Aspect:
We report the first implementation using the Leidenfrost effect to perform accelerated synthesis of organo-functionized POMs in droplets.

References:
656 - A NOVEL PTR-TOF REACTION CELL SUPERPOSING DC AND RF FIELDS LEADS TO TEN-FOLD IMPROVEMENTS IN SENSITIVITIES AND LODS

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Keywords: PTR-MS, time-of-flight, sensitivity, reaction cell, VOCUS

Introduction:
The working principle of Proton Transfer Reaction - Mass Spectrometry (PTR-MS) is chemical ionization upon reactions between H3O+ ions and the analyte VOCswithin a reaction cell. A DC field within the cell moves the primary and product ions towards the detector. In conventional reaction cell ion losses occur at the reaction chamber cell walls because of diffusion and scattering. This problem affects the instrumental sensitivities and limits of detection (LOD).

Methods:
The solution involves adding oscillating RF fields on top of the linear field in the reaction cell. The ions are not only moved along the cell by the conventional DC field but are also simultaneously focused by the oscillating RF fields, so that more ions can reach the detector. The chemistry of ion-molecule reactions within the cell is similar to that of the old design, while the spatial distribution of primary and product ions is changed to achieve the extra focusing.

Results:
The additional ion focusing which is reached in this way greatly improves the transmission of ions towards the detector region. The net gain in sensitivity exceeds one order of magnitude and previous boundaries in LODs are overcome thus providing unprecedented performances for a PTR-MS. The new reaction chamber has been coupled to state-of-the-art mass analyzers such as time of flight mass spectrometers (ToF-MS) reaching 15,000 (m/Δm FWHM) in mass resolution. This new instrument has been denominated VOCUS PTR-TOF. In the case of xylene, the achieved sensitivity is 20000 cps/ppmv and the LODs are <1 pptv and <10 pptv in 1 s and 1 min integration time, respectively. Another problem with is overcome by the new reaction cell is the dependence of calibration factors on the sample humidity. Calibration factors for VOCUS PTR-ToF are independent of sample humidity.

Conclusions
A new PTR-MS reaction cell design is introduced adding oscillating fields to the linear field. Ion losses due to scattering and diffusion are avoided, strongly improving the instrument performance. Limits of detections and sensitivity are improved by more than a factor of ten. The dependence of concentration calibration factors on sample humidity, which is a problem in conventional PTR-MS, is overcome.

Novel Aspect:
The addition of an oscillating field to the PTR-MS reaction cell leads to a sensitivity gain of more than ten-fold.
Determining Acetylcholinesterase Activity Using Paper Spray Mass Spectrometry

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Introduction:
Paper spray (PS) is an ambient ionization technique that has been used to detect and quantitate a wide range of small molecules from complex matrices. Recently, PS mass spectrometry was shown to be a rapid alternative for measuring the results of enzymatic reactions which took place in microcentrifuge tubes. Herein, we present an assay where the enzymatic reaction occurs on the PS substrate to monitor the activity of acetylcholinesterase (AChE) in blood to determine inhibition due to chemical warfare agent exposure. This substrate assay is simple, rapid, and requires minimal sample volume.

Methods:
The paper substrate required vapor application of silanes to generate a hydrophobic surface. The reaction was generated in a vial and spotted onto the surface of the PS cartridge. Due to the hydrophobicity of the substrate surface, the reaction solution formed a ‘reaction sphere’. The loaded PS cartridges were then incubated at 37°C for 30 minutes. The cartridges were then analyzed on a Thermo Scientific Orbitrap Elite mass spectrometer for AChE activity.

Results:
Enzymatic reactions were successfully performed on-substrate using PS-MS to determine AChE activity. AChE activity was determined using the synthetic substrate 1,1-dimethyl-4-acetylthiomethylpiperidinium (MATP), m/z of 202.0836, which is cleaved by AChE generating a resultant fragment at m/z of 160.0837. The change in ratio of these 2 ions demonstrates AChE activity, with corresponding increases in signal as additional substrate was reacted. It was determined that a 30 min incubation at 37°C resulted a log difference in the ratio. The nerve agent VX was used as a control for the reaction to identify AChE as the functioning enzyme, via direct inhibition. A method was then designed that allowed for not only the determination of AChE activity but to also identify the toxic inhibitor, VX, with tandem mass analysis. This method was used to analyze blood from animals exposed to VX. The results were compared with analysis of the same samples utilizing the standard Ellman’s Assay. There was consensus between the Methods:

Conclusions:
PS-MS is suitable for determination of AChE activity in blood. The assay requires only MTAP as a reagent, and no secondary reaction is required. Using a hydrophobic PS cartridge, the reaction may be performed on-substrate with both high and low resolution instruments being utilized for analysis. The assay performed similarly to standard laboratory procedures when analyzing samples from exposed animals, even at a dose a log less than is required to observe symptoms.

Novel Aspect:
An assay has been developed to measure AchE inhibition using PS-MS. The assay requires a single reagent and also allows for mass based identification of the toxicant.

Funding for this project is provided by the Defense Threat Reduction Agency – Joint Science and Technology Office for Chemical and Biological Defense.
Introduction:
We provide a workflow to identify characteristic signatures of superfoods, a poorly defined marketing term referring to food with high levels of nutrients that may benefit health.
A set of 500 plant-based and superfood samples were analyzed by using untargeted UPLC-QTOF-MS/MS and new computational tool. We identified distinct molecular signatures including the diversity of polyphenols, flavonoids and microbial peptides that can be used to categorize superfoods.

Methods:
Samples were extracted and chromatographically separated by UPLC-QTOF system and C18 column separation. Spectra were acquired in positive data dependent acquisition and the five most abundant ions were selected for fragmentation spectra acquisition. Greater than 500 plant-based samples were analyzed by UPLC-MS/MS in DDA and processed on the GNPS webplatform [1].

Results:
Untargeted MS analysis was used to obtain an overview of all features, focusing on compounds with anti-inflammatory, antioxidant or antimicrobial properties across a broad range of plant-based foods. Spectral library search was performed against public and commercial libraries (GNPS, MassBank, Metlin, NIST), and molecular networks were generated to expand spectral annotations. In addition, computational mass spectrometry strategies were employed to annotate bioactive compounds such as polyphenols, flavonoids and phenolic acids. Those include: (1) the automatic network annotation propagation of in silico annotations, (2) the dereplication of bioactive peptides using the Dereplicator workflow [2], (3) the annotation of sub-structures using MS2LDA [3], (4) the computation of the molecular formula using Sirius and CSI:Finger [4] and (5) the prediction of the chemical class using Canopus fingerprint.

Conclusions
Unique microbial peptides with antimicrobial, anti-inflammatory or antioxidant properties and putative unknown cyclolinopeptide variants were found in some superfoods (score>15, p values<1E-11, FDR<1%). The presence of unique compounds, as well as the proportions of polyphenols, flavonoids and bioactive peptides between superfoods and common fruit and vegetables is proposed to define criteria underlying the chemical characteristics of superfoods.

Novel Aspect:
We are presenting criteria to classify superfoods based on the number and the diversity of bioactive Compounds.

References

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HIGH-RESOLUTION MASS SPECTROMETRY IN COMBINATION WITH IN SILICO PREDICTION MODELS TO IMPROVE ACCURACY FOR COMPOUND IDENTIFICATION

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Keywords: compound identification, high-resolution mass spectrometry, complex mixture analysis, linear retention index prediction

Authors:
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Introduction:
Chemical characterization remains a major hurdle for analytical chemists when dealing with non-targeted screening analyses. This work focuses upon the identification of unknown volatile and semi-volatile compounds using gas chromatography coupled to high-resolution mass spectrometry (GC-HRMS) operating in electron ionization (EI) acquisition mode.

Methods:
552 reference compounds, including n-alkanes used as chemical markers, were analyzed by GC-HRMS. Accurate mass spectra (MS) were registered in a Personal Compound Database Library, which enabled targeted screening for these compounds. These compounds were randomly split into training (n=401) and test (n=151) sets, and linear retention index (LRI) values were used to build LRI prediction models [1].

Results:
These models were used to predict LRI values for several thousand chemicals reported to be present in tobacco and tobacco aerosol, plus a range of specific flavor compounds [2]. These predicted LRI values were used in conjunction with EI nominal MS available from commercial libraries to enhance the confidence in structural elucidation.

These models were also used to predict LRI values for hits proposed from the comparison of deconvoluted spectra with commercial MS libraries. The similarity between calculated and predicted LRI values added an additional layer of confidence for compound identification. A final score was calculated as a combination of MS similarity score and the accuracy of experimental versus predicted LRI values.

Recently, a set of 350 reference compounds were analyzed and used to validate the accuracy of the models and highlight any limitations.

Conclusions:
The combination of accurate LRI prediction and MS similarity improved the ranking of compound proposals, thus demonstrating that computational LRI prediction facilitates the evaluation of non-targeted data. This workflow has been used to improve the confidence in structural elucidation for the analysis of complex matrices.

Novel Aspect:
Combination of two LRI prediction models with EI acquisition mode for identification of unknown chemicals.

References
HIGH-THROUGHPUT QUAN/QUAL LC-MS ANALYSIS: WHERE IS THE SWEET SPOT?

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Keywords: Quan/Qual; UHPLC-QTOF-MS; high-throughput; metabolomics; drug metabolism

Introduction:
High-throughput simultaneous quantitative and qualitative (Quan/Qual) analysis is attractive to combine targeted with non-targeted analysis, e.g. in pharmacometabolomics and drug metabolism studies. But, when using a Q-ToF, where is the ‘sweet spot’, viz. an optimal balance between quantitative and qualitative data and optimal use of instrumental capabilities? That is what we investigated in this study.

Methods:
An ultra-high performance liquid chromatography (UHPLC) method with high chromatographic resolution (peak capacity of 266 with a gradient time of 2.25 min) and a Synapt G2S were used to investigate and optimize mass spectrometric parameters for Quan/Qual analysis. The optimized method was applied to a rat metabolomics study investigating the effect of the fasting state and the administration of a dosing vehicle on the rat plasma metabolic profile.

Results:
The scan mode and scan rate proved to be highly important parameters for high-throughput Quan/Qual analysis. The amount of qualitative information that a method can provide correlated negatively with its quantitative performance (accuracy, precision, sensitivity, linear dynamic range). The MSE scan mode with a short scan time of 30 ms provided the optimal balance. The resulting 4.25-min Quan/Qual analysis method enabled quantification with accuracy and precision values ≤20% at the lowest quality control (QC) level and ≤15% at higher QC levels for 16 out of 19 tested analytes. It provided both parent m/z values and fragmentation spectra for compound identification with limited loss of chromatographic resolution. It revealed biologically relevant metabolites in its application to the rat metabolomics study.

Conclusions:
High-throughput Quan/Qual method development requires balancing between the amount of qualitative data, the quality of the quantitative data and the analysis time. We will share recommendations for MS resolution, scan mode, scan rate and smoothing in Quan/Qual method development and analysis.

Novel Aspect:
This work highlights the importance of optimizing high-resolution mass spectrometric parameters for Quan/Qual analysis, opposing the general idea of their insignificance.
Introduction
As more and more new food compounds are being developed and deployed around the world, contaminants not in target lists can appear in products through environmental or manufacturing contamination or misuse. This makes it essential that food safety laboratories stay vigilant for an ever-increasing number of analytes to screen for, and for new, unexpected and unwanted compounds[1] [2].

At the moment, the LC triple quad MS and GC triple quad MS systems are the workhorses of the pesticide laboratory. Most labs in Europe still use these technologies more than any other. But there’s a trend toward implementing high-resolution accurate mass (HRAM) technology, because analysts are no longer satisfied with just finding the expected compounds in the samples; now, the aim is to identify everything (or at least as much as possible). In other words, there is a steady move away from targeted analysis. Screening allows us to discover what is in the samples with less bias – at odds with simply looking for what we already suspect is present.

This poster will show an efficient workflow for a non-targeted acquisition MS-mode for different compound groups and demonstrate a next-generation workflow for more efficient control of unexpected and unwanted compounds in food. The Swedish Ethyl acetate multiresidue method (SweEt), coupled with a high-resolution mass spectrometer offers:
- The ability to screen for several compound groups at the same time, in the same sample
- Advanced software tools for screening evaluation and identification
- A validated and accredited method to screen for around 500 pesticides in fruits and vegetables

Methods
The main extraction techniques used today are making high-moisture food sample preparation faster and more straightforward. SweEt [3][Swedish Ethyl acetate], is amenable to a wide range of matrices and offer a number of advantages over the traditional sample preparation techniques. With a range of polar and non-polar contaminants potentially present in food samples, it is essential that the sample preparation approaches are able to extract a very broad range of analytes. However, it can be challenging to develop methods that are both comprehensive as well as convenient to use. This presentation will describe the workflow for SweEt with around 550 pesticide analytes in targeted mode and the screening workflow for semi/non-targeted mode.

Results
With a generic method it is possible to screen and identify several compound groups in the same sample. The poster will present examples of findings of aflatoxin, benzophenone and pesticides.

Conclusions
Pesticides alone account for around 1000 analytes and can be found in different food commodities. Meanwhile other types of unwanted analytes can also be found such as mycotoxins, alkaloids, food additives, veterinary drugs and food contact materials. The broad variety of compound groups has limitations regarding extractability with different solvents. Therefore it is important to have a generic method and if possible to extract suspect samples in various ways. [4]
Novel Aspect
This poster presents an efficient screening workflow which can make laboratories better equipped to manage what the future may hold. There is a need to stay alert and anticipate what’s beyond the conventional and expected findings, for example food frauds, contaminations or simply misuse of substances.

References


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For information please contact: scientific@imsc2018.it
BRIDGING THE GAP BETWEEN IMAGES AND MOLECULES: PARALLEL ACQUISITION OF FTMS MASS SPECTROMETRY IMAGING AND MS/MS DATA COUPLED WITH AUTOMATED AND HIGH CONFIDENCE LIPID IDENTIFICATIONS

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Keywords: mass spectrometry imaging; lipids; tandem mass spectrometry; Fourier transform mass spectrometry; automated identification

Introduction:
Mass spectrometry imaging (MSI) enables visualizations of lipid distributions throughout tissues. Yet, it has struggled to translate images to underlying lipid biochemistry. A significant contributor is the lack of automated and broad MS/MS acquisition and structural identifications tool. We address this limitation via an innovative MSI approach enabling parallel acquisition of MS/MS and FTMS-MSI of every ion without prolonging MSI acquisition time.

Methods:
Data were acquired using an Orbitrap Elite and a dual MALDI/ESI source (SpectroGlyph, LLC). Norharmane matrix and a 20x40 µm step size were used for MSI [1]. For DDA-Imaging IT-MS/MS were acquired in parallel with each Orbitrap transient. Acquisition of MS/MS for every m/z was achieved without prolonging the FTMS-MSI acquisition. ALEX123 software was used to assign both sum-composition and molecular-lipid identifications utilising both FTMS and IT-MS/MS data [2].

Results:
Exploiting the parallel operation of the Orbitrap Elite our DDA-Imaging-method yielded both high mass resolution FTMS (encoding both the MSI data and sum-composition level lipid assignments) and high quality IT-MS/MS of nearly every ion observed in the MSI experiment. Both a 240,000 resolution FTMS scan and an IT-MS/MS spectrum could be obtained in parallel from each 40 um region of tissue. Even for low abundance lipids having relative intestines less than 1% high contrast images and high quality MS/MS data were acquired.

We used ALEX123 to perform automated identification of detected lipids using both the high mass accuracy (+/- 2 ppm tolerance) and IT-MS/MS data. We automatically identified 165 unique sum-composition-level lipid species and 113 unique molecular lipid species that were present in all replicates of rat cerebellum tissue. All IDs are supported by both FTMS and MS/MS and in relevant cases, cross-correlation of positive- and negative-ion mode data. For all lipid species their spatial distribution is also acquired via the FTMS data.

Conclusions
Our method overcomes the current limitations of broadband molecular identifications in MSI. By exploiting a hybrid ion trap/Orbitrap instrument, MS/MS data area acquired without expanding additional experiment. To fully exploit the information-rich datasets, ALEX123 software provides automated and high confidence lipid identifications in parallel to MSI experiments, allowing broad assignment of MS-images to identified lipids.
Novel Aspect:
Parallel FTMS-MSI and MS/MS combined with ALEX123 software provides both MSI and broad structural identification data in a single acquisition.

References

1105 - UHPLC-HRMS METHOD FOR TARGETED ANALYSIS OF CYANOTOXINS IN FRESHWATER

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Keywords: cyanotoxins, microcystins, high-resolution mass spectrometry

Introduction:
Cyanobacteria are the components of regular microbial succession in periphyton formation, however, some species are known to produce toxic secondary metabolites (cyanotoxins), which vary in structure and harmful properties. During the past decades, an increase of cyanobacterial blooms was noticed in freshwater bodies. To monitor cyanotoxins' levels and prevent both human poisoning and wildlife damage, suitable analytical methods need to be developed.

Methods:
For sample pretreatment, SPE using 2 cartridges in sequence for multiple toxins has been optimized and employed. The chromatographic separation was achieved using a C18 analytical column (150x2.1 mm, 2μm) with acidified acetonitrile and water as mobile phase. The chromatographic separation was coupled to a Q-ExactiveOrbitrap instrument (Thermo Fisher Scientific) and the MS data was acquired in both full scan and parallel-reaction monitoring modes (PRM).

Results:
Ultra-high performance liquid chromatography coupled to high-resolution mass spectrometry (UHPLC-HRMS) was developed. This method provides targeted analysis of cyanotoxins (microcystins, nodularin, cylindrospermopsin, and anatoxin-a) with instrumental limits of detection between 0.1 ng/L (4 toxins) and 1 µg/L (2 toxins). Application of PRM gave an opportunity to identify the most abundant fragments for each toxin. Additionally, HRMS provides an assessment to the potential presence of transformation products and other non-targeted toxins in the samples. The developed method was applied for the study and characterization of cyanotoxins concentrations in Catalonia freshwater reservoirs.

Conclusions:
The developed method can be applied for identification and quantification of cyanotoxins of different chemical classes.

Novel Aspect:
A sensitive, fast and robust method for the analysis of cyanotoxins in freshwaters based on UHPLC-HRMS was developed.
Large-scale Ring Trial metabolomics study of human blood plasma using diverse FT-ICR-MS systems

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Keywords: FT-ICR-MS, Ring Trial, human blood plasma, metabolomics

Introduction:
A ring trial study of main blood plasma metabolites, in collaboration with over 17 different FT-ICR-MS laboratories worldwide, was organized and administrated at the Helmholtz Center Munich in a strong cooperation with Bruker Daltonics. The aim of this study is to investigate and to validate the implementation of several important ultra-high resolution FT-ICR-MS instruments for performing phenomics studies.

Methods:
A novel standard operating procedure (SOP) was developed at the Helmholtz Center Munich, to assess deep metabolic information on human blood plasma samples[1]. Spiking studies with mixtures of chemical standards were performed to challenge the instruments' performance.

Results:
The overlap of known and unknown metabolites (features), reproducibly detected in all labs, is highlighted. In addition, key instrumental specificities between different FT-ICR-MS mass spectrometers are investigated, such as reproducibility, resolution, FWHM, peak shape, measurement sensitivity, dynamic range and mass measurement accuracy.

Conclusions:
The study explores the capabilities of different FT-ICR-MS instruments with diverse ICR cells working under different magnetic field strengths, and with different operational cyclotron frequencies (1 versus 2 ion frequency measurements).

Novel Aspect:
This study provides an unprecedented large-scale investigation to reliably perform accurate mass measurements for serving phenomics studies in the future.

References
1297 - SIMPLE WORKFLOW FOR PROTEOFORM SEPARATION AND ANALYSIS USING 2D GEL ELECTROPHORESIS-HIGH RESOLUTION MASS SPECTROMETRY.

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Keywords: 2D gel electrophoresis, mass spectrometry, proteoforms, post translational modification

Introduction:
In the early days of proteomics, it was assumed that each spot on a 2-dimensional electrophoresis (2DE) gel represent a single protein1 due to the limitations of MALDI peptide mass fingerprinting for protein identification2. The aim of this study was to answer the question how many proteins can be identified within one 2DE spot applying state of the art LC-MSMS techniques and if additional information about proteoforms can be obtained due to separation of proteins by 2DE.

Methods:
Different 2DE-spots from a differential proteomics approach comparing human breast cancer cells before (control) and after perturbation (inhibition of mannosidase 1 with kifunensine) were analyzed. 60 spots were selected for further analysis after comparing the spot patterns of the 2DE gels. Selected spots were cut out and the proteins were proteolytically cleaved by trypsin. The peptides were analyzed by nano RPLC-ESI-MS/MS on a Q Exactive mass spectrometer.

Results:
Thousands of proteins and proteoforms were separated on the 2DE gel seen visually on the gel as multiple spots. The 60 spots subjected to bottom up LC-MS/MS analysis resulted in having greater insight into the number of proteins behind each spot and the distribution of proteoforms across 2DE gels. On average 125 proteins were identified per spot of the control 2D gel. Using this strategy, proteins could be identified unambiguously. About 42 % of the identified proteins were found in more than one gel spot which suggest that the proteins have varied in the post translational modifications (PTMs). Putative annexin A-2 like protein, desmoplakin and junction plakoglobin were proteins which are most commonly found in multiple spots. These multiple protein spots represent proteoforms having varied isoelectric points and molecular masses but are derived from same parent gene.

Conclusion:
The identification of proteins with state of the art LC-MS/MS from stained 2DE spots, which differ in their intensity in the comparison control vs. perturbed state gives information of proteoform changes associated with response to the perturbation. Combination of 2D gel separation and LC-MS/MS offers great potential in the proteome research focusing on proteoforms.

Novel aspect:
The workflow of 2DE followed by high resolution mass spectrometry represents a simple yet effective approach for identifying changes in the proteome on the level of proteoforms.

References:
RAPID AND EFFECTIVE MULTI-CLASS ANALYSIS OF ANTIBIOTICS IN FEEDINGSTUFFS AT CARRY-OVER LEVEL BY LC-MS/MS TECHNIQUES

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Keywords: antibiotic, mass spectrometry, carry-over, feed.

Introduction
The rough clean up of pipelines after medicated feedingstuffs production can result in a cross-contamination of feed, known as “unavoidable carry-over for non target species”. This can expose farmed animals to undesirable administration of antibiotics, increasing the risk of induced resistance to microbials. Therefore, LC-MS/MS based multi-class methods have been developed and compared to determine up to 38 antibiotics in feed for target and untargeted species.

Methods
Purification of up to 38 antibiotics from feed (tetracyclines, sulphonamides, quinolones, macrolides, pleuromutilins, diaminopirimidines, streptogramines) was performed by solid-liquid extraction. Determination was carried out by three different analytical systems: LC/ESI-QTRAP-MS/MS, UHPLC-Orbitrap-MS and UPLC-Xevo-TQ-MS. In all cases, chromatographic separation was performed on reversed phase columns, using linear gradient elution.

Results
The three test methods were in-house validated according to requirements of Commission Regulation 882/2004/EC [1] and Decision 657/2002/EC [2], evaluating the analytical performance parameters specificity, linearity, limit of quantification (LOQ), limit of determination (LOD), trueness, precision and ruggedness for each analyte. Specificity was assessed analyzing uncontaminated feed samples for poultry, cattle, pig, rabbit, sheet, goat and equine; no matrix interference was observed in the chromatograms at analyte retention times. Trueness as mean recoveries (%), and precision as RSD (%), were evaluated for each method by analyzing blank feed samples spiked at least at three concentration levels and were, respectively, in the range 76.3-107.7% and 1.6-22.8%. Linearity of each detector was verified by the correlation coefficient (r2 ≥ 0.950) of linear regression calibration curves of each analyte. The LOQ for all the antibiotics ranged from 0.010 to 0.100 mg/kg depending on the equipment; likely, LOD ranged from 0.002 to 0.030 mg/kg.

Conclusions
The multi-class and multi-drug methods we developed are all based on a rapid and simple cleanup, an effective chromatographic separation and a highly sensitive detection, allowing for simultaneous quantification and unambiguous identification of up to 38 antibiotics down to 0.010 mg/kg. These analytical methods fit with the requirements of the European Committee for the official control of antibiotics in feed at carry-over levels.

Novel Aspect
These analytical methods can replace single-class methods resulting in an optimization of work, costs and staff organization and in a more effective control activity.

References
**Introduction:**
Arsenic is unique among trace elements by exhibiting a rich organic chemistry spanning over 100 naturally occurring organoarsenic species in the environment. Organoarsenic species range from simple methylated arsenic acids through to arsenic-bound sugars and even more complex arsenic-containing lipids [1]. Here we report our most recent results employing high resolution mass spectrometry (HR-MS) to identify novel environmental arsenic compounds.

**Methods:**
We characterize the complex organoarsenical compositions in various environmental samples by means of positive ionisation HR electrospray Orbitrap MS/MS determination after separation of individual species by reversed-phase HPLC. The importance of tandem-MS as a powerful tool for the arsenic specific identification of unknown compounds is demonstrated and factors influencing the selectivity and sensitivity of the method discussed in detail.

**Results:**
The composition of the naturally occurring organoarsenic compounds are revealed by molecular HR-MS/MS measurements, whereby most of them show characteristic fragmentation. Among these, the most significant fragments [M+H]+ have m/z of 104.9680 (C2H6As+) and 122.9786 (C2H8AsO+) originating from a common polar dimethylarsinoyl headgroup (CH3)2OAs. Furthermore, there is a variety of other fragmentation products supporting the identification of structural features such as arseno-ribosides with m/z 237.0102 (C7H14AsO4+), or arsenophospholipids m/z 483.0607 (C13H29PAsO12+). In dexterous combination with non-arsenic containing fragments such as e.g. 184.0733 (C5H15O4PN+) or corresponding neutral losses e.g. m/z 183.0660, we also show simple ways to identify even more complex arsenic compounds such as cholines or ethanol-amines.

**Conclusions:**
In our work we provide a sophisticated but still simple way for the determination of a wide range of organoarsenicals using high resolution mass spectrometry. Our method is not dependent on standard-compounds, and therefore has potential to open up the field for other scientists and assist in further research in this rapidly expanding field of environmental research.

**Novel Aspect:**
We present an approach to species independent, arsenic-specific determination without the necessity of an element-specific detector by using HR-ESMS/MS and monitor characteristic organoarsenic fragments.

**References:**
DIRECT GAS ANALYSIS USING A MULTI-TURN HIGH-RESOLUTION MASS SPECTROMETER

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JEOL(EUROPE)SAS, European Application Group, Croissy-sur-Seine (1) · TNO, Defence, Safety and Security, Rijswijk (2) · JEOL(EUROPE)B.V., Sales Executive Science and Measurement Instruments, Nieuw-Vennep, (3)

Keywords: Gas analysis, High resolution, High mass accuracy, Multi-turn

Introduction: (Limit of 400 characters)
Small molecule gases such as CO, NOx and SO2 are analyzed by using a portable gas analyzer or a GC-MS. However, portable gas analyzers are only able to measure specific gases, and GC-MS is able to measure a variety of gases but requires special columns to separate small molecule gases. In this study, we measured complex gas mixtures by using the multi-turn HR-TOFMS without special columns.

Methods: (Limit of 400 characters)
We measured gas mixtures by using the multi-turn HR-TOFMS with three different sampling methods - gas mixing system, headspace and a tedlar bag. For the gas mixing system, a sample was mixed that contained benzene (1250 ppm) and arsine (40 ppm). The gases were introduced by using the MS vacuum system to draw the sample directly into the source with a short deactivated capillary column.

Results: (Limit 900 characters)
Target gases were observed, and their compounds were separated by the multi-turn HR-TOFMS perfectly. The mass error for the peaks were within 3.1 mDa. In the case of gas mixing system, the m/z difference of benzene and arsine is 0.1 Da (benzene, C6H6, M+· 78.04640; arsine, AsH3, M+· 77.94452). The results showed that the overloaded peak for benzene (M+) and the smaller peak for arsine (M+) were perfectly separated and detected by the multi-turn HR-TOF MS. In addition, the arsine (M+) mass error was only 0.5 mDa.

Conclusions (Limit of 400 characters)
The multi-turn HR-TOFMS can separate complex gas mixtures by using high mass resolution and can identify elemental compositions by using the system’s high mass accuracy. This means that the multi-turn HR-TOFMS is a powerful tool for analyzing complex gas mixtures.

Novel Aspect: (Limit of 150 characters)
Complex gas mixtures analysis by using a multi-turn HR-TOFMS.
Linear Dynamic Range Improvement in TOF-MS/MS Mode on X500 QTOF System

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TITLE
Linear Dynamic Range Improvement in TOF-MS/MS Mode on X500 QTOF system

KEYWORDS
HRMS TOFMS LDR QTOF Quantitation

INTRODUCTION
Linear Dynamic Range for MS-MS mode was improved by employing beam modulation. This technology was previously used in MS mode, but has now also been developed for MS-MS mode. This allowed the extension of LDR by increasing the upper limit of quantitation, with no changes to the lower limit of quantitation.

METHODS
17 standards were prepared by making 3-fold serial dilutions of 100 µg/mL Alprazolam/Atorvastatin in 50/50 acetonitrile/water containing 10ng/mL Alprazolam-D5/Atorvastatin-D5, respectively. The analyte concentration covered the range of 0.00077400 to 33,300 ng/mL.

Experiments ran alternating between modulation on and modulation off acquiring spectra in TOF-MS/MS mode.

RESULTS
In each case, the LLOQ was not affected by EDR, and the improvement of LDR is due to extension of ULOQ to higher concentration samples as expected. Accumulation times down to 25 ms were tested and did not show a significant impact on LDR. The average increase in the LDR was about 100 fold, from about 2.5 orders up to about 4-4.5 orders.

CONCLUSIONS
The EDR feature for MSMS improves the LDR performance for tested compounds and workflows by extending the ULOQ to higher concentration without effects on LLOQ.
Shorter accumulation, 25 ms and 35 ms in MSMS mode has not caused any loss of LDR performance comparing with 50 ms accumulation time.

REFERENCE

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Applying a Novel Component Detection Algorithm to Help Accelerate Metabolomics Discovery Workflows

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Key words: Component detection, Peak picking, Metabolomics, Untargeted analysis, Accurate mass

Introduction
In untargeted metabolomics workflows, data mining techniques are designed to identify differences between metabolite profiles from control and test groups, relating to specific clinical challenges or states. To accelerate metabolite detection we’ve developed the novel algorithm ‘FIND’ to locate components using high mass accuracy LC-MS TOF data. Here, it is applied to assess the impact of phenolic acids and avenanthramides in the diets of adult human volunteers.

Methods
Blood was provided before and after a 4-week intervention consuming a daily moderate dose of phenolic acids and avenanthramides in oats or a phenolic-low control. Extracted plasma was analysed using high mass accuracy LC-MS/MS TOF (Shimadzu Corporation, Japan). The FIND algorithm was applied to raw data with only an estimate of typical chromatographic peak width (FWHM) used. A collection of inbuilt logic is used to correctly identify masses of the unknown components.

Results
Untargeted discovery-based metabolomics workflows are designed to detect components from complex sample matrices. To help accelerate component detection in metabolomics workflows a novel algorithm has been designed to locate ion signals that are covariant and related in context (charge-state, isotope distribution, adducts, multimers) so that only a true monoisotopic mass of the component is reported.

Using a food-omics study as a model system, the component detection algorithm was applied to analysis of metabolite profiles from a human volunteer study exploring the impact of phenolic acids and avenanthramides in oats. In this study, the component detection algorithm was used to locate individual components in raw data files and to help verify metabolite detection between each test group following dietary exposure. The application of the component detection algorithm is simple, takes seconds to process, and does not require a complex method with many user parameters.

Conclusions
Adult volunteers provided blood before and after a 4-week intervention consuming a daily moderate dose of phenolic acids and avenanthramides in oats or a phenolic-low control diet in a crossover design. LC-MS/MS TOF was used to analyse extracted plasma and the FIND algorithm was applied to raw data to correctly identify the masses of the unknown components. A collection of inbuilt logic correctly identifies monoisotopic masses and related ions are grouped.

Novel aspect
Applying a novel algorithm to high mass accuracy LC-MS TOF data to locate individual components in complex samples.
Keywords: halogenated chemicals, GC x GC-HRMS, compound identification, GC-QTOFMS, dust analysis

Introduction
Consumer electronics typically contain halogenated flame retardant chemicals to comply with flammability standards. However, various negative health effects have been associated with the aforementioned compounds [1]. The focus of this study was analysis of selected dust samples from an e-waste dismantling facility in Toronto, Canada. The objective was to determine any halogenated additives present in the e-waste, and especially additives which may not yet be described in the literature.

Methods
The samples were extracted using solvent-liquid extraction and ultrasound-assisted extraction and did not undergo destructive clean-up. The extracts were injected on a Waters Xevo G2-XS quadrupole time-of-flight mass spectrometer coupled to an Agilent 7890B GC with atmospheric pressure chemical ionization (APCI) in two configurations:
- “Fast GC”, using an Agilent DB5-HT 15m × 0.25mm internal diameter × 0.1µm film thickness; a (He) carrier gas flow of 3mL/min and a 10 min run time.
- Comprehensive two-dimensional gas chromatography (GC x GC), using a method with a slow temperature gradient and an Agilent DB-5 60m x 0.25mm x 0.25µm as the first dimension column and a Restek Rtx-17SIL (1m x 0.15mm x 0.15µm) second dimension column. The 2˚ oven was offset +15˚C from the 1˚ oven.

Results
In the fast GC screening, the halogenated compounds tentatively detected were mainly flame retardants, such as polybrominated diphenyl ethers (and especially BDE-209, which was highly concentrated in some samples), TBBPA and BEHTBP (both present in very high amounts) and EHTBB. Polychlorinated biphenyls were also detected in the samples, but in lower amounts than the flame retardants.

The GCxGC data was further investigated for the presence of target compounds, by extraction of a selected ion chromatogram (SIC) for all target masses. Non-target compounds were also identified on the basis of visual inspection of Kendrick Mass Defect (KMD) plots, constructed using -H/+Cl substitution (34 Da)[2]. The list of halogenated isotopic clusters generated from the 1D-GC data was searched in the GCxGC data (GC Image HRMS v2.5) where, due to the higher separation of peaks, co-elutions with other analytes are less likely. Consequently, the spectra of the target and non-target compounds of interest were “pure”. Structure assignment was aided by NIST library searches. The (soft) nature of the ionization, i.e. APCI in the positive mode, typically provides the molecular ion peak as the base peak with few fragments. Despite this, the matches obtained with the NIST library (containing electron ionisation spectra) were quite good, because the search algorithm places more weight on the matches of higher molecular mass peaks/fragments.
Using this technique, a methoxy derivative of TBBPA was tentatively identified, as were a few dibromophenyl and tribromophenyl isomers. A few non-halogenated compounds were detected as well, mainly organophosphate esters. Among these, the tricresyl phosphates and other triaryl phosphates were in particularly high amounts.

Conclusions
The dust from an e-waste dismantling site contained a wide variety of flame retardants, so dust collected from these types of sites is a good indicator of which flame retardants are contained in various types of consumer electronics. The approach presented in this study has the benefits of providing quick information through the Fast GC screening. The enhanced separation of GCxGC provided in-depth information (viz. isobarically pure mass spectra and multidimensional GC retention behavior) at the cost of analysis time.

Novel Aspect
To the authors’ knowledge, this is the first study using both a Fast GC screening and GC × GC for identification and confirmation of chemicals identified in e-waste dust samples.

References
Introduction:
In human and environmental exposure, many of the compounds of concern for their environmental persistence and health risks are halogenated. The twelve initial persistent organic pollutants (POPs) included in the Stockholm Convention are all chlorinated. Many of the new POPs added subsequently are brominated, such as hexabromobiphenyl, (HBB), hexabromocyclododecane (HBCD), polybrominated diphenyl ethers (PBDEs, from the Tetrabrominated to Heptabrominated), or fluorinated, such as perfluorooctane sulfonic acid (PFOS), its salts and perfluorooctane sulfonyl fluoride (POSF) [1]. Furthermore, most chemicals listed in studies evaluating the potential of compounds to meet the criteria of being a POP, (persistence, bioaccumulative and ability to undergo long-range transport) are also halogenated [2,3,4].

For this reason, in the past few years there have been several studies trying to identify and highlight previously unknown halogenated chemicals of both natural and anthropogenic sources such as in stream sediments from southern Ontario [5], Atlantic dolphin blubber [6], and Prymnesium parvum haptophytes [7] and others.

In general, for this type of studies, the samples are usually acquired in full scan or data-independent analysis mode. This is followed by a lengthy data processing procedure, typically involving three steps:
1) Target screening / quantitative analysis step, where information about the levels of analytes present are confirmed with standards
2) Suspect screening step, where compounds of interest (for which standards are not available) are monitored
3) Non-target screening step, where the identification of other compounds is attempted.

For the confirmation of chemicals tentatively identified, tandem mass spectrometry information is necessary. To obtain it, an additional targeted MSn analysis is often required, even if the initial acquisition mode was data-independent analysis, if the intensity of the peak of interest is not high enough to be selected for fragmentation. Consequently, the aim of this study is to develop an instrumental method designed to speed up and facilitate later data analysis by:
1) Incorporating an element of target and suspect screening in the acquisition method itself
2) Highlighting chemicals containing more than three atoms of chlorine and bromine, which are more likely to have an adverse effect on human health
3) Obtain MS2 scans of the halogenated compounds of interest to use in their identification, without requiring subsequent instrument time.

Methods:
The instrument employed in the present study was a Shimadzu Nexera X2 UHPLC coupled to an Orbitrap Fusion MS detector (Thermo Fisher Scientific, Waltham, MA, USA). The detector was run at a resolution of 120,000 and was equipped with an atmospheric pressure chemical ionization (APCI) source. As source parameters, the negative ion discharge current was set at 10 µA and the positive at 4 µA. The sheath gas was set at 25 arbitrary units (equivalent to 3.17 L/min), and the auxiliary gas at 5 arbitrary units (corresponding to 5.08 L/min). The sweep gas was turned off. The ion transfer tube temperature was set at 250 °C and the vaporizer temperature at 325 °C.

A volume of 5 µL of extract was injected, and separation was achieved using a Waters Acquity BEH C18 column (100 mm × 2.1 mm i.d., 1.7 μm particle size), fitted with a 5 mm matching Vanguard pre-column, with the same characteristics. The mobile phases employed were A: milliQ water and B: methanol, both with 5 mM ammonium
formate added. The flow rate was 0.3 mL/min and a linear gradient from 5% to 100% methanol in 8 min, followed by a 2 min hold before returning to the initial conditions for 4 min.

Standards were injected both in negative and positive polarity. For testing and development of the method, standards of brominated and chlorinated chemicals – mainly flame retardants – were employed. The “BDE-CM” mixture of PBDE congeners (28, 47, 99, 100, 153, 183 and 209) was purchased from Accustandard (New Haven, CT, USA), Hexabromocyclododecane (HBCD) from Wellington Laboratories (Guelph, ON, Canada) and three mixtures of novel FRs (From the INTERFLAB inter laboratory study [8]) were kindly donated by Wellington Laboratories. The mixtures contained 26 FRs in total: 2,4,6-Tribromophenyl allyl ether (ATE), 2,3,5,6-Tetrabromo-p-xylene (p-TBX), 2-bromoallyl-2,4,6-tribromophenyl ether (BATE), 1,2,3,4,5-pentabromobenzene (PBBz), Tetrabromo-o-chlorotoluene (TBCT), Pentabromotoluene (PBT), Pentabromoethylbenzene (PBE), Bis(2-ethylhexyl) tetrabromophthalate (BEHTBP), 1,2,3,4,5-pentabromobenzene (PBBz), Tetrabromo-o-chlorotoluene (TBCT), Pentabromotoluene (PBT), Pentabromoethylbenzene (PBE), Tris(1,3-dichloroisopropyl) phosphate (TDCCP), 2,4,6-Tribromophenyl 2,3-dibromopropyl ether (DPTE), Hexabromobenzene (HBBz), Tris(2-butoxyethyl) phosphate (TBEP), Pentabromobenzyl acrylate (PBB), Bis(2-ethylhexyl) tetrabromophthalate (BEHTBP), syn-Dechlorane Plus (s-DP), alpha-Dechlorane Plus (a-DP), Octabromotrimethylphenylindane (OBIND), Decabromodiphenyl ethane (DBDPE), alpha-Tetrabromoethylcyclohexane (aTBECH), beta-Tetrabromoethylcyclohexane (bTBECH), alpha-1,2,5,6-Tetrabromocyclooctane (aTBCC), beta-1,2,5,6-Tetrabromocyclooctane (bTBCC), 5,6-Dibromo-1,10,11,12,13,14-hexachloro-11-tricyclo[8.2.1.02,9]tridecene (HCDBCO) and Tris(2,3-dibromopropyl)isocyanurate (T23BPIC; abbreviations are those used by Wellington Labs) considered as current use compounds. All acronyms are as described in the certificates of analysis from the manufacturers and reference [8].

Results:
To achieve the aims described above, the detector needed to be fast enough to cycle between full MS acquisition and data-dependent MS2 acquisition and the cycle time low enough to permit at least two cycles of MS2 acquisition per peak. A cycle time of 0.8 seconds was chosen for this purpose.

Several filters were set after the initial Full MS scan, serving to select only the desired chlorinated and brominated ions to the latter type of acquisition.

The most relevant filters used to select just the ions with >3 atoms of Cl and/or Br for fragmentation and acquisition of MS2 spectra were: 1) “Targeted Mass Difference” and 2) “Targeted Isotopic Ratio”. In the first filter, it was specified to select pairs of ions with a mass difference of 3.9941 Da 3.9959 for chlorinated and bromine-containing substances respectively. These values were chosen instead of the typical A/A+2 pair because of having the benefit of decreasing the number of false positives of non-chlorinated or brominated ions being selected for MS2.

For the second filter, “Targeted Isotopic Ratio” the input was the typical ratios of isotopomers in a halogenated cluster of 3-14 atoms of Cl or Br. The ratios were calculated theoretically as described in a previous publication [9]. Some tests have also been done using an average value for these ratios and higher tolerance values, but this setup was less than ideal, as it generated higher numbers of false positives.

An exclusion list was defined by monitoring the most abundant ions coming from the mobile phase and the LC system. The list was uploaded into the “Targeted Mass Exclusion” filter, to prevent these ions from being selected for MS2 fragmentation.

An “Intensity” filter was used as well, to eliminate ions close to background levels (filter set at 5E3). The “Precursor Selection Range” was set at 250-1,200, to eliminate the 79/81 cluster and the 159.83 characteristic to many brominated analytes and thus highly unselective.

The “Dynamic Exclusion” filter had the purpose to ensure that ions from more than one halogenated cluster was selected for the data-dependent MS/MS scan, if more than one such cluster is present at the same retention time. This is accomplished by preventing an ion from triggering a second data-dependent scan, for 20 seconds after it had triggered the first one.

To increase the likelihood of obtaining high quality MS/MS data, the “Apex Detection” filter was used, to trigger the subsequent data-dependent MS/MS scans after the apex of the peak, when the intensity of the clusters of interest is at its highest. For this purpose, an expected peak FWHM of 3 seconds was found be adequate, with a desired apex window of 15%.

A second data-dependent MS/MS was added, preceded by a “Targeted Mass Trigger”. This particular scan event was triggered whenever one of the masses on the list was detected. The ions observed in APCI negative mode for the analytes specified in the Materials and methods section were added to this list (targeted screening) and also accurate masses for other relevant analytes from the literature [9,10], such as 2,4,6-tris(2,4,6-tribromophenoxy)-1,3,5-triazine (TTBP-TAZ), for which standards were not available (suspect screening).
At the present stage of the method development process, the method correctly selects chlorinated and brominated clusters for data-dependent MS2 in over half of the cases examined. This can be easily improved by increasing the tolerances of parameters such as the isotope ratio, targeted mass difference, decreasing dynamic exclusion time and adjusting the parameters apex detection (or removing it entirely). However, doing so increases the number of false positives obtained. For example, for BEHTBP, the full scan MS spectrum was obtained, the most abundant isotopomer from the main cluster was automatically selected for the first data-dependent MS2 scan event and the same one was successfully selected as being on the list of the targeted mass trigger. But the same cannot be said for the second, less abundant isotopic cluster from the mass spectrum.

Conclusions:
As a conclusion, the method is accomplishing most of what it was intended for, but more fine tuning is needed for it to be fully suited for its intended purpose. Further studies on highly complex samples with this method are planned, after an additional tuning using indoor dust and waste water stream samples.

Novel Aspect:
To the authors’ knowledge, this is the first study describing a method which provides targeted and suspect screening information, highlights halogenated chemicals in the chromatograms and provides MS2 information for the identification and confirmation of halogenated analytes, all in one run.

References:
1. http://chm.pops.int/TheConvention/ThePOPs
Introduction: (377)
Despite the great variety of ionization sources available, there is no single analytical technique for fully analyze a crude oil. Ion discrimination and ion suppression are limiting the compound detection [1,2]. While ESI is the method of choice for the detection of polar compounds, APPI and APCI favors the non-polar and low-to-medium polar compounds respectively. Until now, there is few information on the combination of different sources.

Methods: (394)
A light crude oil was dissolved and diluted in toluene/methanol (1:1) at a final concentration of 250 ppm. Mass spectrometric analysis was performed on an Orbitrap Elite. The spectra were collected in SIM mode with a mass resolution of 480000 (at m/z 400, 1.5s transient). ESI positive, APPI and APCI as well as the combination of ESI with APPI, or APPI with APCI were selected as ionization Methods: For APPI, a Krypton VUV lamp emitting radiation of 10 and 10.6 eV was used.

Results: (761)
The results obtained with different ionization methods show clearly that under ESI the ionization of nitrogen containing compounds is favored, while under APPI hydrocarbon species as well as oxygen containing compounds in both protonated and radical form were efficiently ionized. The combination of these two methods acted as an intermediate, allowing the ionization of non-polar to polar compounds. An overall increase was observed mainly in the O1 and O2 classes compared to the results obtained only from electrospray, while the classes of OS1 and S1 showed the highest population abundance after the combination of these Methods: On the other hand, in case of APCI, the hydrocarbon species as well as the N1, O1, O2, OS1 and S1 classes showed a higher abundance in their protonated form compared to the APPI. After their combination, an increase of the radical species in all the classes was observed.

Conclusions: (383)
Although each ionization method favors the detection of specific types of compounds, the combination of these methods gives the opportunity to utilize all the benefits of the individual Methods: The use of different ionization methods provides complementary information about the crude oil constituents. To obtain information on a molecular level, high resolution mass spectrometry is the method of choice for the analysis of such complex mixtures.

Novel Aspect: (123)
The effect of combining different ionization methods together for the analysis of complex mixtures is investigated on a molecular level by HRMS.

References:
1178 - ABERRATION-BASED NUMERICAL OPTIMIZATION OF ION MIRRORS

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Keywords: Mathematical modeling, multireflectron geometry optimization

Introduction: Synthesis and optimization of a multireflectron system based on ion mirrors
Mass-spectrometry systems based on ion mirrors have been introduced in 1973 [1] and now represent a perspective class of high-resolution MS equipment [2, 3]. The aim of this work is computer-aided optimization of aplanar-symmetric systems of meshless ion mirrors with the use of different numerical approaches.

Methods: Numerical approaches to optimization of planar-symmetric meshless ion mirrors
A part of the Matlab PDE Tollbox program code oriented to solve the Laplace equation was updated and tested. One of the main features of the newly developed code is a possibility of preforming numerical optimization as applied to ion mirror simulation based on aberration theory.

Results:
The classes of ion mirror geometries satisfying the prescribed requirements have been found
The possibility of further minimization of the aberrations of interest has been shown
The alternative geometries have been studied and simplified

Conclusions
As a result of optimization calculations, a new geometry of the "stairs" type has been designed, which represents a compromise between different geometries based on rectangular electrodes
On the other hand, it is shown that the geometries with rectangular electrodes possess fairly small aberrations of interest and are the simplest among the geometries explored

Novel Aspect:
Computational optimization algorithms based on aberration theory to synthesize ion multireflectron systems with prescribed characteristics have been implemented.

References
APPLICATION OF ETHCD FOR LEUCINE/ISOLEUCINE DISCRIMINATION IN PEPTIDES

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Keywords: EThcD, Orbitrap, peptidomics, leucine/isoleucine discrimination

Introduction:
For many years Edman degradation was a golden standard for peptide sequencing. However since 1990s mass spectrometry (MS) has become an effective alternative with significant advantages like possibility to uncover non-standard amino acids and post translational modification. One problem for MS in this field is isomeric leucine / isoleucine discrimination. The aim of our work is to develop reliable and efficient method exploiting ETD to overcome this difficulty.

Methods:
Experiments were carried out on Orbitrap Elite mass spectrometer with electrospray ionization source. Tryptic peptides of the human serum albumin, bacterial protein gp188, natural peptides isolated from skin secretion of Rana ridibunda frog and several synthetic peptides were introduced into the ion source using a syringe pump. Mass spectra were measured in a positive ionization mode in Orbitrap mass analyzer with 120,000 resolving power.

Results:
As a starting point we improved MS3 sequential ETD-HCD activation developed by our group earlier. To achieve maximum spectral quality and fragmentation efficiency all parameters of ion collection and fragmentation energy in MS3 ETD-HCD mode with maximum possible ion isolation width (600 Da) for peptides were optimized. The resulting ETD-HCD method, with "broad-band" activation made it possible to identify structure of 83 out of 93 leucine / isoleucine residues. Interfering process of radical site migration may be suppressed by a careful choice of the lowest possible energy for w-ion formation. The main disadvantage of "broad-band" activation is low efficiency of z-type ion formation for long peptides (with m/z more than 2000 Da). In the advanced version EThcD was used to accelerate data acquisition. Besides it improved the spectra quality and had a greater sensitivity. Application of new method to a non-separated mixture of skin secretion peptides of frog Rana ridibunda allowed correctly identifying 60 of 66 Leu / Ile residues.

Conclusions:
New EThcD method exhibiting nearly 90% efficiency was developed for reliable Leu/Ile discrimination in peptides. Aspects of ion optics tuning were studied and ETD, ETcaD, and EThcD modes compared. Radical site migration interfering with Leu/Ile discrimination can be suppressed by careful choice of minimal NCE. The present version of the developed approach may constitute a basis for the creation of automatic sequencing protocol.

Novel Aspect:
The developed method is advantageous compared to existing ones. It has a potential for automation and coupling to LC to become a routine proteomics protocol.
Keywords: apixaban, monolithic silica column, LC-ESI-MS/MS, pharmaceutical application

Introduction:
Apixaban (APX), a selective, reversible and direct inhibitor of factor Xa, is in use to reduce the risk of stroke and systemic embolism in patients with non-valvular atrial fibrillation[1]. In spite of some published methods for biological samples [2,3], there are very few methods on its determination in pharmaceutical preparations. The aim of this study was to develop a validated LC-MS/MS method for determination of APX in tablets.

Methods:
Analyses were performed using a Shimadzu LCMS-8040 (Japan) series LC-ESI-MS/MS instrument. APX and irbesartan (internal standard; IS) were separated on a monolithic high-resolution silica column using isocratic elution. The detection was performed in positive ion mode and the precursor-to-product ion transitions were monitored in MRM mode at 460.15→443.20 m/z for APX and 429.15→207.15 m/z for IS. The developed method was validated as per ICH Q2(R1) guideline.

Results:
For APX, linearity could be demonstrated over the concentration range of 2–20 μg/mL (R^2>0.996), and the calculated limit of quantification were about 1 μg/mL. Results for inter- and intra-day assay precision and trueness were obtained using internal quality control samples and remained within the acceptance criterion. External quality control samples were measured at the specified nominal values with inter- and intra-day precisions. Matrix effects were fully compensated by co-eluting IS, which in turn did not relevantly influence ionization efficiency.

Conclusions:
The method development was focused on the optimization of both LC and MS/MS conditions to obtain the best possible sensitivity and future applicability on regular LC-PDA systems. The proposed method was successfully applied on the real samples, possessing very high chromatographic efficiency.

Novel Aspect
The utilization of a monolithic high-resolution silica column, lower quantitation limits and use of IS are novel aspects of the study.

References:
MULTIPLEXED OPERATION OF HIGH RESOLUTION ORTHOGONAL MULTI-REFLECTING TIME-OF-FLIGHT MASS SPECTROMETER.

Boris Kozlov (1) - Jeffrey Brown (1) - Vyacheslav Artaev (2)

Waters, Research, Wilmslow (1) - LECO, LECO, St Joseph (2)

Keywords: multi-reflecting TOF MS, duty cycle, encoded frequent pulsing, folded flight path, artifact-free inverse transform

Introduction:
Multi-reflecting TOF MS incorporate extended ion flight paths to increase resolution, however this is at the expense of duty cycle. Upstream trapping to accumulate ions results in loss of mass accuracy. Alternatively, multiplexing of TOF spectra has been proposed by numerous researchers [1,2,3]. Here we describe the implementation of Encoded Frequent Pushing (EFP) [4, 5], where a repeated sequence of unique time interval pusher pulses is applied.

Methods:
In contrast to Hadamard transform methods, EFP involves a pattern of TOF start pulses where it’s shifted replica does not coincide at more than one point (with a precision comparable to TOF peak width). Decoding by robust statistical verification of all data points produces no artefacts. The main parameter of the decoding algorithm is the ion population of multiplexed spectrum.
Data were acquired using a MR-TOF MS experimental system having a 46m long Folded Flight Path.

Results:
The 2 ms long time-of-flight spectra were acquired by a 1.8 Giga-samples/sec 12-bit ADC (X6 GSPS Innovative Integration), with on the fly decoding performed using computational resources of the GPU card (NVIDIA). They demonstrated two orders improvement of duty cycle and hence sensitivity with no loss of resolution and with dynamic range reaching 5 orders of magnitude in a single ESI-spectrum.
There was no degradation of the mass accuracy when using EFP recorded over the mass to charge range 300 to 3000 (using 2-points for internal calibration). In fact, for weaker peaks within the data, inclusion of more ions helped to improve the accuracy of the mass measurement.
We also found that EFP does not introduce deviations to the relative intensities of various spectral peaks, except when the signal was at the minimal detectable level.

Conclusions
The method is artefact free and provides duty cycles comparable with conventional, non MR-TOF systems without affecting MR-TOF resolution and mass accuracy.

Novel Aspect:
High duty cycle multi-reflecting OA-TOF-MS using artefact-free multiplexed data acquisition method without compromising mass accuracy and resolving power.

References:
Keywords: skin fibroblasts, aging, metabolomics, N-glycans

Introduction:
Fibroblasts are cells that play an important role in wound healing. They are responsible for excreting extracellular matrix at the site of injury and in their myofibroblast form, cause wound contraction and deposit collagen. Delayed wound healing is commonly observed with aging and a major contributing factor is the dysfunction of the fibroblasts. In this study, we explore the metabolic and protein glycosylation changes in skin fibroblasts with aging.

Methods:
Skin fibroblasts were collected from young (YSF) and aged (ASF) donors, cultured to ~1 x 10⁶ cells, and extracted by sonication with 80% methanol. The supernatant was obtained and analyzed using reverse phase (RP) and HILIC LC-MS in positive and negative ESI modes. Precipitated proteins were separated from cell debris, deglycosylation was performed and the N-glycans were labeled using 2-AB. The samples were also analyzed using RP and HILIC LC-MS in positive ESI mode.

Results:
Initial multivariate analysis using Principal Components Analysis (PCA) of metabolomic LC-MS data from positive ESI HILIC and RP modes showed distinct differentiation of ASF and YSF. Orthogonal Projection to Latent Squares-Discriminant Analysis (OPLS-DA) was also performed to identify features that are significantly contributing to the differentiation of the ASF and YSF. Identification of differentiating features revealed that certain phospholipids were lower in ASF samples while acylcarnitines were found to be in higher levels in ASF. This could be indicative of higher rate of lipolysis and reduced mitochondrial fatty acid oxidation in ASF. N-glycan LC-MS analysis also revealed differences in the glycan profile for the ASF and YSF samples. Identification of the significantly differentiating glycans is on-going.

Conclusions:
Preliminary results revealed significant differences in the metabolic and N-glycan profile of skin fibroblasts from young and aged donors. Skin fibroblasts from aged donors were found to have lower phospholipid levels and higher acylcarnitine levels indicating higher rate of lipolysis and reduced mitochondrial fatty acid oxidation function.

Novel Aspect:
We characterized the metabolic and N-glycan profiles of skin fibroblasts from a single set of sample extraction revealing significant changes in skin fibroblasts with aging.

References:
ANNEGRET LAUB (1) - ANN-KATRIN SENDATZKI (1) - GÖTZ PALFNER (2) - NORBERT ARNOLD (1) - LUDGER WESSJOHANN (1) - JÜRGEN SCHMIDT (1)

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Keywords: HPTLC-negative ion DESI-HRMS/MS, mushrooms, anthraquinones

Introduction:
Desorption electrospray ionization mass spectrometry (DESI-MS) is a powerful ambient ionization mass spectrometric technique [1]. Its coupling with high-performance thin-layer chromatography (HPTLC) provides a simple and robust methodological approach for separation and highly-sensitive detection of natural compounds in plants and fungi [2]. Moreover, high-resolution mass spectrometry (HRMS) ensures an outstanding selectivity and specificity of this technique. This methodology can be also be used in natural product research for a fingerprint analysis in crude extracts [3]. We have applied a combination of a normal phase HPTLC with DESI-HRMS for profiling of anthraquinones in Chilean Cortinarius species.

Methods:
Crude methanolic extracts of seven Cortinarius species were investigated by thin layer chromatography using 10x10 cm Silica 60 F254 HPTLC plates. The plates were developed in a horizontal CAMAG chamber using toluene/ethyl formiate/formic acid (10:5:3 v/v/v) as solvent system. The DESI-MS analysis was performed by anOrbitrap Elite mass spectrometer (Thermo Scientific, Bremen, Germany) combined with a 2D-DESI-source (Omnispray System OS-3201, Prosolia Inc., Indianapolis, USA) and operated in the negative ion mode. The MS and MS2 spectra were acquired with the resolution of 30000 by scanning of individual TLC bands in the x-direction at a surface velocity of 200 µm/s. The data were evaluated using Xcalibur 2.2 SP1 software.

Results:
We present HPTLC-negative ion DESI-HRMS results of the analysis of complex crude extracts from seven Chilean species of the genus Cortinarius concerning the occurrence of anthraquinones. These compounds are known as potential chemotaxonomic markers in fungi [4]. Using this method physcion, emodin, dermolutein, hypericin, skyrin, 7,7'-emodinphyscion, and flavomannin C were identified by their elemental composition and partly by their characteristic fragmentation patterns in comparison with reference compounds. It should be pointed out, that the HRMS approach also allows a mass spectral distinction of isobaric ions. This was demonstrated for the detection of some investigated anthraquinones whose nominal mass is accompanied by other compounds in the crude extracts.

Conclusions:
HPTLC-negative ion DESI-HRMS was shown to be a powerful method for the analysis and profiling of anthraquinones in complex crude extracts of different Chilean fungi of the genus Cortinarius. Using this method six anthraquinones were identified by their elemental composition and partly by their characteristic fragmentation patterns in comparison with reference compounds. High-resolution mass spectrometry (HRMS) also allows a mass spectral
distinction of isobaric ions which could be demonstrated for the detection of some investigated anthraquinones whose nominal mass is accompanied by other compounds in the crude extracts.

Novel Aspect:
HPTLC-DESI-HRMS/MS as a powerful tool for chemotaxonomic analysis of Chilean mushrooms

References:
Katharina List (1) - Dorothee Wetzel (1) - Martin Pauers (1)

Boehringer Ingelheim GmbH & Co. KG, Biologicals Development, Biberach, Germany (1)

Keywords: Automation, Sample Preparation, Intact Protein, Handling Robot, Biotherapeutics

Introduction:
The preparation of samples for LC-MS analyses is elaborate and time consuming. Due to more routine utilization of LC-MS in biotherapeutics development, the amount of samples rises, raising the need to automate sample preparation. An automated process will enable faster sample preparation and higher reproducibility and repeatability of the sample.

Methods:
A manual sample preparation protocol, consisting of dilution steps, was transferred to a robotic liquid handling system (Tecan, Fluent). An internal standard was used and added into the samples before the dilution steps for protein concentration determination by LC-MS (Waters Acquity UPLC coupled to a Waters SYNAPT G2-S). Additionally, the protein concentration was determined by UV-photometer.

Results:
LC-MS data confirms the UV data and shows that automated sample preparation presents higher quality in repeatability, reproducibility and comparability in contrast to manual sample preparation. Regardless of the initial concentration of the samples, the robotic system is able to generate dilutions fitting more precisely to the target concentration. Furthermore, it manages to reproduce the same concentration of a sample that was diluted earlier with only minimal deviation and still more exactly as if diluted manually. In addition, the concentrations of the automated prepared dilutions are more accurate from sample to sample meaning an increase of comparability between the samples compared to manual sample preparation. The robotic liquid handling system from Tecan is capable of handling complex and high numbers of protein samples.

Conclusions:
The sample preparation protocol was successfully transferred to a robotic liquid handling system. LC-MS data demonstrated that automated sample preparation is highly reproducible, repeatable and comparable. Further complex sample preparation, such as peptide mapping protocol, is planned to be transferred to the robot.

Novel Aspect:
Reproducible implementation of automated sample preparation on a robotic liquid handling system was achieved.
Introduction:
GC/Q-TOF mass spectrometry (MS) with low-energy electron ionization (EI) and chemical ionization (CI) have developed into versatile analytical tools for the compound identification due to the enhancement in the relative abundance of the molecular ions or their adducts, respectively, as compared to the standard EI. The latter technology offers additional advantages as the ionization process can be controlled with various reagent gases. The use of combination of CI and GC/Q-TOFMS will be shown here with several compound classes including organochlorine and organophosphorus pesticides.

Methods:
Experiments were done with an Agilent 7250 GC/Q-TOF equipped with a low-electron energy EI and an interchangeable CI source. The test compounds included: organochlorine (OCP) and organophosphorus (OPP) pesticides, chlorinated acids and a few conazoles. The GC separation was on a 30 m x 0.25 mm id x 0.25 μm HP-5MS capillary column using He at 1.2 mL/min. The injector temperature and the MS interface were set at 280°C. Methane (99.99%) was used as reagent gas. For positive CI, the methane flow was set at 20% and for negative CI at 40%. For NCI, the source and the quadrupole temperatures were set at 150°C. For PCI, the source temperature was set at 280°C and the quadrupole temperature at 150°C. The spectral data were acquired at 5 Hz and the mass range was 50-650 m/z. 2H-Perfluoro-5,8-dimethyl-3,6,9-trioxa-dodecane (PFDTD) was used to tune the mass spectrometer in the CI mode. Data analysis was performed using Agilent MassHunter (MH) Qualitative Analysis software version B.08 as well as MH Quantitative Analysis software version B.09.

Results:
Low-energy EI provided an increase in relative abundance of molecular ions for halogenated dienes like hexachlorobutadiene and hexachlorocyclopentadiene, halogenated aliphatic ethers, and some phthalate esters. The interchangeable prototype CI source was evaluated with selected OCPs and OPPs in negative mode using methane as reagent gas. Significant decrease in fragmentation was observed for the OCPs, OPPs, and the chlorinated acids as methyl esters, when using negative CI, although the molecular ion was not always the base peak for these compounds. Nonetheless, fragmentation in CI leads to larger m/z fragment ions that helps in both compound identification as well as targeted analysis. The NCI spectra of a series of 14 conazoles including aza-, eta-, propi- and difenoconazole will be presented. NCI has a different fragmentation mechanism than EI and with high resolution Q-TOF such differences between the two ionization mechanisms can be easily explained.

Conclusions
Benefits of the 7250 GC/Q-TOF system equipped with a low energy-capable EI source as well as an interchangeable prototype CI source were explored for targeted and untargeted analysis applications. Chemical ionization alone or in combination with low-energy EI and a high-resolution GC/Q-TOF provides new opportunities in compound identification.

Novel Aspect:
Use of high-resolution GC/Q-TOFMS with interchangeable low-energy EI or prototype CI sources in positive or negative mode
Keywords: Alkylphenolpolyethoxylates (APEOs), Alcohol polyethoxylates (AEOs), Orbitrap, High resolution mass spectrometry, Textiles

Introduction:
Non-ionic surfactants such as alkylphenolpolyethoxylates(APEOs) and alcohol polyethoxylates (AEOs) are widely applied in textile industries. They are potential sources of environmental pollution. In recent years, APEOs and AEOs have been detected and reported frequently in environmental samples, such as water and sediments. In this study, we describe a high-throughput and sensitive method for textiles analysis, using liquid chromatography coupled to quadrupole-Orbitrap high resolution mass spectrometry (LC-Q-Orbitrap HRMS), for the simultaneously quantitative analysis of 40 target APEO oligomers with reference standards and screening of 160 AEO oligomers without standards in textiles.

Methods:
The APEOs contain nonylphenol ethoxylates (NPEOs) and octylphenol ethoxylates (OPEOs) with an EO number of ethylene oxide of 1-20, while AEOs focus on C11EOs-C18EOs with an EO number of 1-20. After ultrasonic extraction in methanol, the extract was directly separated using a core-shell CORTECS C18-column and analyzed by Full MS/dd-MS2 (data dependent acquisition) scan in ESI positive mode. Two best sensitivity experimental conditions for APEOs with short EO chains (AP(EO)1-2) and long EO chains (AP(EO)3-20) were investigated, respectively.

Results:
Most APEO oligomers had wide concentration ranges and the correlation coefficients (R2) were higher than 0.999. The limit of quantitation (LOQ) values for NP(EO)3-20 oligomers ranges from 16.00 to 52.80 µg/kg and for OP(EO)3-20 oligomers is from 2.40 to 8.00 µg/kg. LOQ for NP(EO)1 and NP(EO)2, OP(EO)1 and OP(EO)2 was 2.40 mg/kg and 0.24 mg/kg, 1.20 mg/kg and 0.16 mg/kg, respectively. The average recovery for each APEO oligomer in cotton and polyester matrix was between 78% and 110% at three spiked levels and the relative standard deviation (RSD %) was below 10%. Theoretical m/z and assignments for MS/MS product ions of AP(EO)1-20 were predicted, the fragmentation regularity was divided into 7 groups according to different structures and three typical ions were listed. At the same time, based on the similarity of APEOs and AEOs in structure as well as previous reports on these two kinds of non-ionic surfactants, three typical product ions of APEOs were selected for confirmation of AEOs without reference standards. Then for AEOs suspects, a HRMS compound database containing 160 AEO oligomers was built and several parameters such as exact m/z, isotopic patterns, predicted product ions and predicted retention time were used for screening and confirmation. The established method was successfully applied for analysis of 40 commercial textile samples. Compared with OPEOs, NPEOs, especially NP(EO)3-15 oligomers, were widely detected in samples and the total concentration ranged from 1.56 to 1376.31 mg/kg. AEOs were also found in most samples, among which C12-14, C16 and C18 compounds appeared more frequently and the EO chains mainly ranged from 3 to 15.

Conclusions:
A high-throughput and sensitive LC-Q-Orbitrap HRMS method has been established for simultaneously quantitative analysis of 40 APEOs and screening of 160 AEOs in textiles. Under two optimized chromatographic and mass spectrometric conditions, all oligomers with EO chains from 1 to 20 can be analyzed with high sensitivity. The method has been successfully applied to analyze 40 textile samples. NP(EO)3-15 and AEO homologs of C12-14, C16 and C18 with EO chains ranging from 3 to 15 were frequently detected in samples. This method is rapid, high throughput and sensitive. Moreover, any other potential APEOs and AEOs oligomers interested can be added into the compound database and retrospective analysis of them is feasible for present method.
Novel Aspect:
40 APEOs targets and 160 AEOs suspects in textiles were simultaneously analyzed. 
APEOs with both short and long EO chains can achieve high sensitivities. 
Exact m/z and fragmentation pathways of main APEOs fragments were predicted. 
Fragmentation regularities of APEOs were used for confirmation of AEOs suspects. 
Method was applied on 40 textile samples and oligomers detected were concluded.
Introduction: (388/400 characters without spaces)
FT-ICR MS offers at least 10X times higher mass resolving power than any other mass analyzer, and is thus the mass analyzer of choice for "omics" applications. 21 tesla FT-ICR MS [1] enables ultrahigh mass resolving power at on-line HPLC speed (< 1 second per acquired transient) for high-throughput analysis of complex organic mixtures: e.g. assignment of >450,000 distinct elemental compositions from a single LC FT-ICR mass chromatogram of Atjabasca bitumen.

Methods: (399/400 characters without spaces)
We perform protein top-down and middle-down MS/MS (front-end ETD [2], CID, or UVPD; in-cell IRMPD). Our ICR cell is dynamically harmonized, with 120º azimuthal excitation/detection (for improved S/N and elimination of 3rd harmonics) and segmented electrodes (for 3X ICR frequency detection, and thus mass resolving power equivalent to 3X magnetic field strength). Broadband phase correction yields absorption-mode mass spectra (for 2X LC/MS mass resolving power).

Results: (804/900 characters without spaces)
Based on various front-end extraction/chromatography procedures, we identify up to 10X more elemental compositions in petroleum than from any single broadband mass spectrum. For intact proteins, we report monoisotopic mass measurements 10x more accurate than commercial averagine-based Methods: For example, we identify the class, sequence variants, and multiple glycoforms of monoclonal antibodies from multiple myeloma patient serum, with 100% match to DNA germline sequencing, and without false negatives due to incomplete bone marrow aspiration. For human hemoglobin, we are able to identify sequence variants as well as thalassemia (from, e.g., the abundance ratio of delta chain/beta chain). Work supported by NSF Division of Materials Research through NSF DMR-11-57490 and DMR-1644779, The Gulf of Mexico Research Initiative to the Deep-C Consortium, the Florida State University Future Fuels Institute, and the State of Florida.

Conclusions (395/400 characters without spaces)
FT-ICR mass resolving power and data acquisition speed increase directly with magnetic field strength, B0. Also, mass accuracy, dynamic range, and peak non-coalescence scale as B0^2. This talk will present new benchmarks for FT-ICR MS performance, based not only on 21 tesla magnetic field (highest in the world), but also on recent advances in front-end separations and MS/MS, ICR excitation and detection, and data reduction (mass calibration; phase correction).

Novel Aspect: (132/150 characters without spaces)
21 tesla FT-ICR MS [1], with front-end ETD [2], dynamically harmonized ICR cell with 120º azimuthal excitation/detection and tripled frequency detection

References
THE ADVANTAGES OF USING AN MRMHR ACQUISITION FOR INCREASED SENSITIVITY FOR TARGETED ANALYSIS USING A QTOF INSTRUMENT

Daniel McMillan (1) - Phil Taylor (2) - KC Hyland (3) - Chris Borton (4)

SCIEX, Business Development, Warrington (1) - SCIEX, Marketing, Warrington (2) - SCIEX, Application Development, Redwood Shores (3) - SCIEX, Application Development, Framingham (4)

Keywords: QTOF, Targeted, MRM, Sensitivity

Introduction:
When attempting to quantify with a QTOF instrument using a fragment ion instead of the signal from the TOF acquisition is that the collision energy for a given fragment is not formed at an optimum collision energy. A novel feature of the SCIEX X500R QTOF System is the ability to acquire specific MRM pairs just as are acquired in a triple quadrupole instrument thus improving the sensitivity of the method. This presentation discusses the concept of an MRMHR experiment and demonstrates the advantage of this technique.

Methods:
A set of standards were analyzed using a standard data dependent scan where the parent ion of each compound is selected for fragmentation in Q1 and the ion is fragmented using a collision energy of 35 volts. A quantitation method was created using the two most intense fragment ions and an instrumental detection limit was determined. The same set of standards was re-analyzed using an MRMHR scan. The instrumental detection limit from the MRMHR data was determined and compared to the previous fragmentation data. The SCIEX X500R QTOF system has the unique ability to acquire MRM transitions with optimized collision energies resulting in triple quadrupole sensitivity but allows for the acquisition of full scan MS/MS for compound identification without sacrificing cycle time, and we will discuss the features of MRMHR.

Conclusions:
A limitation of traditional triple quadrupole mass spectrometers for quantitative applications is that compound identification relies on a ratio of two ions from the compound fragmentation. Interference from the matrix frequently causes this ration to be unreliable for compound identification resulting in both false positive and false negative results. The slow speed of acquiring full scan MS/MS spectra from a triple quadrupole instrument prohibits the acquisition of useful spectra for compound identification due to degraded cycle times. One of the advantages of QTOF instruments is the speed of acquiring full scan MS/MS spectra during sample analysis without the slow cycle times observed for triple quadrupole instruments. Unfortunately, quantitation is accomplished using fragments from a full scan MS/MS where the collision energy is not optimized for a given transition.

Novel Aspect:
This is the first demonstration of using a QTOF to acquire MRM transitions with optimized collision energies.
CONTEXTUALIZATION OF DARK MATTER IN MS1 FT-ICR MS\(^1\) DATA.

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Contextualization of Dark Matter in MS1 FT-ICR MS\(^1\) Data.

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Keywords: FT-ICR-MS, Dark Matter, Annotation, Metabolomics

Introduction(Limit of 400 characters without spaces): Major proportions (70-95%) of ultra-high resolution MS-signals with known molecular formula cannot be found in metabolomic databases. This mass spectrometric dark matter [1, 2, 3] exhibits statistical significance and is usually not identifiable via MS/MS. This lack of biological MS1-signal contextualization deems major parts of data useless. Interpretation methods that go beyond mere m/z-to-database matching are required to tackle this problem.

Methods(Limit of 400 characters without spaces): Ultra-high resolution MS data on Populus X. canescens were annotated by virtue of mass difference network propagation. Mass difference enrichment analyses (MDEAs), mapping to databases via MDs and compositional similarity and PLS regression were used to extract molecular patterns in association to isoprene synthase knockdown and experimental perturbations. SIM-STITCH MS2 scans were employed to investigate the plausibility of predictions [4].

Results(Limit of 900 characters without spaces): 4,335 m/z peaks that obtained molecular formula annotation occurred non-randomly across 211 mass spectra. Merely 129 molecular formulas (3%) matched to KEGG pathways, while several hundreds of peaks discriminated wild type plants from ISPS knock-downs. MDEAs highlighted the molecular building blocks that were used to build up either class’s biomass. The usage patterns of the building blocks implied by MS1 were consistent with SIM-STITCH MS2 results. MDEA confirmed prior knowledge on the effects of ISPS knock-downs. The highlighted mass difference building blocks (MDBs) were used to connect the mass spectrometric dark matter to KEGG database entries, which resulted in consistent transformation patterns, explaining how the different genotypes transform KEGG compound classes and metabolites from other databases into biomass. Compound class conserving MDs were elaborated. Compound class distributions were in-line with results from compositional similarity approaches with the power to contextualize almost 100% of the obtained data.

Conclusions(Limit of 400 characters without spaces): The mass-difference space in MS1 spectra contains valuable information on usage patterns of molecular building blocks. MDEA can highlight the processes that drive the synthesis of biomass after fundamental perturbations to systemic carbon flux. Dark matter contextualization will help narrowing down fruitful fields of research by highlighting compound classes that change systematically. References:


Novel Aspect (Limit of 150 characters without spaces): UHR-MS peaks are considered as being altered versions of well-known metabolites. We introduce parametric- and non-parametric ways to search for such systemic alterations.
1376 - COMPREHENSIVE ANALYSIS OF MULTI-CLASS HAZARDOUS CHEMICALS IN PLASTIC PRODUCTS USING HIGH RESOLUTION MASS SPECTROMETRY

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Keywords: multi-class, hazardous chemicals, plastic products, high resolution mass spectrometry

Introduction:
With the increasing awareness of the safety of consumer goods, the relevant restrictions on the number of hazardous chemicals are increasing. Simultaneous detection for multi-class targets and non-targets is becoming a more popular and difficult topic.

Methods:
Present work established a novel method for the comprehensive analysis of 41 chemical hazards (20 targets and 21 non-targets) in plastic products (including plastic toys, electrical and electronic plastic parts, polymeric food contact materials, etc.). Compounds studied here belong to four classes, mainly including phthalic acid esters (PAEs), organotins (OTs), perfluorochemicals (PFCs) and flame retardants (FRs), et al. According to the sample material, appropriate organic solvents were selected for ultrasonic extraction, and methanol was used to precipitate the polymer. After standing for some time, the extract was separated by the core-shell particle column and analyzed by the Orbitrap high resolution mass spectrometry.

Results:
The results were confirmed by multidimensional identification of the parameters such as the m/z of the parent ion, the isotope ratio, and the exact m/z of fragments. Method validation for 20 compounds was evaluated. The limits of quantitation ranged from 2 mg/kg ~ 200 mg/kg, the recovery was 74% ~ 120% and the precision was less than 13%. Present method was applied for simultaneous detection both targets and non-targets in 34 plastic samples, among which di(2-ethylhexyl) phthalate (DEHP), dibutyl phthalate (DBP) and diisobutyl Phthalate (DIBP) were detected quantitatively and tetrabromobisphenol A (TBBPA) was detected qualitatively.
Keywords: high resolution mass spectrometry; screening and identification; dyes; textiles; data-dependent acquisition mode

Introduction:
Various prohibited dyes are often misused or even deliberately used for pursuit of economic interest in textile industries. Owning to their widespread use, toxicity on human health, as well as destruction to the ecological environment, some international authoritative consumer laws have strictly limited their use. Dyes used in textiles often contain synthetic intermediates, dispersants and other impurities, making their composition very complex. While traditional analysis usually focus on single group of compounds, and false positive identifications might be possible due to the low selectivity of methods.

Methods:
The currently presented work firstly developed a high-throughput screening and identification method for simultaneous analysis of 48 prohibited dyes, belonging to 5 different groups, in textiles. After extraction in pyridine/water (1/1, V/V) water bath at 95 °C, the extract was filtered with 0.22 μm membrane, then directly separated using a C18 column and analysed by Full MS/dd-MS2 (data-dependent acquisition) scan using electrospray ionization probe (ESI) operating in positive and negative mode. The retention time, exact mass of each precursor and MS2 fragment, as well as the isotopic pattern of precursors were used for the simultaneous analysis of targeted dyes. Moreover, for the identification of complex components within certain dyes such as disperse blue 35, software of ACD/Labs were also used for the prediction of MS2 fragment assignments and calculation of the theoretical accurate mass, which greatly improve the identification reliability and accuracy of the presented method.

Results:
All 48 dyes had wide concentration ranges and the correlation coefficients (R2) were higher than 0.99. The limit of quantitation (LOQ) values ranged from 0.01 mg/kg to 10 mg/kg. The average recovery for each analyte in representative cotton and polyester matrix was between 62% and 120% at three spiked levels and the relative standard deviation (RSD%) was below 15%. The established method was successfully applied for analysis of 40 commercial textile samples with various fiber types, among which disperse orange 37 was detected. This method is rapid, sensitive and accurate, which could be applied on the high throughput analysis of prohibited dyes in textiles.
SIMULTANEOUS DETERMINATION OF NINE WATER-SOLUBLE SYNTHETIC COLORANTS IN COSMETICS BY LC-Q-TOF/MS AND LC-MS/MS

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Keywords: synthetic colorants, cosmetic, LC-Q-TOF/MS, LC-MS/MS

Introduction:
In this study, LC-Q-TOF/MS and LC-MS/MS method were developed to identify and measure the illegally adulterated synthetic colorants (Acid yellow 9, Brilliant black BN, Orange GGN, Yellow 2G, Red 2G, Green S, Carmoisine, Erythrosin B, Patent blue V) that may be found in cosmetics circulated in Korea.

Methods:
We have established and optimized the simultaneous analysis of nine water-soluble synthetic colorants in facial toner using LC-Q-TOF/MS and LC-MS/MS. Colorants-spiked facial toner samples (0.2 g) were prepared with 50 mL of 20% methanol:chloroform (9:1) into a Teflon centrifuge tube. Sample extraction was performed by vortexing the sample for 2 min and ultrasonating for 30 min. The extract was centrifuged for 5 min at 3000 rpm and upper layer was collected in volumetric flask of 50 mL. All solutions were filtered through a 0.22 μm syringe filter (PVDF) prior to LC-Q-TOF/MS and LC-MS/MS analysis.

Results:
The method was validated for specificity, linearity, limit of detection (LOD), limit of quantification (LOQ) and recovery. The linear correlation coefficients were good (>0.99), the limits of detection and quantitation of the method ranged from 0.0039 to 0.0625 μg/mL and from 0.0117 to 0.1875 μg/mL, respectively. The recovery for this LC-MS/MS procedure was 82.5–104.7%, with an RSD less than 15% (n=3).

Conclusions:
In this study, a fast, simple, and accurate method was developed for the identification and quantification of nine water-soluble synthetic colorants in facial toner by using LC-Q-TOF/MS and LC-MS/MS. The method was completely validated showing satisfactory data for specificity, linearity, limit of detection, limit of quantification and recover. Therefore, this study will be helpful for comprehensive analysis and monitoring synthetic colorants in cosmetics.

Novel Aspect:
In this study, we have developed analytical methods for illegally adulterated synthetic colorant in cosmetics circulated in Korea. The high sensitivity, high resolution and accurate mass determination, LC-Q-TOF/MS method is a useful analytical tool for synthetic colorants. Also, the results indicated that the method can be used for rapid and accurate screening of synthetic colorants present in cosmetics.

References:
Introduction:
The ability to visualize complex metabolic behavior of living organisms in situ and at the single cell level represents a bioanalytical grand challenge that has yet to be fully realized. Herein, we present the combination of fiber-LAESI with fluorescence microscopy and ultrahigh resolution 21T-FTICR-MS to address these needs.

Methods:
To provide simultaneous spatial distributions for hundreds of metabolites in biological tissues, an LAESI ion source was coupled with a 21T FTICR-MS system. Utilizing the ultra-high mass resolving power, isotopic fine structure readout was used for metabolite annotations. Single-cell spatial resolution was achieved by delivering mid-IR laser pulses through a sharpened optical fiber, while a dual modality microscopy allowed for precise targeting of cells.

Results:
Spatial heterogeneity was tackled using LAESI coupled with the 21T-FTICR-MS. This unique instrumental configuration provides exquisite mass and spatial resolution, down to the single cell level. A dual channel microscope capable of both simultaneous brightfield and fluorescence imaging was combined with the fiber-LAESI-MS to enable selection of specific cells. For instance, cells in a soybean root nodule infected by GFP labeled soil bacteria were targeted for analysis in a heterogeneous population of infected and uninfected cells. The superior mass resolution and accuracy offered by the 21T FTICR-MS facilitated identification of differentially abundant metabolites in soybean root nodule cells infected by wt and nifH- rhizobia. Notably, this novel capability facilitated characterization of organometallic molecules due to their natural isotope patterns (e.g., 57Fe isotopologues). Preliminary data indicates significantly lower abundance of heme b, disaccharide, and S-adenosylmethionine in the nifH- mutant.

Conclusions
We demonstrate that LAESI coupled with the 21T FTICR-MS holds tremendous potential for in-situ single cell metabolomics as illustrated by the study of plant-microbe interactions.

Novel Aspect:
In situ single cell analysis by fiber-LAESI-MS promises new insights into cellular heterogeneity and metabolic noise.
Introduction:
To assess chocolate quality and authenticity, a combination of various analytical procedures is involved, thereby making the process time-consuming and costly.[1] Thus, we investigated the potential of supercritical fluid chromatography coupled to quadrupole-time of flight mass spectrometry (SFC-QTOF-MS) to replace ‘classic’ Methods: Its specific separation character and selective detection make this technique suitable for authentication of fatty foods.

Methods:
Samples were prepared by sequential extraction using hexane in the first step, followed by water extraction of the residue. Analysis of combined extracts was performed by Waters SFC-QTOF-MS with electrospray ionization (ESI). To obtain most comprehensive information about sample composition, the MS method comprised a full MS1 scan to detect potential unpredicted compounds, and several time-scheduled targeted MS/MS functions (parallel reaction monitoring).

Results:
A single 8 minute analytical run enabled us (i) to determine the presence of cocoa butter equivalents and milk fat according to triacylglycerols, (ii) to calculate dry non-fat cocoa solids based on theobromine and caffeine content, and (iii) to screen sugars, i.e. the key components involved in legislation. Optimizing parameters in the individual MS/MS functions (scan time, target enhancement, collision energy) led to a significant adjustment in analyte responses according to their concentration range in samples, e.g. decreased response of 3 major triacylglycerols of cocoa butter in contrast to increased response of minor triacylglycerol specific for milk fat. Attention had to be paid to background contamination (e.g. phthalates) to avoid the suppression of target ion intensity. Thus, in addition to an effort to minimize such contamination, it was useful to acquire a full MS scan not only to obtain comprehensive information about samples, but also to prevent quantification errors caused by abrupt changes in background intensity.

Conclusions:
SFC-QTOF-MS has proved to be a powerful tool to assess chocolate quality and authenticity, enabling fast, simultaneous determination of cocoa butter equivalents, non-fat cocoa solids and sugars. We have demonstrated a unique separation (compared to reversed-phase liquid chromatography) and advantages of using parallel reaction monitoring for targeted analysis. In addition, we have shown the importance of acquiring a full MS scan to aid interpreting the results.

Novel Aspect:
Fast and cost-effective method for the assessment of several chocolate quality and authenticity parameters that are usually analyzed by a combination of standard Methods:

References:
Keywords: Microchannel plate (MCP), Atomic Layer Deposition (ALD), detector

Introduction:
A microchannel plate (MCP) is an array of identical parallel open channels with diameters typically between 5 and 25 microns in a glass plate with a thickness that is typically 40-60 times the channel diameter. MCPs are made of a special lead-silicate glass that, when properly processed, makes the MCP an excellent ion detector and electron multiplier, capable of high gain and low noise. While it has long been possible to coat the input and output surfaces of MCPs to enhance their performance, historically it has been prohibitively difficult to use a coating to affect the inner channel surface that is responsible for electron multiplication. Atomic layer deposition (ALD), which is a process that builds conformal coatings one atomic layer at a time using a sequence of self-terminating gas-solid reactions, offers an opportunity to coat these high aspect ratio surfaces with high uniformity and enhance some aspects of the MCP performance as a result.

Methods:
Standard and coated MCPs were fabricated and their relevant performance characteristics were characterized using industry standard test methods for MCPs. These characteristics include overall gain, gain stability (lifetime), and linear current output. In addition, coated and uncoated MCPs were assembled in detector assemblies intended for mass spectrometry and their instrument-specific characteristics were evaluated using the instrument tune sequences.

Results: Coatings of high electron yield materials such as aluminum oxide can result in higher overall gains when the MCPs are operated at high voltage and lower gains when the MCPs are operated at lower voltages. This is in line with expectations based on the dependence of the kinetic secondary electron emission yield curves as a function of primary electron energy. Coated MCPs have good gain stability during operation at typical vacuum levels (<1E-5 Torr) similar to uncoated MCPs. When used in time of flight detector assemblies, aluminum oxide coated MCPs generally tune at a lower voltage. Aluminum oxide coatings can also be used to form an inert barrier layer on standard MCPs. This can affect long-term resistance stability when the MCPs are exposed to air or dry nitrogen. It is typical for the overall resistance of uncoated MCPs to increase gradually over time. Coating of the MCPs can reduce this effect.

Conclusions: ALD can be used to uniformly coat the high-aspect ratio inner channel surfaces of MCPs and affect their electron multiplication behavior. This provides an additional method for tailoring MCPs to meet the required performance characteristics of particular applications.

Novel Aspect:
An alternate electron multiplication coating can be applied to MCPs to enhance performance.

References
For information please contact: scientific@imsc2018.it
Introduction:
The characterization of trace polar species in fuels is important due to their influence on the fuel’s physical properties, which has given rise to the field of petroleomics [1]. Due to the complex nature of diesel and other crude-derived fuels its chemical composition is often investigated by considering classes of compounds rather than individual chemical species [2,3,4].

Methods:
The chromatographic separation of diesel fuel extracts using hydrophilic interaction liquid chromatography (HILIC) was combined with high resolution mass spectrometry (TOFMS). Selective ionization of trace polar species was achieved with electrospray ionization and atmospheric pressure chemical ionization. Furthermore, ion mobility mass spectrometry was used to obtain additional chemical information.

Results:
The separation of the polar fraction of diesel into classes of compounds, i.e. based on functionality, was achieved. Complementary information was obtained by considering both positive and negative ion mass spectra. Separation of species that readily formed positive ions was influenced by heteroatom content as well as degree of unsaturation (or DBE). Visualisation of the multidimensional data was explored in order to facilitate the interpretation of chemical information obtained. More than half of the detected m/z values were observed to show isomers separated by drift and/or retention time. A large amount of structural information is therefore gained by pre-separation despite the loss in sensitivity. A novel graphical representation has been introduced where Kendrick mass defect (instead of m/z values) is plotted against drift time to visualise information regarding heteroatom content and double bond equivalents (DBE) against structural information, resulting in an orthogonal system.

Conclusions:
Hydrophilic interaction liquid chromatography was used to separate methanol extracts of diesel fuel in terms of heteroatom content (i.e. group-type separation). Ion mobility mass spectrometry provided additional chemical information of species in diesel fuel extracts. The multidimensional data obtained from hyphenated techniques can be visualized in unique ways to facilitate interpretations.

Novel Aspect:
The group-type separation of diesel fuel extracts was achieved with hydrophilic interaction chromatography. Mobilograms were modified to incorporate mass defects.

References:
Keywords: evolved gas analysis, high resolution mass spectrometry, Petroleomics, atmospheric pressure ionization, photo ionization

Introduction:
Petroleomic materials remain to be one of the greatest challenges in chemical analysis. The conversion of heavy oils or exploration of alternative sources raises new challenging issues, such as the formation of deposits or efficient processing of residues. Thermal analysis using evolved gas analysis coupled to mass spectrometry is one powerful approach for deciphering these materials.

Methods:
For this purpose, several thermal analysis systems coupled to different mass spectrometric analyzers, such as ultra-high resolution FT-MS [1,2] and TOF-MS, were applied to study low-viscous and solid petroleomic materials. The setups varied in ionization techniques covering soft vacuum and atmospheric pressure approaches, and hard electron impact allowing in combination an in-depth-chemical description of the desorbed and pyrolyzed material. [3]

Results:
Besides the description of heavy oils and bitumen, asphaltenes were in the primary focus of the study. Generally, for the intended pyrolysis of the asphaltenes, an ultra-complex molecular pattern is revealed. This molecular space could be comprehensively analyzed by ultra-high resolution mass spectrometry equipped with atmospheric pressure chemical and photo ionization revealing the highly aromatic and Sulphur-containing core structures, whereas vacuum single photon ionization was able to analyze the thermally fragmented side chain distribution. Moreover, a vacuum direct inlet probe equipped with electron ionization was able to broaden the picture by evaporating larger components prior thermal decomposition and thereby allow for additional structural information.

Conclusions
Summarizing, mass spectrometric evolved gas analysis has shown to be an efficient technique for Petroleomics. Most importantly the data combination of various setups enabled an in-depth chemical and structural analysis and hypothesizing of macromolecular structural features. Future work will focus on data integration as well as on structural information by adding a chromatographic separation between thermal release and mass spectrometric detection.

Novel Aspect:
Data integration of several mass spectrometric evolved gas analyzer allowing comprehensive chemical analysis and structural motives of complex petrochemical materials.

References:
828 - APPLYING HIGH-RESOLUTION MASS SPECTROMETRY TO ELUCIDATE THE FORMATION AND FATE OF OZONATION TPS IN WASTEWATER TREATMENT

Jennifer Schollee (1) - Rebekka Gulde (1) - Marc Bourgin (1) - Moreno Rutsch (1) - Julian Fleiner (2) - Marc Boehler (2) - Juliane Hollender (1) - Urs von Gunten (3) - Christa McArdell (1)

Eawag, Environmental Chemistry, Duebendorf (1) - Eawag, Process Engineering, Duebendorf (2) - Eawag, Water Resources & Drinking Water, Duebendorf (3)

Keywords: Non-target screening, Ozonation, Transformation products, Advanced wastewater treatment

Introduction: (Limit of 400 characters without spaces)
Ozonation has increasingly been applied for micropollutant abatement in wastewater and high removal rates have been observed for most known micropollutants. However, the formation and fate of ozonation transformation products (OTPs) is not well understood. High resolution tandem mass spectrometry (HRMS/MS) with non-target analysis was applied to study unknown OTPs in real wastewaters in Switzerland.

Methods: (Limit of 400 characters without spaces)
Samples were collected from two wastewater treatment plants (WWTPs) before and after ozonation and measured with Orbitrap HRMS/MS coupled to reverse-phase liquid chromatography (RPLC). Hierarchical cluster analysis grouped features based on intensity patterns and the fate of likely OTPs was investigated. Additionally, a method was developed for HRMS coupled to hydrophilic interaction liquid chromatography (HILIC) to measure highly polar OTPs.

Results: (Limit 900 characters without spaces)
Out of approximately 8000 HRMS features detected at one WWTP, 600-700 features (9%) were determined to be likely OTPs based on their intensity patterns. In subsequent post-treatment, 54-83% of likely OTP features were well-removed, with granular activated carbon (GAC) filters (run up to 40,000 bed volumes) performing slightly better than the biofilters investigated. It is speculated that sorption in the GAC filters accounted for the higher OTP abatement compared to biofilters, where biodegradation was the primary mechanism for OTP abatement. It was also found that OTPs that were well-removed in post-treatment had significantly higher retention times in RPLC (p<0.05, Mann-Whitney U-test), suggesting recalcitrance of polar OTPs. To get information on highly polar OTPs, a new method combining HILIC with HRMS/MS was developed and preliminary results indicate that previously undetected OTPs can now be investigated with this method. Formation of OTPs from 50 micropollutants is being further studied with HILIC- and RPLC-HRMS/MS.

Conclusions (Limit of 400 characters without spaces)
The combination of HRMS with non-target analysis provides a more comprehensive picture of the formation and abatement of OTPs. By developing a HILIC-HRMS/MS method, unknown highly polar OTPs can also be evaluated. The ability to monitor OTPs in WWTPs is critical to elucidate persistent OTPs that might make their way to the aquatic environment.

Novel Aspect: (Limit of 150 characters without spaces)
Novel analytical and data analysis methods applied to HRMS/MS data were used to evaluate the formation and abatement of OTPs during advanced wastewater treatment.
858 - ANALYSIS OF CATIONIC POLAR PESTICIDES IN BABY FOOD USING ION CHROMATOGRAPHY AND MS/MS OR HIGH RESOLUTION ACCURATE MASS SPECTROMETRY

Frans Schoutsen (1) - John E Madden (2) - Jeffrey S Rohrer (2) - Jonathan Beck (3)

Thermo, takkebijsters 1, Breda (1) - TFS, CA, Sunnyvale (2) - TFS, CA, San Jose (3)

Keywords: LCMS, HRAM, Ion Chromatography, Chlormequat, QuPPe

Introduction:
In this publication we demonstrate analysis of quaternary amine pesticides in baby food samples using cation-exchange chromatography. Polar cationic pesticides such as chlormequat, diquat, mepiquat, paraquat, morpholine, trimethylsulfonium, and propamocarb may occur as residues in food, but may be excluded in pesticide monitoring programs due to challenges using generic multi-residue Methods:

Methods:
Cation-exchange chromatography with serial detection by suppressed conductivity and mass spectrometry. These techniques are demonstrated by targeted MS/MS on a triple quadrupole mass spectrometer and by high resolution accurate mass spectrometry (HRAM MS)

Results:
A chromatographic separation was demonstrated on a prototype column. The six pesticides had good accurate mass, meeting the SANTE requirements of <5 ppm. Native cationic pesticides of interest were not found in the samples. Sensitivities in the low µg/L were determined by spiking pesticides in the samples. Good accuracy was found, with recoveries between 80-120%.

Conclusions
Ion chromatography coupled with HRAM mass spectrometry demonstrated clear benefits for challenging cationic pesticide separation and identification.

Novel Aspect:
Ion chromatography coupled with HRAM mass spectrometry demonstrated clear benefits for challenging cationic pesticide separation and identification.
AN AUTOMATED WORKFLOW FOR HIGH-THROUGHPUT NICOTINE QUANTITATION USING HIGH PERFORMANCE LIQUID CHROMATOGRAPHY COUPLED TO HIGH RESOLUTION ACCURATE MASS SPECTROMETRY FOR PROTOTYPE TESTING.

Sandra Sendyk (1) - Quentin Dutertre (1) - Arno Knorr (1) - Julia Hoeng (1) - Mark Bentley (1)

Philip Morris, Science & Innovation, Neuchatel (1)

Keywords: Mass spectrometry, high-throughput, automation, method validation

Authors: S. Sendyk, Q. Dutertre, A. Knorr, J. Hoeng, M. Bentley

Introduction: (Limit of 400 characters)
Human organotypical 3D tissue cultures are routinely used for in vitro toxicological assessment of the biological impact upon exposure to smoke from a reference cigarette 3R4F and aerosol from heat-not-burn products at the air-liquid interface using the Vitrocell® system. Nicotine, one of the major constituents of tobacco can be used as biomarker of exposure.

Methods: (Limit of 400 characters)
Cigarette smoke or aerosol of heat-not-burn products was deposited at Phosphate Buffer Saline solution (PBS), serving as a surrogate matrix in the Vitrocell® system. A reverse phase high-performance liquid chromatography coupled to high resolution accurate mass spectrometry was used. The procedure was automated using a protocol developed in Pipeline Pilot (Biovia®) from acquisition to reporting.

Results: (Limit 900 characters)
The method was designed enabling analysis of a large number of samples within a short time. Selectivity of chromatographic separation was proven for nicotine and its internal standard (nicotine-methyl-d3), separated from other interferences. A high-throughput method was designed and enables quantitation of nicotine within a dynamic range of three orders of magnitude. The method was validated within a working range from 50 ng/mL to 2000 ng/mL in PBS. Linearity of the method was achieved by an 8-point calibration curve. Furthermore, accuracy, precision and instrumental repeatability were assessed. Quantitation was performed by isotopic dilution technique using stable isotope labelled nicotine as internal standard. The method is reliable for the analysis of subsequent sample batches and demonstrated high robustness in practical applications.

Conclusions: (Limit of 400 characters)
An automated workflow for high-throughput nicotine determination was established delivering robust and accurate data. The method was validated testing accuracy, linearity, instrument repeatability and stability matching acceptance criterion according to standard regulation [1, 2]. It shows suitable performances for routine based application to accurately determine nicotine concentration in PBS.

Novel Aspect: (Limit of 150 characters)
An automated workflow for high-throughput PBS exposed samples analysis by liquid chromatography coupled to high resolution accurate mass spectrometry.

References:
Committee for Medicinal Products for Human Use, European Medicines Agency Science Medicines Health, 2011
CHARACTERIZATION OF STRESS DEGRADATION PRODUCTS OF FOSAMPRENAVIR CALCIUM USING Q EXACTIVE™ PLUS ORBITRAP AND LTQ XL™ MASS SPECTROMETERS, AND PREDICTION OF THEIR TOXICITY USING ADMET PREDICTOR™

DILIP KUMAR SINGH (1) - Mayurbhai Kathadbhai Ladumor (1) - Chinmaya Narayana Kotimoole (2) - Kedar Balaji Batkulwar (2) - Abhijeet Yashwantrao Deshpande (2) - Sanjeev Giri (2) - Saranjit Singh (1)

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Keywords: Fosamprenavir calcium, Stress studies, Degradation products, Orbitrap, ADMET PredictorTM

Introduction:
Stress testing is an essential part of a regulatory-compliant stability program for all types of drugs [1]. It involves i) subjecting the drug to a variety of conditions, ii) separation of degradation products (DPs) by a stability-indicating method (SIM), and iii) characterization of DPs with the help of advanced spectroscopy tools [2]. These studies were systematically accomplished on fosamprenavir calcium (FPV), a protease inhibitor used to cure HIV [3].

Methods:
The drug was subjected to stress under hydrolytic, oxidative, photolytic and thermal conditions. It proved stable in the latter three conditions. The hydrolytic DPs were separated using a SIM, and were initially characterized from mass results obtained by employing UHPLC-Thermo Q-Exactive™ Plus Orbitrap and LC-LTQ XL™ spectrometers. Toxicity of each DP was predicted using ADMET PredictorTM software (Simulation Plus).

Results:
Six DPs (DP 1-DP 6) were formed on acid and base stress. DP 1, DP 2, and DP 4-DP 6 were generated in acid (2N HCl, 75 °C, 6 d), while DP 3 was the lone DP in basic (2N NaOH, 75 °C, 1 d) condition. DP 2 was a common product. Mass studies revealed that the drug was protonated at two different sites, each following a separate fragmentation route. HRMS and MSn data allowed suggesting comprehensive fragmentation pathways of the drug as well as its DPs. The information allowed successful delineation of structures of all DPs. Major hydrolytic routes were cleavage of carbamate ester of the drug to form DP 2, and dephosphorylation of both the drug and DP 2 to form DP 5 and DP 1, respectively. Methylation of the drug in presence of methanol resulted in DP 6 (at aniline nitrogen) and DP 3 (upon replacement of THF moiety). DP 6 underwent carbamate ester cleavage to result in DP 4. Toxicity predictions highlighted that DP 1 was associated with propensity for phospholipidosis, DP 2-DP 4 had susceptibility towards skin sensitization, while DP 5 was associated with reproductive toxicity.

Conclusions:
Stress degradation study on FPV provided significant information on its degradation behavior. The drug was found to be susceptible only to acid/base hydrolysis. A total of six DPs were formed, which were characterized using HRMS and MSn data. The DPs were also evaluated for their in silico toxicity using ADMET PredictorTM software.

Novel Aspect:
Degradation profile of FPV was established and justified through relevant mechanistic explanations. In silico prediction studies highlighted added toxicities of DPs.

References:
1231 - QUANTITATION OF SERUM CREATININE IN MICE USING HYDROPHILIC INTERACTION LIQUID CHROMATOGRAPHY (HILIC) COUPLED WITH HIGH-RESOLUTION ACCURATE-MASS (HRMS) ORBITRAP MASS SPECTROMETER

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Keywords:
Creatinine
HILIC
HRMS
Biomarker
Cyanoacetic acid

Introduction:
Serum creatinine level (SCR) is widely used as a biomarker of renal function. The SCRs are lower in normal mice than in human, dogs and rats. Colorimetric methods (Jaffe method and enzymatic method) commonly used in laboratory and clinic are insufficient from perspectives of accuracy and sensitivity for determination of the mouse SCR. A highly accurate and sensitive analytical method using LC/MS/MS for determination of mouse SCR has been developed.

Methods:
Creatinine was separated in HILIC mode using cyanoacetic acid as an acidic additive in LC mobile phase and detected by parallel reaction monitoring (PRM) in positive ESI mode using hybrid quadrupole-orbitrap mass spectrometer (Q Exactive Focus, Thermo Fisher). The SCR in mice with renal ischemia-reperfusion (I/R) and sham operation were determined using the novel HILIC/HRMS method and the colorimetric methods and compared with blood urea nitrogen (BUN).

Results:
In the HILIC/HRMS method, cyanoacetic acid, whose decarboxylation occurs by heating at temperature higher than 160°C and produces acetonitrile with carbon dioxide, resolved the matrix effect with decrease in MS response of creatinine in mouse serum observed using formic acid as an acidic additive. The selectivity of creatine in the blank serum was evaluated using peak area ratio of quantifying ion mass (m/z 86.07) to qualifying ion mass (m/z 72.04) (Quant/Qual ratio), and the issue was minimal. The intra- and inter-assay accuracy and precision, and the results in stability testing (storage at −20°C for 95 days and freezing and thawing) were preferable. In I/R model and sham-operated mice, the correlation of SCR by the HILIC/HRMS method with BUN was better than those of SCRs by colorimetric methods (Jaffe method and enzymatic method) with BUN. In particular, the deviations from the correlation of SCR by the HILIC/HRMS method were smaller at lower levels in normal mice or mice with mild renal failure than that of SCR by enzymatic method.

Conclusions:
The novel analytical method for determination of serum creatinine in mice has been developed and utilizes separation in HILIC mode using cyanoacetic acid as an acidic additive of LC mobile phase and HRMS detection in
positive ESI mode. Cyanoacetic acid resolved the matrix effect in MS response of creatinine in mouse serum. The
developed method showed potentials of highly accurate determination for lower SCR (≤ 0.3 mg/dL) in normal mice
or mice with mild renal failure.

Novel Aspect:
The novel HILIC/HRMS method showed accurate and precise determination of mouse serum creatinine. Cyanoacetic
acid showed a novel potential for LC/MS detection in ESI mode.
Introduction:
For pesticides screening, untargeted data collection utilizing LC/MS High Resolution Mass Spectrometry allows labs to continually evolve the scope of targets without the need to change a base method. Measurement of fragment ions can sometimes suffer from interferences, effecting the reliability of ion ratio and ultimately the ability to detect much reduced fragments. To overcome this, precursors are resolved into smaller bands of ions on a prototype QTOF.

Methods:
The quadrupole isolation windows are adjustable by changing DAC values ranging from 0 to 255, effecting the applied RF values. By using calibrant ions, the isolation window width and rectangularity could be determined. Multiple acquisition methods were generated, dividing the mass range into sections between 10amu and up to several hundred amu. For proof of concept, avocado and black tea QuEChERS extracts, spiked to 2 and 10 ppb with pesticides, were evaluated.

Results:
The rectangularity of the quadrupole window width needed to be determined, because the shape of the isolated window determines the necessary overlap when moving from one window to the next. Generating empirically the needed DAC values for a 30 amu and a 50 amu window, and scanning the quad in 0.1Da steps, less than 1 amu was needed to reach FWHM on both sides of the isolation window. Calibrant mass 322 was used to establish beginning, end and half maximum of the window.

In the case of Imazalil, however, the All Ion(AI) spectra showed a matrix interference of one of its fragment ions, leading to a mass shift as well as an EIC height which was overlaid with the matrix contribution. The effect of matrix contribution went up with lower concentration of the pesticide. By using 30 amu isolation window width, the matrix interference was completely removed, allowing the reliable quantitation of Imazalil in both avocado and black tea matrices with identical ion ratio compared to the standard in neat solvent matrix.

Conclusions:
Comparing the analyses of AI MS/MS alone with quadrupole resolved All Ions MS/MS on the spiked pesticide samples, we have noticed that the fragmentation spectra looked substantially cleaner compared to All Ions MS/MS spectra. Nevertheless, in multiple cases ion-ratio of fragment ions remained the same. The dependence of pesticide concentration, quadrupole window width (fixed and variable) as well as matrix complicity will be further evaluated and presented.

Novel Aspect:
Increased confidence in compound qualification and quantitation on a Q-TOF mass spectrometer by utilizing TQ-like post-processing criteria using untargeted acquisition.
INTRODUCTION

In recent years, pharmaceuticals are becoming more frequently found in the aquatic environment[1] which indicates limitations of their removal in conventional wastewater treatment plants[2]. Therefore, new treatment processes that are more efficient and notharmful for the environment are developed. Among different technologies for advanced water treatment belongs boron doped diamond (BDD) [3,4].

METHODS

Residual pharmaceuticals and their metabolites produced during the electrochemical degradation process were determined using TSQ Quantiva LC/MS/MS (Thermo Fisher Scientific, San Jose, CA, USA). For the identification of electrochemical degradation products high-resolution mass spectrometers Q-Exactive MS (Thermo Fisher Scientific) and IT-TOF-MS (Shimadzu, Kyoto, Japan) were used.

RESULTS

Electrochemical degradation of selected pharmaceuticals was performed in electrochemical cell with two parallel BDD electrodes. Based on our preliminary results, different types of BDD electrodes (grown with different deposition parameters, like CH4/H2, B/C in gas phase) were used for water treatment in the first part of this study. Obtained results indicate that this method is able to effectively degrade a wide spectrum of pharmaceuticals that are insufficiently removed by traditional processes at WWTP. Micropollutants such as caffeine, sulfamethoxazole, diclofenac, carbamazepine, valsartan, trimethoprim and venlafaxine were degraded with more than 50% efficiency. In the second part of our study we focused on the identification of electrochemical degradation products. To identify potential electrochemical degradation products two different high resolution mass spectrometers were used.

CONCLUSIONS

This work deals with the possibilities of boron doped diamond electrodes for electrochemical degradation of different pharmaceuticals in wastewater. Using high resolution mass spectrometry, it was possible to identify the products of electrochemical degradation of selected pharmaceuticals.

ACKNOWLEDGEMENTS

This work was supported by the grant of the Slovak Research and Development Agency (APVV-16-0124).

NEW ASPECT

Identification of electrochemical degradation products of selected pharmaceuticals by high resolution mass spectrometry.
References
867 - REMOVAL OF ISOBARIC INTERFERENCES BY LASER RESONANCE IONIZATION MASS SPECTROMETRY FOR DETERMINATION OF 126Sn/121mSn RATIO

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Keywords: Laser resonance ionization mass spectrometry; Isobaric interference; Isotope ratio; Fission product; Spent fuel reprocessing solution

Introduction:
As fission product, 126Sn and 121mSn are considered as important nuclides to monitor the nuclear contamination. The common methods for determination of 126Sn/121mSn ratio are inductively coupled plasma mass spectrometry (ICP-MS), thermal ionization mass spectrometry (TIMS), and accelerator mass spectrometry (AMS). However, the isobaric interferences caused by 126Te and 121Sb are the main difficult problem in these methods. A complicated sample preparation procedure is commonly adopted to separate the interfering species, which may cause a waste of sample and introduce new interferences. Laser resonance ionization mass spectrometry (LRIMS) has both advantages of laser resonance ionization spectroscopy and mass spectrometry. By selectively ionizing only one element at a time through a resonance excitation process, isobaric interferences are essentially eliminated. In this paper, a LRIMS method of measurement of 126Sn/121mSn ratio in the sample extracted from the spent fuel reprocessing solution was proposed. The removal of isobaric interferences in this method was confirmed by analysis of a tin-antimony-tellurium mixture sample.

Method:
Our LRIMS device developed indigenously mainly includes laser system, atomization source, electromagnetic analyzer, data acquisition and processing system. The basic process of the proposed LRIMS method is as follows: firstly, the sample is atomized by electric heating; secondly, the atoms are selectively ionized by the dye lasers with specific wavelengths; lastly, the ions are drawn into the electromagnetic analyzer for isotopic ratio analysis. The laser system consists of three tunable dye lasers pumped by 532 nm Nd:YAG solid-state lasers with pulse frequency of 10 kHz. The tunable wavelength range is 550 ~ 850 nm, which can be extended to shorter wavelength (275 to 425 nm) by frequency doubling method. The atomization source is designed by electric heating tantalum filament with 18 mm long, 0.7 mm width, and 0.045 mm thickness. A grid electrode is mounted behind the atomization source to shield the ions produced by the thermal surface ionization. The detection efficiency of the sample with and without graphene oxide solution are compared. Three laser beams are coupled into the 3×1 fiber combiner, and focused by achromatic lens. Through carefully adjustment, the special overlap between the laser beam and atomic beam is maximized. The electromagnetic analyzer with the mass resolution of 500 and the abundance sensitivity of 2×10^-6 is used to measure tin isotopic ratios.

In order to evaluate the isobaric interference in LRIMS method, a mixed sample of Sn, Sb and Te with a ratio of 1:1:1 is prepared to evaluate the isobaric interference. The 126Sn/121mSn isotopic ratio in a tin sample extracted from the spent fuel reprocessing solution is determined.

Results:
The auto-ionization energy spectrum of tin atom is obtained by laser resonance ionization spectrometry. According to the LRIMS signal of tin ions as a function of each laser wavelength, inefficient three-color-three-photon resonance ionization scheme of tin is confirmed. The laser wavelengths of each excitation/ionization step are optimized as \( \lambda_1 = 286.42 \text{ nm} \), \( \lambda_2 = 811.63 \text{ nm} \), \( \lambda_3 = 823.72 \text{ nm} \). In order to achieve saturated resonance excitation and ionization, the laser average power are set as 18 mW, 230 mW, and 1700 mW respectively. The linewidth of each laser is tuned as about 10 pm to reduce the isotopic discrimination effect. The temperature of the filament required for adequate neutral tin species production is optimized as 600-700°C. The total detection efficiency of 1 µg tin sample is measured as about 3×10^-5 by mixing samples with graphene oxide solution, which is as 4.5 times higher than the sampling without graphene oxide solution. The results show that a layer of graphene oxide gives better control of evaporation and reduces loss of sample as molecular oxide species.

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The tin isotopic ratios in a tin-antimony-tellurium mixture with a ratio of 1:1:1 are measured. The major tin isotope ratios in the sample are determined and the relative standard deviations of $^{116}\text{Sn}/^{120}\text{Sn}$, $^{117}\text{Sn}/^{120}\text{Sn}$, $^{118}\text{Sn}/^{120}\text{Sn}$, $^{119}\text{Sn}/^{120}\text{Sn}$ are all less than 1%. There are no signal at M/Z=121 (121Sb) and 126 (126Te) during the experiment indicating a selectivity factor for Sn exceeding 108. Results show that the isobaric interferences from Sb and Te are effectively eliminated in the LRIMS method.

A tin sample extracted from the spent fuel reprocessing solution is measured by the LRIMS method. The ratio of $^{126}\text{Sn}$ and $^{121m}\text{Sn}$ is measured as 49.78 without sample preparation chemistry procedure.

Conclusions:
Based on the LRIMS device developed in the laboratory, a new attempt has been made for measurement of $^{126}\text{Sn}/^{121m}\text{Sn}$ ratio in the spent fuel reprocessing solution. The isobaric interferences are eliminated in this method. The results show that LRIMS may be the most appropriate technique to correctly determine isotope ratios if isobaric interferences exist from other elements in the sample.

Novel Aspect:
Based on the LRIMS device developed in the laboratory, the auto-ionization energy spectrum of tin atom is obtained, and an efficient three-color-three-photon resonance ionization scheme of Sn is confirmed. A LRIMS method of measurement of $^{126}\text{Sn}/^{121m}\text{Sn}$ ratio in the sample extracted from the spent fuel reprocessing solution is proposed, in which the isobaric interferences caused by $^{126}\text{Te}$ and $^{121}\text{Sb}$ are eliminated.

References:
ULTRASENSITIVE DETECTION OF PROTEIN BIOMARKERS BY SIGNAL AMPLIFICATION MASS SPECTROMETRY

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Keywords: biomarker, membrane protein, mass spectrometry, signal amplification

Introduction:
Proteins are essential biomolecules in revealing biological activities, while abnormal expression of proteins is often related to various diseases.[1, 2] However, protein biomarkers expressed in cells can be exceedingly rare. Moreover, unitary biomarker is usually expressed by more than one type of disease.[3, 4] Thus, sensitive and accurate detection of multiple biomarkers is indispensable for clinical diseases diagnosis.

Methods:
Herein, we propose a novel mass spectrometry (MS) signal amplification method to detect low-abundance protein biomarkers using mass labels (MLs)-modified Au nanoparticles (AuNPs), based on our previous work.[5] AuNPs modified with specific antibodies against protein biomarkers and multiplexed MLs were introduced to recognize the biomarkers through a sandwich protocol.

Results:
By analyzing the abundant MLs with laser desorption ionization time-of-flight (LDI-TOF) MS, presence of the biomarkers can be confirmed. As the types of ML increased, a variety of protein biomarkers could be analyzed simultaneously. In this assay, protein biomarkers of epithelial cell adhesion molecule (EpCAM) and cytokeratin 19 (CK19) were selected as exemplified, 2 zmol of which could be specifically and accurately identified. Meanwhile, utilizing this method, EpCAM and CK19 with the amount of 20 zmol were simultaneously detected.

Conclusions
In conclusion, a novel MS-based approach has been developed to co-detect multiple biomarkers using numerous MLs-labeled AuNPs. Protein biomarkers, EpCAM and CK19, were simultaneously identified when their amount were lower to 20 zmol. In view of the advantages of ultrahigh sensitivity and specificity, the protocol possesses promising value in cell characterization, cell signaling behavior measurement, and the monitoring of disease formation and progression.

Novel Aspect:
By analyzing various MLs with LDI-TOF MS, multiple protein biomarkers could be analyzed simultaneously and sensitively.

References


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1010 - IMPROVING COVERAGE IN TRANSMEMBRANE DOMAINS OF INTEGRAL MEMBRANE PROTEINS WITH ULTRA-VIOLET PHOTODISSOCIATION

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Keywords: membrane protein, FTMS, electron-capture dissociation, electron-transfer dissociation

Introduction:
The most common structural feature of membrane proteins is the bilayer embedded alpha helix. Mass spectrometric coverage during dissociation experiments is frequently limited due to lack of charged residues and retention of structure in the gas phase. In this presentation we explore new strategies for improving coverage in transmembrane domains using FTICR and other high-resolution instruments, including ultra-violet photodissociation (UVPD).

Methods:
Samples were typically dissolved in formic acid immediately prior to separation by reverse-phase chromatography on polymeric stationary phase at 40 °C (PLRP/S, Agilent) or size-exclusion in chloroform/methanol/aqueous formic acid (4/4/1). Samples were selected for high-resolution top-down mass spectrometry directed by low-resolution MS data and analyzed by static infusion freeing us from the constraints of chromatographic timescales.

Results:
Samples were subjected to positive-ion nano-electrospray ionization mass spectrometry using either a 7 Tesla FTICR instrument (LTQFT Ultra with ECD and IRMPD) or an orbitrap (Lumos ETD with a solid state UV laser (213 nm) for UVPD). Addition of supercharging reagents shifted the predominant molecular ion to higher charge states, improving yield of product ions in ECD though it is unclear whether this was due to increased abundance of the precursor or denaturation by the supercharging reagent. We tested dissociation of the two transmembrane helix c-subunit of the ATP-synthase. The 7997.36 Da (monoisotopic) protein isolated from S. oleracea retained its initiating formyl-Met residue and yielded either 4- or 5-charge ions (m/z 2001 and 1334 respectively) both of which were analyzed by static nano-electrospray to yield a precursor intensity of 1e6. These ions were subjected to various dissociation techniques including higher energy collisional (HCD), electron transfer (ETD), the binary mixture these two (EThcD) for comparison with UVPD.

Conclusions
Modern dissociation strategies are giving excellent coverage in transmembrane domains of integral membrane proteins. While EThcD enabled coverage above 50% for single charge states compared with CAD, HCD and ETD, UVPD gave 70 - 80% coverage. Summing UVPD data for two charge states gave close to 100% coverage.

Novel Aspect:
UVPD and EThcD appear especially effective for detailed structural characterization of the transmembrane domains of integral membrane proteins.
Study for Mass Resolution of Quadrupole Mass Spectrometers According to the Rod Electrode Alignment

Keywords: Quadrupole Mass Spectrometers, mass resolution, multipole electric field, rod electrode alignment, BEM

Introduction: (Limit of 400 characters)
Radio frequency (RF) quadrupole mass spectrometers (QMS) have come to be used in various fields because of their compactness. Ionized specimens are separated while passing through a QMS in accordance with their m/z values. The mass resolution of QMS must be raised to identify species with high accuracy. The mass resolution is related to the alignment of a QMS’s four rod electrodes [1].

Methods: (Limit of 400 characters)
We developed our original simulation program PISA-QMS, in which ion motions in an RF electric field can be calculated using the BEM [2],[3] for an electric field and using the Shanks method for ion motion [4] within 0.01% error. PISA-QMS was applied to a QMS varying r/r₀ from 1.063 to 1.145, where r and r₀ indicate the radius of one rod and the inscribed circle between four rods. The relationship between mass resolution and r/r₀ values was investigated using PISA-QMS.

Results: (Limit 900 characters)
The relationship between mass resolution (FWHM) and r/r₀ values showed a range of r/r₀ values with high mass resolution (FWHM ≤ 0.5). The range of r/r₀ values giving high resolution (FWHM ≤ 0.5) was 1.124 ≤ r/r₀ ≤ 1.13. To clarify the mechanism of high mass resolution in such r/r₀ values, we calculated the electric fields between four rod electrodes and expanded them into multipole components (n=1 – 6). According to the values, the generation % of quadrupole components (n=2) and others (n ≠ 2) changed. When r/r₀ was 1.127, the generation % (n=2) became almost 100% because the other multipole components totally canceled each other. The values of r/r₀ giving high resolution (FWHM ≤ 0.5) show good agreement with the range of r/r₀ giving a high % of quadrupole component generation (≥ 99.8%). High resolution could be obtained even when the rod electrode surfaces were not hyperboloid by generating a quadrupole electric field component greater than 99.8% and by canceling other components to no less than 0.2%.

Conclusions (Limit of 400 characters)
An electric field, ion motions, and mass spectra were calculated by applying our simulation program PISA-QMS to QMS varying r/r₀ values. The results showed the mechanism of mass resolution is determined by quadrupole components % among the generated electric field. High resolution could be obtained even when the rod electrode surfaces were not hyperboloid by generating an electric field so as to make the quadrupole component ≥ 99.8% and the other components ≤ 0.2%.

Novel Aspect: (Limit of 150 characters)
High resolution can be obtained by generating an electric field so as to make the quadrupole component ≥ 99.8% and the other components ≤ 0.2%.

References

For information please contact: scientific@imsc2018.it
B.04 INSTRUMENTATION AND METHODS - DEVELOPMENTS AND APPLICATIONS IN ION MOBILITY MS

624 - A HIGH-RESOLUTION ION MOBILITY SPECTROMETER COUPLED TO A TIME-OF-FLIGHT MASS SPECTROMETER

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Keywords: High-resolution ion mobility spectrometer; Gated IMS-MS interface

Introduction:
Over the last years, we have developed compact high-resolution drift tube ion mobility spectrometers (IMS) combined with different ion sources reaching high resolving power up to \( R = 250 \) [1,2]. High-resolution IMS allow fast separation of ion species by size, shape and charge, yielding structural information complementary to molecular mass. To keep such high IMS resolving power when coupling the IMS to a time-of-flight mass spectrometer (TOF-MS), here a Bruker micrOTOF II, several aspects have to be considered, such as the ion transfer and IMS-MS synchronization.

Methods:
Usually, when coupling IMS to TOF-MS data acquisition of both instruments is synchronized resulting in two-dimensional data containing ion mobility and mass spectra. However, due to the short temporal peak width of just 60 µs in our high-resolution IMS with \( R = 100 \), this technique is not applicable due to significant peak broadening during the ion transfer into the MS and an insufficient data acquisition rate of the MS. Thus, a simple gated interface has been designed to transfer selected peaks of the ion mobility spectrum into the TOF-MS. The interface is realized by replacing the Faraday plate of the IMS with a Faraday grid that is shielded by two additional aperture grids. By directly inserting the interface into the capillary shield of the MS, the IMS can be operated at ambient pressure and easily adapted to the MS inlet capillary.

Results:
Coupling our high-resolution IMS to a TOF-MS as described above does not affect IMS resolving power. The novel interface allows both, recording full ion mobility spectra and transferring selected ion peaks or time domains of the ion mobility spectrum into the MS to analyze selected ion species formed in the IMS. Furthermore, due to high grid transparency, ion losses in the interface are minimized. The investigation of the positive reactant ions, ketones and alcohols shows the occurrence of ion cluster dissociation and ion fragmentation processes inside the transfer stage of the Bruker micrOTOF II. As proof-of-concept study, the presented IMS-MS is used to investigate the product ions of benzene, toluene and m-xylene in dry, purified air using a 3H source. Besides the well-known product ions \( M^+ \) and \( M-NO^+ \), for high concentrations a dimer ion is also observed for benzene and toluene, consisting of two molecules and three further hydrogen atoms.

Conclusions:
A simple interface to couple high-resolution IMS to APCI-TOF-MS while keeping full IMS resolving power is presented. Due to its fast gating capability this interface can be used to investigate ion species of a certain peak in the ion mobility spectrum and to explore the ionization processes in IMS. In particular, coupling our IMS to a Bruker micrOTOF II allows investigating the ion chemistry of different ion sources.

Novel Aspect:
Simple interface for coupling high-resolution IMS operated at atmospheric pressure to TOF-MS, minimizing ion losses, recording full ion mobility spectra and enabling a gated transmission of ions into the MS.

Acknowledgement:
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References:
Peptide mapping is an essential method for evaluating monoclonal antibodies (mAb) heterogeneity and their response to stress conditions. The demands placed on this assay have drastically evolved from initially checking the primary sequence to the localization of post translational modification and the determination of low level heterogeneities such as amino acid misincorporation. Because of this, there is a lot of interest in developing platform methods that are faster or able to provide more information.

Parallel serial fragmentation (PASEF) scans use ion mobility as a method to accumulate and select precursors for data dependent scans. This makes it possible to separate peptides that do not separate in the chromatography dimension, leading to improved sensitivity and new opportunities to improve peptide maps.

Methods:
The NISTmab reference material (SRM8671) was incubated under high pH for 24 and 48h. The stressed samples and a control were alkylated and digested with trypsin before UHPLC separation using gradient lengths from 5 to 45 min. The data acquisition was conducted on a timsTOF Pro (Bruker) using the PASEF scan mode. The resulting data was processed with Byonic/Byologic (Protein Metrics) and the ability to detect deamidated peptides was systematically evaluated for the various gradient lengths.

Preliminary Data:

Rapid gradient:
Sequence coverage upward of 95% was achieved for the unstressed NISTmab sample with an HPLC gradient length of 5 min and detection using the PASEF scan mode. Despite the short run time, multiple deamidation sites were detected.

Detailed characterization:
The traditional peptide map method using an extended 120 min gradient combined with the PASEF scan mode allows the detection of low level heterogeneities down to 1-10 ppm of the NISTmab such as beta-microglobulin.

Now ongoing:
We are now running a systematic evaluation of the performance of the method with gradient lengths of 5, 15, 30 and 45 min, while optimizing of the mobility and MS/MS parameters to take into account the increasing amount of coeluting precursors as the HPLC gradient length is reduced.

Novel Aspect:
Impact of PASEF scans on the ability to detect deamidation in peptide mapping
821 - A CLIP ON ION MOBILITY DEVICE. ENABLING GAS PHASE IONS SEPARATION AND COLLISION CROSS SECTION MEASUREMENTS TO ANY MASS SPECTROMETERS.

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University of Manchester, Manchester Institute of Biotechnology, Manchester (1) - MS Vision, MS Vision, Almere (2)

Keywords: Ion mobility, Instrumentation, Retrofitted

Introduction:
Ion Mobility is a powerful gas phase separation technique that is used to distinguish ions as a function of their sizes/shapes; leading to molecule identification directly from the measurement of their mobilities. When coupled to mass spectrometry, IM-MS technique brings in a new dimension and numerous applications. We are developing a clip-on device that would be adaptable to any mass spectrometer to enable ion mobility separation and CCS measurements.

Methods:
The developed prototypes utilise a resistive glass tube to generate an absolute and linear electric field, needed for direct collision cross sections (CCS) measurements. The entrance and the exit caps have been specially designed to minimize hardware modifications on the host MS instrument. A single gas inlet is needed along with a total of only eight simple electrical connectors.

Results:
The first generation will be fitted to a WATERS QToF-Ultima. The only necessary modification will consist into shortening the second ion guide, prior to the quadrupole region; retaining the original footprint of the instrument. An ion funnel has been designed and mounted on the exit cap allowing efficient refocusing of the ion beam post drift. A second generation will embed an ion mobility gating region (necessary for ion pulsing) along with the ion funnel region. Both have been developed to minimize the imprint on the host system and will be mounted and part of the entrance and exit caps. SIMION simulations have been successfully run on various types of model systems ranging from Leucine Enkephalin (low mass singly charged) to Cytochrome C (High mass, multiple charges). The maximum theoretical resolving power at different drift voltages at ambient temperature is evaluated with a maximum operating voltage of 20 V/cm.

Conclusions
A portable ion mobility device has been developed to enable gas phase separation and CCS measurement on any mass spectrometer (Q-ToF, FT and Ion Trap). The device is designed to minimize its imprint on the host, embedding trapping, drifting and refocusing regions in a single assembly. The simulations undertaken show very promising results for a large range of ions.

Novel Aspect:
The novelty resides in the device flexibility. The length of the ion mobility drift region is easily adjustable and the device can be fitted to any MS instruments.
Introduction:
Applications of proteomics technologies to biomedical research require further developments of mass spectrometry (MS) technology to overcome longstanding limitations in speed, sensitivity, and robustness. Trapped ion mobility spectrometry (TIMS) coupled to a quadrupole time-of-flight (qTOF) analyzer in combination with the ‘Parallel Accumulation – SErial Fragmentation’ (PASEF) scan mode has shown much promise in this regard. [1]

Methods:
TIMS separates ions based on their mobility and releases them sequentially into the MS. PASEF synchronizes the MS2precursor selection with the TIMS elution time - multiplying sequencing speed and sensitivity by multiple precursor selection per TIMS scan [2]. An algorithm that detects precursors in 4 dimensions was developed. Data analysis is implemented in MaxQuant. We evaluated the TIMS-TOF Pro performance with regard to sensitivity, speed, and reproducibility.

Results:
In 2h single-shot measurements of 200ng HeLa digest, our setup detected more than350,000 multiply charged features and identified more than6,000 protein groups with an high quantitative reproducibility between replicates (R2 = 0.97) and a scan speed of >120 Hz. From only 10 ng of HeLa digest, which converts to approximately 30 HeLa cells, we identified more than2.400 protein groups in 60 min and more than2.450 protein groups in only 30 min, highlighting the sensitivity and sequencing speed of our setup. [3] We demonstrate the combination of the exceptional scan speed and high sensitivity of our mass spectrometry setup for turbo protein-protein interactome studies in yeast pulldown experiments. We identify all known interaction partners from protein complexes distributed across the whole protein copy number range and from only 50-100 ng of input material in 23 min measurements.

Conclusions
The additional separation of co eluting ions and signal compression in sharp ion mobility peaks in the additional TIMS dimension increases sensitivity and dynamic range. This allows high speed and high sensitivity applications, and routine large-scale measurements of peptide collisional cross sections (CCS), reflecting their 3D structure, which adds a new and yet underexplored level of information to conventional bottom-up proteomics workflows.

Novel Aspect:
Robust, high sensitivity and high speed shotgun proteomics on a novel TIMS-qTOF platform combined with the PASEF scan mode.

897 - COLLISION CROSS SECTIONS OF PHOSPHORIC ACID CLUSTER ANIONS AND THEIR USE AS CALIBRANTS

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Keywords: IMS-MS, CCS, clusters, phosphoric acid

Introduction:
The coupling of ion mobility and mass spectrometry (IMS-MS) is spreading as a supplemental method of structural characterization to mass spectrometry. Several IMS-MS techniques necessitate the use of reference ions and calibration to measure the collision cross section (CCS) of the but the pool of reference ions is limited. Since a first study in 2014[1], we study phosphoric acid cluster ions in view of investigating their potential as calibrants.

Methods:
Phosphoric acid solutions (5 to 50 mM) in water and acetonitrile were analyzed using electrospray ionization in the negative ion mode. Collision cross section were measured in helium drift gas using an Agilent 6560 drift tube ion mobility (DTIMS) Q-TOF instrument, equipped with an electrospray ionization source. Ion mobility mass spectrometry measurements were also carried out on a SYNAPT G2 HDMS (Waters), that operates a travelling wave ion mobility device.

Results:
Collision cross sections from drift tube ion mobility spectrometry in helium gas were obtained for singly, doubly and triply charged phosphoric acid cluster anion. We studied the relation between collision cross sections and the aggregation number. Phosphoric acid clusters were modelled by non-overlapping spheres that adopt a tight three dimensional packing and collision cross sections were calculated using the projection approximation method. Using such minimal clusters of spheres as rough models for the cluster of phosphoric acid molecules, we could reproduce the growth of the CCS of the clusters by adapting the radius of the spheres that represent the phosphoric acid molecules.
Collision cross sections in helium were correlated to the collision cross sections in nitrogen, and their spherical models compared.
Both collision cross sections in helium and nitrogen were used to calibrate a travelling wave ion mobility instrument, and the resulting accuracy were compared using published reference compounds.

Conclusions
As phosphoric acid cluster anions are isotropic clusters that can be regarded as nanodroplets of a very accessible and simple compound, they could be used as universal calibrants for collision cross section measurement for ion mobility mass spectrometry.

Novel Aspect:
Universal calibrants for ion mobility mass spectrometry using isotropic clusters.

References
EXPLORING THE CHEMICAL COVERAGE AND STRUCTURAL ASSIGNMENT OF NATURAL PRODUCTS BY ION MOBILITY SPECTROMETRY COMBINED WITH ULTRA-PERFORMANCE LIQUID CHROMATOGRAPHY/MASS SPECTROMETRY

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Keywords: Ion mobility-mass spectrometry, Natural products, CID fragmentation, Collisional cross section.

Introduction:
The major goal in Natural Products (NP) research is to interrogate complex biological extracts for the purposes of metabolic exploration and natural products discovery. In order to achieve this objective, NP studies rely on accurate and selective acquisition of all possible chemical information, which includes maximization of the number of detected metabolites and their correct molecular assignment [1-3].

Methods:
In this work, we investigated the capabilities of liquid chromatography coupled to ion mobility high-resolution mass spectrometry in the context of natural products. In other words, we addressed the use of comprehensive four-dimensional data (chromatographic retention time, drift time, mass-to-charge and ion abundance) as a way of detect and elucidate NP in complex biological extracts at nanoscale.

Results:
In order to demonstrate the performance of ion mobility for larger chemical coverage, we investigated the behavior of distinct NPs (or NP-inspired molecules) on UHPLC within hybrid Travelling Wave Ion Mobility (TWIM) QTOF system, considering sensitivity and selectivity, LC-deconvolution and conformation space (correlation between m/z and drift time) filtering. The procedure focused on two types of MS/MS data acquisition; data-dependent acquisition (DDA) or auto-MS/MS, and data-independent acquisition (DIA) or all-fragment-ion technique. We evaluated the influence of the “precursor ion isolation width” on chemical coverage, using both approaches, and the impact of additional ion mobility dimension on molecular detection and spectral-quality information. The observations were further explored in a partially characterized sample from a bank of marine bacterial extracts, spiked with the mixture of STDs at the two ranges of concentrations.

Conclusions:
The use of ion mobility (IMS) as orthogonal information on LC-MS method can improve both metabolite detection and chemical characterization for natural products by separating ions in the gas-phase and adding coarse-grained structural information based on shape and collision cross sectional area (CCS).

Novel Aspect:
The application of IMS-MS can improve both metabolite detection and chemical characterization of complex samples in the field of Natural Products.

References
315 - INFLUENCE OF PROTOMERS ON DISSOCIATION OF SEQUENCE-DEFINED OLIGO(ALKOXYAMINE AMIDE)s

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Keywords: Protomers; dissociation mechanism; ion mobility; sequence-defined polymers; MS/MS

Introduction:
Poly(alkoxyamine amide)s are sequence-defined synthetic polymers in which binary information can be written thanks to a 0/1 coding system based on the mass of two amide synthons that alternate with a nitroxide moiety [1]. They can be easily sequenced by MS/MS since they mainly dissociate via homolysis of all fragile C–ON bonds between a coding unit and a nitroxide moiety. Surprisingly, each of these cleavages does not produce the two expected complementary products.

Methods:
Oligo(alkoxyamine amide) trimeric models were ionized in positive ion mode electrospray. Tandem mass spectrometry and ion mobility spectrometry (IMS) experiments were performed with a Synapt G2 HDMS instrument. Geometry optimization was done after DFT calculations using M06-2X functional with the 6-31G(d) basis set implemented in the Gaussian-16 program. The MOBCAL software was used to convert Cartesian coordinates in collision cross section (CCS) values.

Results:
Production of only one fragment upon homolysis of one X-T bond in a protonated α-X-T-X-T-X-ω trimer (where X stands for the coding amide moiety and T the TEMPO nitroxide) strongly suggeststhat, although not directly involved in the mechanism of this charge-remote reaction, the adducted proton has an influence on its occurrence [2]. Protonation of the nitrogen atom in nitroxide was demonstrated to induce an increase of the dissociation energy of the C–ON bond [3]. Accordingly, homolysis of the first X-T bond would occur only when the second T nitroxide was protonated, and vice versa. This assumption implies that each precursor ion would exist as two protomers. Ion mobility experiments indeed showed two distinct conformations for all protonated trimers but those with a 1 coded unit in the second monomer, which were shown to have the same CCS regardless of the proton location. However, although a single C–ON bond homolysis was expected for each protomer, MS/MS spectra recorded after IMS separation still showed homolysis of the two C–ON linkages.

Conclusions
Positive ion mode electrospray ionization was shown to produce different protomers of sequence-defined oligo(alkoxyamine amide)s. By combining ion mobility data and molecular modeling, conformation of these protomers was demonstrated to be sequence-dependent. However, the actual influence of proton location on the occurrence of alkoxyamine bond homolysis is still unclear and is currently investigated with theoretical calculations.

Novel Aspect:
Protomer-specific conformations and their influence on dissociation of small sequence-controlled polymers

References:
Introduction
One of the most challenging problems in biochemistry involves understanding the structures of non-native proteins that are sometimes intermediates during protein folding and unfolding events. After more than 50 years of work, experimental characterization of protein folding and unfolding usually leads to results which are described as a cooperative, two phase, transition between the folded and unfolded states – i.e., the protein appears to melt and no information about non-native states is obtained.

Methods:
Here we present new data from an IMS-MS analysis of simple proteins that are electrospayed from a temperature controlled source. In this approach the source temperature is varied and IMS-MS is used to read out changes in conformations as a function of temperature. Droplets undergo an evaporative cooling phenomenon during shrinkage and this appears to trap solution structures that are not easily observed with other techniques. The IMS distributions change as the shapes of protein conformers change as the MS charge state distributions.

Results:
The results suggest that the cooperative two state behavior involves other states that are captured in the IMS-MS analysis. In some examples, we find evidence for at least 10 structures that arise at slightly different transition temperatures. To date we have investigated several peptides, proteins, and protein complexes.

Conclusions:
The ability to experimentally capture information about new states that are involved in folding and unfolding events may help guide theoretical efforts to model folding and unfolding processes.

Novel Aspect:
IMS-MS techniques are used to capture information about non-native protein populations and structures from solution.
Introduction:
Electrochemistry/mass spectrometry (EC/MS) has evolved as a valuable and purely instrumental approach in mimicking the phase I metabolism of xenobiotics. Due to the formation of isomers after the electrochemical treatment, the development of a separation method in order to distinguish between them is required. For this purpose, trapped ion mobility spectrometry (TIMS) enables a novel and faster approach for the separation of oxidation products.

Methods:
The generation of metabolites was performed in an electrochemical cell equipped with a boron-doped diamond working electrode applying a potential ramp. The EC cell was coupled online to ESI-HR-MS. The results were plotted in three-dimensional mass voltammograms. Mobilo voltammograms for each m/z were recorded and visualized as a contour plot, which shows the signal intensity versus the applied potential and the inverted reduced mobility (1/k0) of the analyte ions.

Results:
With the recorded mass voltammograms different oxidation products of the model compound metoprolol were identified via their accurate masses, e.g. N-dealkylated or hydroxylated products. Especially hydroxylations are typically possible at different molecular positions. The mobilo voltammograms for each m/z reveal isomer formation regarding hydroxylation of metoprolol. Two different hydroxylation products can be distinguished in the mobilo voltammograms via differences regarding their reduced mobility referring to different collisional cross sections. Additionally MS/MS experiments after TIMS separation were carried out in order to give structural information of both compounds. Further fragmentation experiments after HPLC separation show the same results in fragmentation pattern and isomer distinction. Accordingly, by coupling EC with TIMS the analysis time can be reduced by at least the factor of five compared to conventional HPLC separations.

Conclusions:
The ability to distinguish between different electrochemical metabolite isomers proves that EC/TIMS/ESI-HR-MS is a fast and powerful tool for the simulation of xenobiotic phase I metabolism including identification of the metabolites via MS/MS experiments. The time-consuming HPLC separation development process was replaced by the implementation of TIMS resulting in a faster analysis.

Novel Aspect:
The new and fast separation technique TIMS has been implemented in the electrochemical simulation of phase I metabolism in order to investigate isomer formation.
44 - PRION PROTEIN CONFORMATIONAL LANDSCAPE STUDIED BY MASS SPECTROMETRY AND ION MOBILITY.

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Prion protein conformational landscape studied by mass spectrometry and ion mobility.
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Keywords: prion, native mass spectrometry, ion mobility, conformational landscape

•Introduction: MS has proved to be an efficient way to analyze “native” complexes. More recently, the commercial availability of instruments coupled to ion-mobility devices has led to renewed interest of structural biologists with access to conformational landscape of proteins. Recent studies suggest that different prion conformers might coexist in solution. We therefore decided to screen the ovine and human PrP monomers using ion mobility coupled to mass spectrometry.

•Methods: Shortly, ion mobility and MS data were recorded under direct infusion of the prion protein in TEAA 20 mM pH 3.3 buffer on a Synapt G2-Si. Then collision cross section calibration was performed following the procedure of Bush et al for data comparison. For data mining we used an automated data extraction pipeline for which we developed a Python/Qt script base interface.

•Results: Data showed that at least three PrP conformers are observed in the gas phase. PrP monomers are known to lead to the formation of oligomeric species in specific conditions not compatible with MS. We developed a SEC-IMS-MS setup with the aim to study the oligomers produced in these conditions. The development of this methodology allowed us to achieve CCS calibration with standards and successfully observed the small (O3) oligomeric species (O3 ~9-mer). One key observation in this process was that the relative abundance of the gas phase monomeric conformers changed upon SEC elution. This suggest that conformer family abundances were related to protein concentration. Thus we probed the effect of protein concentration upon conformer relative abundances. Results allowed us to clearly demonstrate that monomer concentration has an effect on the ratio of conformers present in solution, which could be related to the occurrence of structural information transfer occurring through the formation of discreet polymeric objects.

•Conclusions: Here we demonstrated that ion mobility coupled to mass spectrometry can be a highly efficient tool, not only to qualitatively describe the conformational landscape but also to quantitatively follow conformers dynamic. Our results, opened new ways for biological interpretation of prion pathologies.


•Novel Aspect: First evidence of conformers in the gas phase that keep part of solution properties and helped deciphering prion pathologies.
Introduction:
Peptoids are peptidomimetic polymers of N-substituted glycine units that differ from amino acid building blocks by the side chain position present on the nitrogen atom [1]. Peptoids can adopt secondary structures, such as helix, which are dependent on cis/trans amide conformation, itself related to the composition [2]. Nowadays, a great care is raised to the peptoid structure especially since the helicoidal folding possibly induces enantioselective properties [3].

Methods:
(S)-phenylethyl peptoids are synthesized using a well-described solid-phase protocol involving a series of repeated acylation and nucleophilic displacement steps [4]. Mass spectrometry experiments have been completed using the Waters Synapt G2-Si instrument integrating the traveling wave ion mobility technology. The interpretation of experimental collisional cross sections is supported by Molecular Modeling with optimized force field.

Results:
To try deciphering the gas-phase secondary structure of ionized peptoids, Ion Mobility Mass Spectrometry (IMMS) and Molecular Modeling techniques are combined. The helicoidal structure adopted by (S)-phenylethylamine homopeptoids (Nspe) is confirmed in solution justifying the choice of this side chain as model compound [5]. The Collisional Cross Sections (CCS) of protonated Nspe peptoids from one to fifteen monomer units are measured. Interestingly, when the CCS values are reported in function of the polymerization degree, a linear correlation appears, potentially attributable to the helicoidal conformation. However, comparing the CCS st and analyzing the relative energies, a charge-induced loop structure seems to be a more relevant gas phase structure. Acetylation of terminal amine function is therefore envisaged to hinder the specific charge positioning at the amine terminal function. The IMMS results obtained for AcNspe peptoids show a totally new trend.

Conclusions:
Peptoids are an emergent class of biomimetic polymers [1]. Numerous in-solution studies describe their ability to fold as a helix despite the fact that the amide conformational equilibrium drastically complicates the data interpretation [2]. Mass spectrometry represents an elegant alternative although the ionization/desolvation processes may denature the solution phase structures.

Novel Aspect:
In contrast to peptides, the H-bond suppression in peptoids induced by the side chain position questions all the considerations about the structure conservation upon ESI.

References:
Introduction: (Limit of 400 characters without spaces)

Ion mobility coupled to mass spectrometry (IM-MS) allows the separation of molecular species based on their mass, charge and shape, providing an additional separation dimension to chromatography. In this work, we compare the reproducibility of collision-cross section (CCS) measurements for analytes separated using different liquid chromatography (LC) mobile phase conditions to evaluate the robustness of LC-IM-MS for extractables identification.

Methods: (Limit of 400 characters without spaces)

In order to study the impact of pH on adduct formation of known impurities from pharmaceutical packaging material by IM-MS, several aqueous mobile phases with different pH were evaluated. IM-MS detection was performed on a Waters VION in positive and negative modes between m/z 80-1000. Each mixture of standards analysed in triplicate was bracketed by QC analysis (n=5) and CCS values for each compound were collected.

Results: (Limit 900 characters without spaces)

The reproducibility of CCS values has been measured for analytes originating from different LC mobile phases. Different pH were evaluated (10 mM ammonium acetate + 0.05% CH₃COOH, 0.1% HCOOH and 0.001% HCOOH) using methanol as organic mobile phase during a 35 min gradient method. 10 µL of standards or QC samples were injected into an Agilent Zorbax Eclipse Plus, 2.1 x 100 mm at 40°C with a 0.5 mL/min flow. All molecules were detected in negative mode as [M-H]-. In positive mode, each analyte was detected as [M+H]+, [M+Na]+, [M+K]⁺, [M+NH₄]⁺, or different combinations of these adducts. The precision of CCS values was ≤1.13% and ≤0.57% for QC and samples, respectively. The difference between expected and observed CCS (ΔCCS) ranged between [-1.60; 1.02%] and [-1.94; 1.30%] for QC and samples, respectively. A very good precision and “accuracy” were measured independently of the pH of the mobile phase. The results of this study also showed that the relationship between the adduct formed and the measured CCS value is not straightforward.

Conclusions

These results showed that CCS values for small organic ions are pH-independent, unlike the retention time. The very good reproducibility of CCS values highlighted the usefulness of CCS values in identifying compounds and can serve as a complementary fingerprint for the identification of unknown compounds in E&L studies. In the identification process, the adduct formation should be considered as it will impact the measured CCS value.

Novel Aspect: (Limit of 150 characters without spaces)

This work highlights the relevance of using CCS values as an additional dimension to identify unknown compounds in E&L studies.
Introduction:
Ion mobility spectrometry (IMS) in combination with mass spectrometry (MS) provides complementary information to m/z, the so-called collision-cross-section (CCS, \( \Omega \) (Å²)), which may be useful for the identification of human and veterinary drugs [1]. So far, only a reduced number of these drugs has been characterized in terms of CCS, limiting its application as identification parameter [2,3]. Moreover, in the field of metabolomics where a wide number of compounds from different chemical families are detected, the creation of CCS databases is crucial for molecular identification.

Methods:
IMS-MS analysis were carried out on a hybrid quadrupole/traveling wave ion mobility/orthogonal acceleration time-of-flight (ToF) geometry instrument (Synapt G2-S HDMS). CCS characterization of human and veterinary drugs was accomplished under flow injection analysis (FIA) and positive electrospray ionization (ESI) conditions. Nitrogen was used as drift gas in the ion mobility cell. In this work, 7 families of drugs (i.e. 18 benzimidazoles, 11 5-nitroimidazoles, 11 aminoglycosides, 19 quinolones, 18 \( \beta \)-lactams, 10 sulfonamides and 5 tetracyclines) have been identified and characterized in terms of m/z and CCS.

Results:
CCS is a molecular characteristic closely related to m/z, so linear correlation between both parameters is expected for compounds belonging to the same chemical family and with similar structural composition. The developed data set includes the CCS of 173 ions considering both [M+H]+ and [M+Na]+ species. High correlation between m/z and CCS is observed for [M+H]+ (r=0.9756, n=91) and [M+Na]+ (r=0.9558, n=82) ions. As expected, CCS values for sodium adducts are greater than for protonated molecules because they present higher molecular weight. However, sodium adducts of aminoglycosides, \( \beta \)-lactams, and of several quinolones and benzimidazoles, present lower CCS values than expected, that can be interpreted as these ions are more compact than their related protonated molecules. The main fragment ions for most compounds have also been characterized in terms of CCS and included in the database as additional interesting piece of information. Finally, 11 veterinary drugs in bovine urine samples were analyzed showing no influence of matrix on the CCS values.

Conclusions
This work proposes a CCS database for human and veterinary drugs. It includes 92 compounds of which 37 of them have been characterized in terms of CCS for the first time. In traditional LC-MS workflows, this database can be used for identification proposes together with other well established identification criteria such as precursor ion, accurate mass, fragment ion, isotopic pattern and retention time.

Novel Aspect:
Additional information for the identification of a wide range of contaminants and other residues is provided as required by new trends in global and/or untargeted analysis.

COLLISION CROSS SECTION AS A NOVEL PARAMETER FOR THE CHARACTERIZATION OF STEROIDS

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Keywords: collision cross section, ion mobility spectrometry, mass spectrometry, steroids, biological samples

Introduction:
Steroids play important roles in biological processes, thus, steroid profiling enhances the knowledge about metabolic mechanisms and the effects of chemical exposures on human health [1]. The integration of ion mobility spectrometry (IM) in LC-MS workflows provides additional information to steroid profiling by adding an extra separation dimension and measuring the molecular collision cross section (CCS) [2].

Methods:
A total of 300 compounds belonging to different steroid families (i.e. androgens, estrogens, progestogens and corticosteroids) and involving 1080 ions (i.e. protonated and deprotonated molecules, fragment ions, sodium adducts, dimers) have been identified and characterized in terms of m/z and CCS. A hybrid quadrupole/traveling wave ion mobility/time-of-flight mass spectrometry instrument (Synapt G2-S HDMS) was employed for CCS characterization. Urine samples containing steroid glucuronides and sulfates have been analyzed by LC-ESI-IM-MS.

Results:
High correlation has been observed between m/z and CCS for steroids. However, several steroids with large moieties such as steroid glucuronides and steroid esters resulted in more compact or elongated molecules than predicted from the large set of steroids analyzed. In addition, steroids present different structural properties depending on the number of carbons of their steroid skeleton with consequences on their spatial size as evidenced by the CCSs observed. Structural differences of 22.8 ± 5.9 Å² have been observed between the protonated molecule and the sodium adduct of steroids. Certain protonated molecules and sodium adducts present a CCS deviated from the expected value which results in valuable information for steroid identification in untargeted analyses. Moreover, several isomeric steroid pairs (e.g. 5β-androstane-3,17-dione and 5α-androstane-3,17-dione) have been separated based on their CCS differences.

Conclusions:
CCS provides additional information to retention time and mass spectra for supporting steroid profiling and gives more confidence to the results obtained in the routine analysis of steroids. The CCS of steroids in urine samples can be measured with high confidence considering a variation < 2% in relation to the values obtained in standards.

Novel Aspect:
This work proposes the first CCS database for 300 steroids (i.e. endogenous, including phase I and phase II metabolites, and exogenous synthetic compounds).

References:
398 - APPLICATION OF CO2 DRIFT GAS IN TRAVELLING WAVE ION MOBILITY MASS SPECTROMETRY FOR THE STRUCTURAL ELUCIDATION OF ENVIRONMENTALLY RELEVANT ISOBARIC ISOMER COMPOUNDS

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Keywords: travelling wave ion mobility, isobaric isomers, polarizable drift gases, environmental samples

Introduction:
The oxidation of volatile organic compounds in the atmosphere leads to secondary organic aerosol compounds (SOA) that partition into ambient particulate matters. Owing to lack of authentic standards, their structural analysis mostly relies on MSn experiments though the information tends to be limited to the presence of functional groups. Here we show the application of travelling wave mass spectrometry with polarizable drift gas for their structural analysis.

Methods:
The extracts of PM samples collected in Chengdu, China were analysed using a Waters Acquity UPLC coupled to SYNAPT G2 HDMS (UPLC/(-)ESI-IMS-QTOFMS). An Acquity UPLC HSS T3 column (100 x 2.1 mm, 1.8 micrometer) was used for the separation of the sample. The ability to separate small isobaric isomer compounds was compared for N2 drift gas and CO2 drift gas.

Results:
Among SOA compounds detected in the chromatograms, we focus on a series of isobaric isomer compounds detected at m/z 294 (m/z 294.0647, C10H16NO7S-). Based on the number of carbon and hydrogen atoms, they are thought to originate from monoterpenes. Common m/z values in MS2 spectra were m/z 247 (C10H15O5S-), and m/z 95 (·SO4-). Two of the isomers showed m/z 231 (C10H15O4S-), whereas the one peak showed m/z 233 (C8H11NO5S-) instead. The presence of m/z 95 (·SO4-) in MS2 suggests that precursor monoterpenes contain a tertiary carbon where a sulfate group can be attached. In the drift time of CO2-IMS-MS, the m/z 247 fragment in the peak at 9 min showed a larger drift time (6.8 msec) than the peak eluting at 8.5 min (6.5 msec). This difference was not visible for the N2 drift gas. This indicates that the CCS of the precursor monoterpenes is larger for this peak than the other peak. While this information alone is not sufficient to determine their structures or precursors, it provides an important piece of information to narrow down their possible structures.

Conclusions
In this work, we showed that IMS-MS separation with CO2 drift gas can be useful obtaining additional structural information to MS2 data. The technique is potentially useful in obtaining structural information for other environmental compounds that have no authentic standard compounds.

Novel Aspect:
Application of highly polarizable drift gas in IMS-MS to elucidate structures of unknown small molecules.
815 - PROFILES OF ARRIVAL TIME DISTRIBUTIONS IN DIFFERENT DRIFT GASES AS A TOOL FOR IDENTIFICATION OF ISOMERS

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Keywords: ion mobility, mass spectrometry, isomers, new psychoactive substances, saccharides

Introduction:
Ion mobility coupled to mass spectrometry improves selectivity[1] but available instruments provide relatively low resolving power. Change of drift gas can enhance separation[2] or peak fitting can reveal isomers in their mixture [3]. Although strongly overlapped peaks were distinguished by the fitting very similar peak shapes can limit its efficiency. Three drift gases were used to distinguish isomers comparing not only their drift times but also peak profiles.

Methods:
Experiments were carried out using a Q-TOF instrument equipped with ion mobility (Synapt G2-S, Waters, Manchester, UK). A custom-made gas controller (Chromservis, Prague, Czech Republic) allowed switching between different drift gases (N2, Ar or CO2). Sample solutions were directly infused into an electrospray ion source. Isobaric or isomeric new psychoactive substances and isomeric hyaluronan-derived oligosaccharides were used as testing compounds.

Results:
Using three drift gases (N2, Ar, CO2), ion mobilograms of standards were acquired to get the reference drift time values and arrival time distribution (ATD) profiles. In some cases the change of drift gas allowed separation of isomeric or isobaric analytes and individual peaks were observed in mobilograms. Some critical pairs were not separated sufficiently in any tested drift gas. Nevertheless, differences in ATD (peak) profiles of some unseparated analytes were evident which allowed to distinguish them. For example for drugs of abuse, two isomers (3-FMC, 4-FMC) were well separated from isobaric 2C-H in N2 but both isomers showed practically identical drift time values as well as ATD profiles and could not be identified. Applying CO2, the isomers were not separated sufficiently but their ATD profiles were different and helpful for the identification. ATD profiles of some individual compounds could also be extracted from a mobilogram of their mixture and peak areas corresponding to individual compounds were determined.

Conclusions:
In ion mobility, individual analytes are identified using their drift times or collision cross sections. Selectivity can be changed by applying different drift gases but drift times of compounds can still remain too close. Even in such cases, ATD profiles of individual compounds can be characteristic and useful for the identification. Since ATD profiles of analytes can be similar in one drift gas application of different gases can improve the identification ability.

Novel Aspect:
In addition to drift time values, ATD profiles acquired in different drift gases were successfully used to identify isomers.

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References:
Introduction:
Spontaneous aggregation of proteins can be the reason for a diversity of diseases e.g. Alzheimer’s Disease (AD) and Parkinson. The proteins involved in these diseases (beta-amyloid (Aβ42) and α-Synuclein, respectively) show the ability of spontaneous aggregation, leading to fibrils formation [1,2]. It is now generally accepted that the small oligomers rather than fibrils are the toxic species in the emergence of those diseases [3].

Methods:
Laser-induced-liquid-bead-ion-desorption (LILBID) MS is an ionization technique which has the ability to conserve non-covalent interactions. Thereby, tiny droplets are transferred into vacuum and irradiated by IR laser pulses. This leads to release of the analyte-ions which are then mass-analysed by time-of-flight. Electrospray-ionization (ESI) combined with IMS allows to correlate the oligomerization of Aβ42 to structural changes of the amyloid peptide.

Results:
We present the differences, advantages and disadvantages of both methods for studying amyloids. By combining the two complementary techniques it is possible to analyze oligomerization, ligand binding and structural changes of the aggregating proteins in dependence of different ligands for a better understanding of the amyloid’s behavior. We use LILBID to study the kinetics of the oligomerization of Aβ42 and α-Synuclein by time-resolved measurements and the effect of molecules which influence the oligomerization. We show examples of molecules which inhibit or even reverse the oligomerization. Furthermore, we use ESI MS and IMS to study the peptide structure. We are able to correlate experimentally determined CCS values to the structure of aggregating peptides to detect changes of the structure induced by ligand molecules. Thereby we can gain insights into amyloid formation as well as the effect of different kinds of ligands.

Conclusions:
LILBID-MS is a well-suited tool to study the kinetics of amyloid behavior allowing to follow the oligomerization of amyloids in terms of disease-relevant oligomers in dependence of the presence of inhibitors. ESI-IMS can give complementary information on how different binding partners affect the conformation of Aβ42. By correlating both methods we can gain an insight on the mechanism of oligomerization.

Novel Aspect:
Combining two mass spectrometry techniques to gain orthogonal information (structure and oligomeric state) of amyloidogenic peptides

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Differential Mobility Separation (DMS)-based separation of bile acid isomers
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Keywords
Differential Mobility Separation Bile Acids

Introduction
Bile acids are involved in a wide range of biological functions including lipid resorption, immunological functions and metabolic regulation. Through metabolic transformations, isomeric and isobaric variants are generated, which makes the unequivocal identification and quantification of individual chemical species difficult. Here we introduce Differential Mobility Separation (DMS) as a methodology for the separation of bile-acid isomers. DMS is an ion mobility technology which separates molecules based on their dipolar moment. We show DMS separation in conjunction with chromatographic separation (LC-DMS-MS) as well as with direct infusion (DMS-MS). While the combination with chromatographic separation may improve selectivity, the separation power of SelexIon is sufficient for a clear separation of isomers, allowing for infusion-based fast quantification without the need for LC development.

Methods
Bile acid standards were prepared in 10% DMSO at a stock solution of 1 mM. Two groups were used: taurodeoxycholic acid (TDCA), taurochenodeoxycholic acid (TCDCA), tauroursodeoxycholic acid (TUDCA) with monoisotopic mass of (499.2967) and formula (C_{26}H_{45}NO_{6}S) and glycodeoxycholic acid (GDCA), glycochenodeoxycholic acid (GCDCA) and glycoursodeoxycholic acid (GUDCA) with monoisotopic mass of (449.3141) and formula (C_{26}H_{43}NO_{5}). For direct infusion, compounds were prepared in water/methanol 50/50, 0.1% FA at a concentration of 1 µM. For LC-separation preparation was done in the same solvent at a concentration of 0.1 nmol/ml with 1 µl injection volume. The samples were measured on a QTRAP® 6500 LC-MSMS system equipped with a SelexION® (DMS) device and coupled to an ExionLC™ system for the LC experiments.

Preliminary results
Using the DMS technology, we directly infused the bile acid standards individually and as mixtures to determine the CoV values of the different isomers. To this end, the compensation voltage was ramped over a range between -30V to 0V. The results showed almost baseline separation of the different isomers. The next aim was to combine DMS with chromatographic separation. The combination of DMS with chromatography has several advantages: Firstly, DMS showed a marked reduction of chemical background for better quantification, resulting in improvement of the signal to noise ratio (give a value NNN). A further advantage is less requirement for development of chromatographic separation, which was shown by the elimination of isomers from extracted ion chromatograms. This also eliminated the requirement for retention time assignment and less reliance on compound specific fragments which may be low abundant. For example, DMS allows to use a highly abundant fragment without the need for compound specificity.

Novel aspects
Differential mobility separation, increased selectivity of bile acid separation by LC-DMS-MS
Addition of Collision Cross Sections to Searchable Libraries: A Robust Approach to Enhance Cumulative Specificity In Screening Assays

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Keywords:
Collision Cross Section, Ion Mobility, Steviol Glycosides, Screening Libraries, CCS Fingerprints

Introduction

CCS data bases are being developed across multiple application areas.1-6 A method using Ion Mobility Mass Spectrometry has been developed to screen/distinguish steviol glycoside isomers. A quantitative assessment of long-term CCS measurement stability and an intersite comparison is presented. In addition to illustrating how CCS fingerprints can be produced to enhance cumulative identification specificity within screening libraries.

Methods

Reversed phase separation was performed (2µl/min), using a microfluidic PCA (post column addition) separation device (40°C), incorporating a 1.7 µm, ACQUITY UPLC BEH C18 stationary phase in a 150 µm diameter separation channel, with integrated ESI emitter (-2.6 kV). The source enclosure was interfaced to a SYNAPT® G2-Si mass spectrometer and generic ion mobility separation performed in negative ion HDMSE mode, at 20000 FWHM (m/z 50-2000).

Results

55 food commodity samples labeled as containing stevioside sweeteners were screened using a steviol glycoside CCS library. Analyses produced CCS values, enabling the unequivocal identification of steviol glycosides and isomeric pairs. Retrospective data analysis revealed coeluting isomeric species comprising, (steviol glycosides, formate adducts, labile fragment ions, doubly de-protonated dimers, multiply charged species) and have been differentiated using CCS, generating specific isomeric analyte fingerprints.

Short term precision/repeatability CCS measurement RSD’s between 0.11 and 0.21% were obtained. For an inter-site comparison (UK/Belgium), the CCS difference was <0.7%. For long term performance, over a 45 days (24/7, one calibration), samples comprised of solvent standards (1-1000 pg/µL) and a commodity subset. 18,974 steviol glycoside detections were measured. The determined CCS error was <1%, with RMS error of 0.26%.

Conclusions

Inter-site and short/longterm term precision of CCS measurements indicate that CCS is a reliable metric that can be added to searchable screening libraries, enabling an additional complimentary metric to be used in conjunction with retention time and accurate mass measurement. CCS fingerprints can be used as an additional metric to enhance identification specificity, even where no product ion information is available and retention time shifts occur.
Novel Aspect

Micro UHPLC MS sensitivity and CCS retrospective data review, enabled multiple analyte specific isomeric species (CCS fingerprints) to enhance searchable databases.

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The Use of CCS-enabled Libraries for Drug Metabolism Applications

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Keywords: CCS, Libraries, DMPK, Unknowns, Matrices

Introduction
A primary challenge in characterizing metabolic fate is distinguishing drug-related material in the presence of the complex matrix. A commonly used approach is to filter components based on parent drug properties. However, identification of drug-related, unexpected metabolites remains a limiting issue. By characterizing the native complement of biological matrices in a library based on m/z, CCS and product ions, we can flag components in samples that are unlikely to be drug metabolites.

Methods:
Hepatocyte incubations were analysed using ion mobility enabled data independent acquisition (HDMSE) LC/MS data were acquired with two different reversed phase gradient Methods: The data were co-detected across all samples in the m/z, RT, and CCS dimensions. An aggregate peak list file was developed from which an initial query library was created. Through an iterative search and (Kendrick) filtering process, the content of the known-unknown library was curated and appropriate query parameters derived.

Results:
Five technical replicate LC-MS experiments of each incubation were conducted per gradient method. Four of the five replicates were used for library creation and the remaining replicate was used for testing and evaluation. The initial library comprised components that were consistently detected across all runs with regard to m/z, retention time and drift time reproducibility. No restrictions were applied at this stage with respect to intensity and/or number of fragment ions per product ion spectrum. Appropriate search tolerances were readily obtained by considering every possible match parameters, resulting in a set of parameters that are equivalent to those typically applied in studies were the structure and/or elemental composition of the target molecules are known. The curation process also allowed for the estimation of the contribution of each analytical parameter, either individually or combined, to the overall specificity of a compound search. In addition, two methods were considered and evaluated that express normalized specificity metrics either as a function of the number of detections or curated library entries, respectively.

Conclusions
The application of the known-unknown library and the ability to estimate FDR will be demonstrated by hepatocytes incubation matrices spiked with known compounds and metabolites. The development and evaluation additional known-unknown libraries of other biological matrices is currently underway as well as the identification of commonly detected unknown compounds in different biological drug incubation matrices.

Novel Aspect:
Our approach shows the benefit of integrating CCS values even for unknown compounds in known-unknown libraries which has the potential to accelerate metabolite identification in DMPK workflow
Introduction:
Native electrospray offers the ability to maintain structural characteristic of the proteins prior to MS analysis. In recent years, the coupling of conventional mobility has also proven to be useful at interrogating the various conformation that may exist in the gas phase associated with intact proteins. More recently, differential mobility separation (DMS) has also been used to effectively separate isoforms and isomers prior to analysis. Here we report of the use of DMS to augment the separation of intact protein under native- LC conditions.

Methods:
All proteins and peptide standards were acquired from SIGMA and used without any further purification. LC was performed using a Shimadzu Nexera UFLC system operated at a flow rate of 400uL/min with a 2x150mm Yara 3u SEC-3000 (Phenomenex) over 15 minutes. All samples were analysed using a TripleTOF ® 5600+ system equipped with a SelexION® (DMS system) using a Turbo V source.

Results:
Cytochrome-c was used as model protein to assess the DMS and source condition required for successful detection of various conformations. Under ‘native like’ infusion conditions, it was found that the DMS can be operated at medium temperature (225°C) and varying SV values to reveal multiple transmission optima (n=3) for the +8 charge state. Prior publications have observed and attributed multiple conformers to this particular charge state. These conformers were also observed under LC conditions as long as the source temperature was kept below 300°C. Though CCS measurement may not be directly obtained from the CoV values, HDX in the gas phase after the DMS separation confirmed that the conformations had differences in exchange rate that are consistent with compact and extended structure. When the spray and solution conditions were modified to induce conformational changes in the solution phase, the CoV optima for that charge state changed. These results are consistent with conformational changes that can be detected by DMS separation.

Conclusions
Differential mobility provides an orthogonal separation to mass spectrometry and LC technology. The work presented here opens up an avenue of structural biology, with the interrogation of compact and extended conformations. The addition of gas phase HDX provides further evidence that the ions are entering the gas phase with a level of solution phase structure. Importantly the observed species are supported through previous publications.

Novel Aspect:
‘Native like’ structure analysis with differential mobility and LC separation
1045 - ION GUIDE WITH VERY STRONG AXIAL TRANSPORTING FIELDS

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Keywords:  
Ion transport, ion transport, CollisionCell with axial field

Introduction:
The main task of this investigation is the searching of geometrical and RF power suppling modes for RF-only stacked ring ion guide with traveling waves for providing very high axial transporting fields. It can be attractive for 1) high-speed, high-pressure collisional cell, 2) the lifting of ions from low to high pressure area for Quadrupole/IMS applications.

Methods:
For a theoretical calculation of effective potentials for the stack of ring electrodes with RF suppling we can use comprehensive work of Berdnikov A. S. [1]. Also we calculate ions trajectories in gas with using SIMION 8.1 with own modified HS1 code. The modification allows to significantly accelerate the calculation of trajectories at the high pressure without loss of accuracy.

Results:
As a basic version of stacked ring ion guide we chose a 4 times the electrode pitch which repeated along axis. Thus we needed only 4 RF power signal for applying to N=1..4 electrodes. RF signal is a combination high frequency Vrf+ and low frequency (LF) signal providing traveling waves field. The LF voltages have an amplitude Vtw and are phase shifted by $\pi/2N$.

The inner diameter of the electrodes was chosen quite small $R_0 \approx 1$ mm, to produce straight axis field caused by TW potentials. The thickness of electrodes was 0.2 mm and the gap between of them is 0.2 mm.

Numerical simulations show the good ion transport efficiency at wide pressure range 0-100 Torr with different RF power supplying regime. At low pressure we have to apply higher amplitude with higher frequency Vrf+ and for high pressure we can leave only LF supplying signals for the traveling waves. Also simulation shows excellent ability for a high speed axial ion transporting.

Conclusions
The ability of ion guide based on stacked ring with traveling waves to operate at wide pressure range 0-100 Torr with high axial transporting speed of 100 m/s (at 100 Torr) is shown. Thus our ion guide can operate as ions passing system from low to high pressure ~ 10-100 Torr for realizing Quadrupole/IMS devices. Also it will be shown that the presented ion guide is effective as a very high speed at operating rate collision cell.

Novel Aspect:
Represented ion guide can be used for 1) ions passing system from low to high pressure for Q/IMS devices or high pressure collision cell 2) very high speed operating rate collision cell.

References
Berdnikov A. S., Nauchnoe Priborostroenie (Scientific Instrumentation), 2011, 21, № 4
Introduction:
Protein folding is difficult to study due to the lack of sensitive techniques that can detect all populated structures on a folding pathway. Ion mobility spectrometry-mass spectrometry (IMS-MS) paired with a variable-temperature electrospray ionization (vt-ESI) source has elucidated multiple folding structures based on changes in collision cross section (CCS) upon protein denaturation[1]. Here, this method is used to study protein folding and assembly.

Methods:
Experiments were carried out using nested IMS-TOF instruments fitted with a custom vt-ESI source that maintains a solution temperature up to ionization and analysis. Wild-type and point-mutated chymotrypsin inhibitor 2 (CI2) were expressed in E. coli and purified by existing techniques. Avidin was purchased from Lee BioSolutions and all other reagents were purchased from Sigma Aldrich. Samples were made to ~30 µM with varying amounts of ammonium acetate buffer.

Results:
We monitored the unfolding of CI2, a single-domain protein previously reported to fold by a two-state cooperative transition[2]. Changes in the charge state distributions suggest the melting transition follows a two-state transition; our melting temperatures (at several solution pH values) are within 1% of calorimetric data. However, the mobility distributions within each charge state reveal several structures that behave differently with increased temperature (i.e., multiple folding states). Point-mutated CI2 variants were melted and compared to the wild-type protein to determine the contribution of specific residues to the overall folding behavior. Preliminary experiments studying the homotetrameric protein avidin show interesting melting behavior. For example, the compact tetramer is favored until ~82 °C; beyond this point the tetramer appears to dissociate into folded monomers. Further increases in temperature result in unfolding of the monomers by what appears as a two-state process.

Conclusions:
Despite the two-state behavior observed in the mass spectral data, CCS structures provide a clear indication that two-state behavior is a composite of many structures. CCS data show a minimum of two native states and four denatured states for CI2. The results for avidin show an example of multiple cooperative transitions hidden within a complex dissociation and unfolding transition – the tetramer first dissociated, followed by monomer unfolding.

Novel Aspect:
Multiple structures are involved in a protein typically thought to unfold via a two-state transition.

References:
Introduction:
In drift tube ion mobility spectrometry (IMS), ions are driven through a drift tube by a homogenous electric field and collide with neutral drift gas molecules. The separation mainly depends on the ion-molecule collision cross section (CCS). Therefore, in more and more applications IMS are used for pre-separation of isomers that cannot be distinguished by their mass-to-charge ratio (m/z) in mass spectrometers (MS). One major benefit of IMS is the fast separation within a few milliseconds revealing the CCS.

Methods:
The ultra-high resolution drift tube IMS used in this work has a resolving power of Rp=250, which is defined as the full width at half maximum (FWHM) divided by the drifttime. To achieve such high resolving power in IMS a drift voltage of 25 kV is used. Nevertheless, the length of the drift tube is just 15 cm. For ionization, atmospheric pressure chemical ionization with a miniature soft X-ray source is used as well as atmospheric pressure photo ionization (APPI) with 10.6 eV.

Results:
In previous work, we demonstrated the separation of several isotopologues with the instrument used. [1] Here, isotopes with small differences in CCS are separated. The percentage difference in CCS is defined as the difference of the CCS divided by the average CCS and multiplied by one hundred. Calculating the percentage difference that can be resolved with Rp=250 yields a minimum percentage difference in CCS of 0.8 %. [2] For example, the isomers 2-picoline and 3-picoline are clearly separated using an APCI ionization source. Whereas these substances can hardly be resolved with GC-MS. [3]

Conclusions:
The separation of isomeric compounds is of major interest, especially in pharmaceutical science. With ultra-high resolution drift tube IMS and IMS-MS small percentage differences in CCS can be resolved.

Novel Aspect:
Separation of isotopes with differences of only 0.8 % in CCS using ultra-high-resolution DT-IMS.

Acknowledgements:
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References:
Ion Mobility-Derived Collision Cross Section for Mycotoxins: an interlaboratory study.

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Keywords: Travelling wave ion mobility spectrometry; CCS database; Interlaboratory comparison; Mycotoxins.

Introduction:
Ion mobility mass spectrometry (IM-MS) is spreading rapidly as a promising technique for simultaneous screening, quantification and identification of a large number of food contaminants. Collision cross section (CCS) measurements resulting from ion IM-MS experiments provide a promising orthogonal dimension of structural information in MS based analytical separations. As with any molecular identifier, interlaboratory standardization must precede broad range integration into analytical workflows [1].

Methods:
More than 60 mycotoxins were analyzed both in standard solution and in matrix to demonstrate the reproducibility of this new molecular identifier. The reproducibility of CCS values was evaluated across two traveling wave ion mobility mass spectrometers (Synapt G2 HDMS and Vion IMS QTOF) located in different laboratories.

Results:
A database containing TWIMS-derived CCS values of parent and modified mycotoxins is hereby presented. CCS measurements showed high intra- and inter-day repeatability (RSD <1.8%), they were not affected by the complexity of the investigated matrices (i.e. malt, corn flakes, maize feed, wheat) (ΔCCS ≤2%) and they resulted independent from the concentration [2]. In addition, they were not influenced by instrument settings. CCS values derived from Synapt G2 HDMS and Vion IMS QTOF mass spectrometers were highly reproducible with interlaboratory RSD <5%. Furthermore, the novel geometry of the Vion system allows to elucidate fragmentation that might occur for labile mycotoxins during different stages of the ESI-TWIMS-QTOF analysis. Indeed, it is possible to state if dissociation occurs before (fragments would present shorter drift times than precursor ion) or after the TWIMS cell (drift time of the fragment ions would match that of the precursor ion). This is useful to understand which parameter has to be properly set to avoid/promote analyte fragmentation.

Conclusions:
Overall, our study demonstrates the reproducibility of CCS across different instrumentations and analytical conditions. The addition of CCS in the mycotoxins screening workflows improves the accuracy and precision of analysis and would provide a higher degree of confidence in the identification workflow compared to traditional analytical approaches. Thus, many false-positive detections could be avoided by applying a maximum ΔCCS tolerance of 2%.

Novel Aspect:
The low RSD observed in this interlaboratory study illustrate the potential of CCS as a molecular identifier that can be used alongside the traditional molecular identifiers.

References


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Introduction:
The combination of ion mobility separation with mass spectrometry offers increased capabilities for determining the structure of molecules. In these scenarios, ion mobility initially delivers a separation based on molecule sizes. Yet, often even simple molecules or molecule mixtures show ambiguous mobilities which cannot easily be resolved further, e.g., by chromatography.

An algorithm has been developed to deconvolute these overlapping signals in the mobility vs. m/z domain of a direct infusion experiment. The result are compounds comprising each of a clean mass spectrum and a mobilogram. Optionally, a fragment mass spectrum is extracted.

Methods:
A mixture of isobaric lipids has been analyzed using direct infusion trapped ion mobility spectrometry (TIMS) coupled with an orthogonal time-of-flight mass spectrometer. The algorithm developed here is evaluating such analyses. It is a further development of an algorithm initially developed for deconvolution of compounds of a chromatographically separated LC/MS analysis. [1] The basic idea of this algorithm is the comparison of all extracted ion mobilograms to detect and deconvolute compounds. The key to success is the usage of fuzzy logic for the decision if any of two ions belong to the same compound or to different, partly overlapping compounds. Each compound is then associated with a clean compound mass spectrum, deconvoluted by any overlapping compounds and background subtracted. In extension to that fragment spectra at the same mobility are also associated to that compound. Finally, the collision cross section of each compound is determined from the m/z ratio of the most intense ion and its charge state.

Results:
The mobility–m/z heatmap of a measurement of a mix of lipids reveals several partly overlapping signals. The direct infusion analysis was acquired over one minute and was performed on the Bruker timsTOF Pro instrument. Applying the algorithm delivers compounds, each of a clean mass spectrum and a mobilogram. Signals are clearly separated and contain no signals from overlapping compounds or any background signals. Since charge state and molecular weight are also determined, collision cross section (CCS) values are calculated as well and the mobilograms can be directly depicted on a CCS scale. Summarizing the results shows that also isomeric lipids were separated, each measured with a distinct CCS value.

Conclusions:
Ion mobility separation has already proved to be a valuable combination with mass spectrometry. In the task of structure elucidation however, even simple mixtures of isobaric compounds can show complex and ambiguous signals in a direct infusion experiment. This algorithm (DissectTIMS) deconvolutes these signals into compounds, each of a clean mass spectrum, a mobilogram and a CCS value.

Novel Aspect:
Deconvolution of overlapping signals in a direct infusion TIMS-MS experiment delivering clean mass spectra, mobilograms and CCS values.

References:

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366 - ION MOBILITY MASS SPECTROMETRY FOR PROFILING AND STRUCTURAL CHARACTERIZATION OF HUMAN HIPPOCAMPUS GANGLIOSIDES

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Keywords: ion mobility separation mass spectrometry; gangliosides; human fetal hippocampus; collision-induced dissociation; cholinergic activity.

Introduction:
The structural analysis of gangliosides (GGs), a particular class of glycosphingolipids highly expressed in central nervous system, experienced a new level due to the rapid advances in ion mobility separation mass spectrometry (IMS MS) [1]. In view of the direct correlation of GGs with neurogenesis and synaptogenesis, in this work we have implemented IMS MSn for the first time in the study of a highly complex native mixture originating from human fetal hippocampus [2].

Methods:
The GG mixture was purified from a 17-th gestational week fetal hippocampus (FH17) using the method of Svennerholm and Fredman and modified by Vukelić et al [4]. The sample dissolved in methanol to 5 pmol/µL concentration was infused at 2µL/min flow rate and 2 kV ESI voltage into the Synapt G2s. To enhance the separation IMS wave velocity was set at 650 m/s, IMS wave height at 40 V and IMS gas flow at 90 L/min. CID was performed in the transfer cell, using energies between 10-45 eV.

Results:
The ionization and detection of low abundant species was possible due to IMS that allowed the differentiation of GGs based on the carbohydrate chain length and degree of sialylation. These findings provided a more reliable insight into the expression of polysialylated structures, which might constitute markers of brain development. By IMS MS, after six minutes of signal acquisition, no less than 134 distinct GGs were identified, a number nine times higher than the number of GGs previously reported in FH following chip nanoESI QTOF MS analysis without prior separation (only 14 structures) [3]. Moreover, IMS MS evidenced no less than 54 different species modified by either carbohydrate or non-carbohydrate type of attachments. Of these, 25 ions correspond to GG structures modified by CH3COO- attachment, novel species never reported before. CID MSn experiments conducted after mobility separation provided a detailed structural characterization of CH3COO- GT1b(d18:1/18:0), a biologically relevant compound.

Conclusions
The screening of FH17 by IMS MS provided data related to the expression and role of GGs in fetal hippocampus, with a particular emphasis on their cholinergic activity by the discovery for the first time of novel species modified by CH3COO- attachment. In view of the present findings, we may conclude that IMS MS and CID MSn proved their high efficiency for unequivocal detection and characterization of these low abundant species, with a significant biological role.

Novel Aspect:
First implementation of IMS MS for GG determination in FH and discovery of CH3COO-- modified species, correlated with the cholinergic activity of GGs in central nervous system.
References
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Institut Lumière Matière, Université de Lyon, Lyon (1)

Keywords: FAIMS, IRMPD spectroscopy, isomers, hyphenated techniques, multidimensional analysis

Introduction:
The analysis of isoforms is challenging by mass spectrometry and ion mobility spectrometry is gaining momentum for this purpose. However IMS technologies suffer from the absence of detailed structural information. On the other hand, IRMPD spectroscopy (Infrared Multiple Photon Dissociation) is able to resolve subtle isomerisms on pure sample. We propose to combine FAIMS separation, mass spectrometry and IRMPD spectroscopy for multidimensional analysis.\[1\]

Methods:
This new instrument consists of an ultraFAIMS (Owlstone) device interfaced to a linear ion trap (LTQ XL Thermo Scientific) which was modified for IRMPD spectroscopy. the combination of FAIMS separation, mass spectrometry and infrared spectroscopy in asingle instrument allows various possibilities of multidimensional analysis, especially for mixture of isomers.

Results:
For a given mass-selected ion, two different modes using FAIMS separation and IRMPD spectroscopy are enabled to characterize the composition of a mixture of isomers. They are demonstrated on an isomeric mixture of paracetamol and 2-phenylglycine. In the first mode, a FAIMS (high-Field Asymmetric waveform Ion Mobility Spectrometry) separation of the isomers is performed with a static compensation field for mass- and isomer-selective ion spectroscopy. In the second mode, the compensation field is scanned while the ions are irradiated at a fixed wavenumber allowing selective photofragmentation of a chosen species. the FAIMS-MS-IR workflow allows separation and identification of mixtures of isomers using an appropriate database of IRMPD spectra and without the use of standards for calibration purpose. Alternatively for such isomeric mixture, IRMPD spectroscopy alone yields a convoluted spectrum. By adding a FAIMS dimension, it is possible to retrieve individual spectroscopic signature for each isomer present in the mixture.

Conclusions:
This configuration of IMS-MS-IR workflow constitutes an attractive alternative to more elaborate approaches for ion spectroscopy of mass-selected and isomer-selected ions. In the field of analytical chemistry, we hope that this type of multidimensional analysis will be a valuable tool for broad range of biological/medical/pharmaceutical applications where the identification of isoforms is critical.

Novel Aspect:
A new multidimensional analysis workflow combining FAIMS separation and IRMPD spectroscopy for analytical purpose or structural analysis.

References
PROTEIN ANALYSIS USING ION MOBILITY MS AND IN-SOURCE ION ACTIVATION COMBINED WITH ECD ION FRAGMENTATION

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Keywords: In-source, Protein, Activation, Mobility, ECD

Introduction:
Tandem mass spectrometry is accepted as a powerful analytical technique and when used with electron capture dissociation (ECD) makes it well suited for protein identification. In-source ion activation can be used to facilitate collision induced unfolding (CIU) of proteins and when coupled to IM-MS offers information about ion energetics and structures [1]. Combining the above technologies may provide top-down protein analysis with improved sequence coverage.

Methods:
An Agilent 6560 IMQTOF instrument was modified to include ion optic elements at the exit of the ion transfer capillary to improve in-source ion activation prior to ion mobility analysis. The instrument was also equipped with a prototype-MSion ECD cell mounted at the exit of the quadrupole mass filter. Proteins were prepared under native and denatured conditions and data were analyzed using IM-MS browser, CIU suite programs, Prosight, and LcMsSpectator.

Results:
Native ESI conditions for carbonic anhydrase and BSA show low charge state precursor ions ranging from +8 to +11 and +15 to +19 respectively. These native low charge state ions have folded compact structures shown by their measured small CCS values, but they can be unfolded by using in-source ion activation. These proteins under denatured ESI conditions provide higher precursor ion charge states, +12 to +18 and +22 to +58 respectively, with correspondingly elongated ion structures and larger measured CCS values. The ECD cell provided nearly complete sequence coverage of denatured proteins as large as carbonic anhydrase with preservation of post-translational modifications. Sequence coverage results will be presented for the above proteins under native and denatured ionization conditions, coupled with CIU to facilitate more extensive fragmentation, and combined with ECD technology. Experimental data from larger proteins like monoclonal antibodies will also be analyzed and presented.

Conclusions:
Electron based ion fragmentation methods, such as ECD, produce extensive ion fragmentation for readily exposed regions of proteins. The fragmentation efficiency for a protein can be enhanced by either using denatured proteins or by unfolding native proteins in gas phase prior to ECD fragmentation. This study focuses on the use of CIU to improve ECD fragmentation efficiency and relates the degree of unfolding and ECD efficiency to protein structure in the gas phase.

Novel Aspect:
In-source ion activation coupled with IM-MS and ECD ion fragmentation for greatly improved sequence coverage and native protein structural analyses.

References
Introduction:
Analyzing peptides such as insulin from dried blood spots DBS represents an urgent task in various fields of analytical chemistry (e.g. doping controls, forensic- or clinical chemistry) due to simplified sampling, transfer and storage. Coupling liquid chromatography, ion mobility separation with high resolution mass spectrometry enables the sensitive detection of insulin and its synthetic analogues from DBS at physiologically relevant levels.

Methods:
The blood spots (20 µL) were extracted from pretreated DMPK cards, immunopurified with magnetic beads and anti-insulin antibodies, separated by reversed-phase liquid chromatography (one-pump-trapping) and detected by high resolution mass spectrometry (time-of-flight) including an ion-mobility separation (T-wave IMS). The method was fully validated considering qualitative result interpretation.

Results:
The evaluation of the diagnostic ion traces were performed under consideration of the respective retention times, accurate masses and specific drift times for each of the target peptides. With this strategy, validation of the method yielded specific and sensitive (< 0.5 ng/mL) results for human insulin and all relevant synthetic analogues. Recoveries range at approximately 30 % with precise (CV<25%) and linear results within the working range of the method (0.5 to 10 ng/mL). The proof-of-principle was shown by further analyzing DBS samples from healthy volunteers at non-fasting level (endogenous insulin detected only) and one diabetic volunteer (Type I). Here the rapid acting synthetic insulin Novolog was clearly detected. Generally, in this study was shown that additional separation by ion mobility combined with the accurate mass enhances the results significantly.

Conclusions
Analyzing insulins from urine or blood samples represents an established approach in doping controls [1]. The present study described now a fit-for-purpose method to analyze insulin from DBS cards. The application of DBS sampling becomes more and more interesting in various analytical fields. Here, the combination of immunoextraction, chromatography and high resolution MS with ion mobility separation offers the required sensitivity (sub ng/mL level).

Novel Aspect:
To best of our knowledge the study shows the first assay to determine insulin and its analogues from dried blood spots in physiologically relevant levels with LC-MS.

References
1344 - LIPID AND FATTY ACID ISOMER ANALYSIS USING A TRAVELLING WAVE CYCLIC ION MOBILITY SEPARATOR

Mike McCullagh (1) - Martin Palmer (1) - Giorgis Isaac (2) - Russell Mortishire-Smith (1) - James Langridge (1) - Johannes Vissers (1)

Waters Corporation, Wilmslow, United Kingdom (1) - Waters Corporation, Milford, United States (2)

Title:
Lipid and Fatty Acid Isomer Analysis Using a Travelling Wave Cyclic Ion Mobility Separator

Authors:
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Keywords:
IM, lipid, isomer, resolution, traveling-wave

Introduction: (400/400 characters)
Lipids represent a diverse group of biomolecules that have an essential role in structural, storage and signaling processes in living systems. Class separation is readily achieved using chromatographic and MS based identification techniques; however, the analysis remains challenging due to the chemical structure diversity and isobaric nature of lipids. The addition of ion mobility (IM) to lipidomic workflows enhances separation and improves isomer resolution.

Methods: (383/400 characters)
Data were collected on a hybrid quadrupole cyclic IM orthogonal acceleration time-of-flight (Q-cIM-oaToF) instrument. Ion mobility separation is achieved using a multi-pass travelling-wave cIM separator, where increasing the number of passes around the device allows of the increase in both mobility resolution and ion residence time. MS and CID fragmentation data were obtained on precursor IM separated lipids followed by ToF mass measurement.

Results: (845/900 characters)
Unsaturated free fatty acid (FA) standards, differing in chain length and number of cis/trans configurations, were chosen to determine the degree of IM separation required to separate lipid isomers. FAs represent the simplest class of lipid components, yet they exist in the structure of each lipid categories. In all direct infusion IM-MS measurements, FAs with cis-double bond orientations, introduced as two component mixtures, were found to be more compact than those with trans-orientations. Moreover, the cis- and trans-orientations for the monounsaturated FAs were distinguishable. A different number of cycles through the cIM separator, thereby increasing the effective path length/resolution, were required to achieve a similar degree of IM separation for mono-unsaturated FAs of differing chain length. Unsaturated FAs with two or more double bonds, separated by two mid-chain carbons, could not be distinguished. The separation of other lipid classes by cIM is currently under study.

Conclusions (399/400 characters)
A Q-cIM-oaToF geometry has been characterized and successfully applied to the IM separation of isomeric, mono-unsaturated FAs. The required IM resolution was found to be a function of chain length. Shorter, structurally more rigid and compact FAs were discriminated at reduced resolution, as could longer chain mono-unsaturated FAs as a
result of partial chain back-folding. Following IM separation, isomeric lipids were successfully CID fragmented and identified.

Novel Aspect: (108/150 characters)
High resolution ion mobility separation of cis/trans lipid isomers using a multi-pass travelling-wave cIM separator.

References

533 - 5 HOUR SINGLE SHOT HUMAN PROTEOME ANALYSIS USING LC-FAIMS-MS/MS

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Keywords: [Proteomics, FAIMS, Ion Mobility, Quantitation, and FTMS]

Introduction:
Limitations in separation peak capacity and dynamic range restrict identifications in single shot proteomics experiments to ~ 5,000 protein groups. Pre-fractionation can skirt these issues; however, it comes at the cost of increased labor and number of samples for MS. Here we demonstrate that High Field Asymmetric Waveform Ion Mobility Spectrometry (FAIMS) coupled with an Orbitrap MS can allow near whole proteome sequencing without the need for pre-fractionation.

Methods:
Proteins were extracted from a human cells (K562) and digested with trypsin. Peptides were loaded onto a nanoflow capillary column (30cm 1.7um BEH C18), ionized, and further separated with a cylindrical electrode FAIMS device prior to injection into an Orbitrap Lumos. The FAIMS system operated with a 3MHZ bi-sinusoidal waveform (5000V peak to peak), electrode temperature of 100°C, and CVs between -35 and -110V dc. Protein identifications were filtered to 1% FDR.

Results:
We describe a compact, cylindrical, helium free, rapid CV stepping, FAIMS source that easily interfaces between the ESI emitter and the MS inlet. This device induces an aerodynamic Coanda effect that entrains and diverts ions away from the inner electrode surface, yielding a high ion transmission efficiency (>50%). 20 ms transit times were obtained by minimizing the ion separation gap and electrode length (2.5mm to 1.5mm and 24mm to 15mm, respectively).

Performance test for single 90 minute analyses with and without FAIMS yielded 6,312 and 5,267 proteins, respectively. Triplicate FAIMS analysis of the same sample with 3 separate CV values (-40V, -60V, -80V) yielded 87,527 unique peptides and 7,427 proteins. To obtain similar performance without FAIMS offline high pH reversed phase chromatography (3 fractions total) had to be performed (95,745 unique peptides and 7,433 proteins). We conclude that FAIMS can accomplish gas-phase separations that offer comparable proteomic depth to conventional two-dimensional approaches.

Conclusions
Beyond these results we present a detailed characterization the z, m/z, mass and sample purity dependence of FAIMS. We compare quantitative (LFQ) reproducibility and identification overlap with and without FAIMS. Lastly we explore the possibility of analyzing with human proteome in a single-shot analysis where we identified 8,023 proteins with intra-analysis CV switching across 4 CVs (-45V, -60V, -75V, and -90V) during a single 5 hour experiment.

Novel Aspect:
A novel FAIMS device enabling gas-phase fractionation as a viable alternative for rapid and deep proteomic analysis.
Introduction: Probing Peptide Conformational Rearrangements with High Resolution SLIM Traveling Wave Ion Mobility-MS.

Multiply charged peptide ions in the gas phase can adopt distinct conformations, trapped in local energy minima [1]. The relative stability of these conformations provides insight into the types of secondary structures adopted by peptide ions and the mechanisms of their conformational rearrangements, such as proline cis/trans isomerization and intramolecular proton transfer. High resolution ion mobility (IM) separations are useful for probing peptide gas phase secondary structures, as they can potentially resolve multiple kinetically trapped conformers of peptides with low activation barriers for rearrangement. In this study we have employed high resolution SLIM traveling wave (TW) IM separations to study kinetically trapped peptide conformations. We investigated the impact of various IM parameters on peptide structural rearrangements during storage and separations in a SLIM IM-MS platform.

Methods: Probing Peptide Conformational Rearrangements with High Resolution SLIM Traveling Wave Ion Mobility-MS.

Preliminary experiments were performed using 1 meter and 13 meter Serpentine Ultra-long Path with Extended Routing (SUPER) SLIM platforms [2,3]. The SLIM utilized 6-5 (6 RF strips interspersed with 5 TW segmented arrays) or similar 4-3 electrode arrangements. Samples were introduced via a nano-electrospray ion source and trapped in the funnel trap (IFT) prior to introduction to the SLIM IM module. Afterwards ions were refocused in the rear ion funnel and passed through a segmented quadrupole to the Agilent TOF instrument. IM separations in Nitrogen and Helium were performed in the 2.5 to 4 Torr pressure range. Simion simulations of ion trajectories through a SLIM IM module were performed with Statistical Diffusion Simulation (SDS) Model.

Results: Probing Peptide Conformational Rearrangements with High Resolution SLIM Traveling Wave Ion Mobility-MS.

We tracked the evolution of the kinetically trapped conformations of Substance P (M+3H)3+ [1] and other peptide standards as a function of IM parameters, such as the TW electric field as well as source conditions, such as RF ion funnel amplitudes. We estimated the extent of collisional heating in the SLIM platform by monitoring ion losses and fragmentation during storage and separation of selected peptide standards. Velocity distributions of peptides during SLIM IM separations have been modeled with Simion simulations. In preliminary experiments we successfully detected multiple kinetically trapped conformations of multiply charged peptide standards, including Substance P (M+3H)3+, Bradykinin (M+3H)3+ and Melittin (M+5H)5+, using both 1 meter and longer path SLIM separations. Impact of average electric field and guard bias voltage on modelled velocity distributions of Substance P (M+3H)3+ correlates with the distribution of the peptide conformations in local and global energy minima in the IM experiments. Collisional cross sections of the peptides under a select range of IM parameters are in close agreement with the values experimentally determined in drift tube ion mobility [4]. We detected no ion losses for Leucine Enkephalin with over 100 meter SLIM separations as well as during storage in the SLIM platform. In future experiments we plan to use different peptide ions as well as peptide noncovalent complexes to investigate separations and possible ion activation with the use of on SLIM trapping and in other novel SLIM modules. We will...
compare IM experiments with molecular modeling to probe the mechanisms of peptide rearrangement during IM separations.

Conclusions: Probing Peptide Conformational Rearrangements with High Resolution SLIM Traveling Wave Ion Mobility-MS.
SLIM TW IM enables high resolution separations of kinetically trapped conformations whose cross sections are in close agreement with the values determined by drift tube ion mobility. It also enables separation and storage of peptides with minimized dissociation. Collisional heating the SLIM platform can be controlled using certain SLIM IM parameters, enabling studies of peptide structural dynamics and the relative stability of secondary structures during IM separations.

Novel Aspect: Probing Peptide Conformational Rearrangements with High Resolution SLIM Traveling Wave Ion Mobility-MS.
High resolution SLIM TW IM separations of kinetically trapped peptide conformations and the characterization of collisional heating during storage and separation in SLIM.

References: Probing Peptide Conformational Rearrangements with High Resolution SLIM Traveling Wave Ion Mobility-MS.

Differentiation of Panax ginseng, Panax quinquefolius, and Ginseng Products by Using Differential Ion Mobility Spectrometry Coupled with Tandem Mass Spectrometry

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Introduction:
Ginseng are highly treasured medicinal herbs in traditional Chinese medicines. However, authentication of the origins of ginseng products is not an easy task [1]. An DMS-MS/MS based method is established for the differentiation of Panax ginseng (Oriental ginseng) and Panax quinquefolius (American ginseng).

Methods:
This method is based on the separation of ginsenosides present in the ginseng extracts using Differential Ion Mobility Spectrometry (DMS), followed by data dependent acquisition with Tandem Mass Spectrometry. Collision-induced dissociation (CID) and electron-induced dissociation (EID) were applied to generate diagnostic fragment ions for the differentiation of structural isomers [2, 3].

Results:
The presence of ginsenoside Rf and pseudoginsenoside F11 in the Oriental and American ginsengs was selected as the criteria of differentiation. A good separation of isomeric ginsenosides was achieved in the Differential Ion Mobility Spectrometry using aliphatic alcohols as polar gas-phase modifiers. Data Dependent Acquisition results of DMS coupled with Collision-Induced Dissociation Mass Spectrometry was highly efficient compared with LC-MS/MS. Continuous ion separation in DMS chamber enabled the utilization of other dissociation methods that required longer acquisition time, such as EID. In comparison with collision-induced dissociation (CID), EID provides more structural informative ions and eliminates glycosyl group migration of Rg1 and Rf.

Conclusions:
The spatial separation of isomeric ginsenosides in DMS and the generation of structural diagnostic ions for isomer differentiation by EID could eliminate ambiguities in differentiating ginseng and ginseng products. This study demonstrates a fast and effective method for differentiation of Oriental ginseng and American ginseng; and for characterization of commercial ginseng products.

Novel Aspect:
High performance DMS-MS/MS method for differentiation and characterization of ginseng and ginseng products.

References:
995 - ULTRA-HIGH THROUGHPUT SCREENING FOR ENGINEERED SYNTHETIC BIOLOGY LIBRARIES BASED ON SAMPLE CHIPS COMBINED WITH DESI MSIMAGING

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Manchester Institute of Biotechnology, University of Manchester, Manchester (1) - Waters Corp., Waters, Wilmslow (2) - Labcyte Europe, Labcyte Europe, Staffordshire (3) - Manchester Institute of Manchester, University of Manchester, Manchester (4)

Keywords: DESI, mass spectrometry, ion mobility, high throughput, synthetic biology

Introduction:
The screening of variant libraries is an important yet costly and time consuming step in full Design-Build-Test-Learn pipelines[1] central to many current synthetic biology strategies. By employing liquid handling robotic platforms for automated sample-chip preparation, our new HTP strategy can generate visualized imaging data on the scale of 12 sample/min with only nL quantities needed for each sample.

Methods:
MSImaging was performed by using a DESI 2D Omni Spray Ion Source combined with SynaptG2 Si HDMS mass spectrometer[2]. Sample chips were prepared using an Echo 550 liquid handling robotics system and cell strain supernatants transferred onto glass slides covered with a nylon membrane. The maximum capacity of each sample chip was 1536 samples/plate. The DESI imaging pixel size was set as 100um X 100um, with a scan rate of 15ms/spectrum. The acquired data were processed with HDImaging software.

Results:
(i) Ambient and direct sampling conditions dramatically reduce liquid handling of samples; (ii) target compound titer measurements are label-free, and can thus be applied to different production pathways for the screening of intermediates and final products from synthetic biological libraries; (iii) the robotic sample chip preparation and low volumes required allow time course tracking and real time monitoring; (iv) the HTP sensitivity can reach uM level (e.g. in the case of pinocembrin flavonoid screening), which is far lower than the normal titres, and demonstrates the potential to apply this approach to other engineered pathways; (v) higher sensitivity is available when triple quadrupole mass spectrometry is applied; (vi) visualized MSImaging measurement provides highly accessible data for upstream Learning and subsequent re-Design-Build.

Conclusions
Our super-high throughput strategy, based on large capacity sample chips combined with MS imaging technology, makes medium and large scale directed evolution synthetic biology library screening possible over reasonable time scales. The superfast measurement data generated can provide sufficient data to allow the application of machine learning to model and debug malfunctioning synthetic biology pathways.

Novel Aspect:
The super-high throughput MS screening method combines the automated production of sample chips and MS imaging that presents a powerful new capability for the screening of large synthetic biology libraries.

References
Introduction:
β-Amyloid (1-42) (Aβ42), the protein involved in Alzheimer’s disease (AD) has been demonstrated to generate β-sheet forming oligomers leading to fibrils and plaques. Whereas fibrils have long been suspected to cause neurodegenerative disorders, recent studies suggest misfolded oligomers as the origin of neurotoxic effects in AD. Hence the early oligomerization stage stands in focus of inhibitor development to suppress the formation of toxic oligomers.[1]

Methods:
Additionally to laser induced liquid bead ion desorption mass spectrometry (LILBID-MS), which gives an insight in the early aggregation kinetics of Aβ42 in absence and in presence of aggregation inhibitors, electrospray ionization mass spectrometry coupled with ion mobility (ESI-IM-MS) is used to reveal the inhibitor’s structural influences on Aβ42.[2]

Results:
Whereas LILBID-MS allows to observe the aggregation kinetics and demonstrates the effect of well-known and novel aggregation inhibitors at the oligomeric level ESI-IM-MS provides a detailed look on several inhibitor binding events. Distinct m/z signals reveal the presence of several gas-phase conformations in their arrival time distribution (ATD) of the same oligomer order. Due to inhibitor interactions, the corresponding inhibitor bound states differ in their collision cross sections (Ω), as well in their gas-phase stabilities. Those indicate structural rearrangements which thereby affect distinct aggregation pathways differing in their kinetic behavior.

Conclusions
The investigation of inhibitors by ESI-IM-MS provides an insight into binding behavior of Aβ42 at the oligomeric level. A closer look at inhibitor bound states reveals differences due to conformational changes which potentially lead to non-toxic oligomeric states. Estimating aggregation kinetics of Aβ42 in absence and in presence of inhibitors by LILBID-MS additionally to the findings by ESI-IM-MS reveals the structural influence on the aggregation behavior.

Novel Aspect:
The combination of LILBID and ESI-IM-MS provides promising results in understanding the aggregation of self-assembling peptides and their prevention by inhibitors.

References:
B.05 INSTRUMENTATION AND METHODS - PETROLEOMICS, HYDROCARBONS AND BIOFUELS

802 - DETAILED LOOK IN SARA FRACTIONATION: SATURATES, NOT ONLY SATURATED COMPOUNDS!

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Keywords: SARA, Saturates, HRMS, direct analysis

Introduction: (348 characters without spaces)
Saturates, Aromatic, Resins and Asphaltenes (SARA) fractionation is a decade old fractionation method of crude oils. Saturates, referring to their chemically descriptive name, are known to contain only aliphatic compounds. To date, there are very few saturates centered studies of which all were performed using GC techniques. Therefore, their direct MS analysis were neglected and never studied in detail.

Methods: (347 characters without spaces)
SARA fractionation was performed on different crude oils using a modified IP-143 method.\cite{1} Saturates were dissolved in Toluene: Methanol (1:1) to the final concentration of 250 ppm. Results of the direct injection HRMS analysis by ESI-(+)-FTMS for all of the samples were compared. In order to get further comparable information, all of the samples were further analyzed using HRGC-EI-MS on a GC-Orbitrap MS.

Results: (772 characters without spaces)
Direct injection HRMS was successfully performed for all of the samples. Comparison of direct injection analysis shows that saturates of different oils, poses different chemical characteristics including their heteroatom containing classes and the length of their aliphatic chains. Results of the HRMS analyses are contradicting the common knowledge about the characteristics of saturates. In this regards, the compounds found in this fraction do not necessarily only contain aliphatic chains and saturated rings, but also many olefinic chains and unsaturated rings. More than 15000 different elemental compositions could be calculated from the data, showing a much wider variety of compounds than expected from a saturate fraction. The GC-MS measurement results show presence of volatile and aliphatic compounds that are obviously only a part of the saturates fraction, which are detectable using GC technique.

Conclusions (290 characters without spaces)
Results of the HRMS analysis prove that the characteristics of saturates fraction differ from the contemporary knowledge consisting of only aliphatic compounds. This is because SARA fractionation is solubility, polarity and adsorption based separation method which cannot give a clear separation and results overlaps between fractions.

Novel Aspect: (138 characters without spaces)
Saturates fraction contains not only GC detectable aliphatic compounds, but many olefinic and aromatic compounds which can be analyzed by direct injection HRMS.

References:
490 - DIRECT INLET PROBE - HIGH RESOLUTION TIME-OF-FLIGHT MASS SPECTROMETRY FOR THE DESCRIPTION OF HIGH BOILING PETROLEUM FRACTIONS

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Keywords: Bitumen, crude oil, direct inlet probe, high resolution mass spectrometry, ionization

Introduction:
High boiling petroleum fractions remain challenging to analyze on a molecular level due to their complexity and physical properties, which make them unsuitable for chromatographic techniques. Therefore, High resolution mass spectrometry has become a key technique for the analysis of these complex materials.
In this study, we used a direct inlet probe (DIP) coupled to High resolution time of flight mass spectrometry for detailed chemical characterization.

Methods:
Crude oil vacuum residues and bitumen were analyzed with a multi-reflection-tof mass spectrometer (LECO Pegasus HRT) equipped with a direct inlet probe. To volatilize the sample components, a temperature gradient from 50°C to 400°C was applied. Beside electron impact Ionization (EI), two soft ionization techniques, namely single photon ionization (SPI) and chemical ionization (CI) were utilized on the same system.

Results:
DIP-HRMS allowed the introduction of petroleum residue samples directly into the ion source, where the temperature was linearly increased via a temperature program. Substances could be separated according to volatility and classified into chemical groups by high resolution mass spectrometry. The decreased pressure in the ion source reduces the effective boiling point, which enabled the destruction-free vaporization of high boiling substances. With the time resolved high resolution mass spectra, elemental compositions could be assigned, and homologues series of typical petro-chemical compound classes could be identified. Moreover, several bitumen samples of different origin and varying physical properties could be discriminated on a molecular level. Preliminary results showed that CI and SPI can be advantageous as complementary ionization techniques to EI because of a reduced fragmentation tendency and different selectivities.

Conclusions:
Summarized, DIP-HRMS showed to be a powerful technique for the detailed description of complex, high boiling matrices. The combination of thermal separation and exact mass information enabled identification of characteristic molecular structures. Moreover, it was possible to use the chemical fingerprint to discriminate between vacuum residues which were derived from different crude oils and refineries.

Novel Aspect:
We present a new approach for in-depth analysis of high boiling samples based on the combination of thermal analysis in vacuum and high resolution mass spectrometry.
Introduction:(367/400 characters)
Lubricating oils are composed of a base oil and various additives. Generally, since complicated pre-treatment is necessary prior to analysis, this extending the time and increases the effort required to obtain the final results. Thus, analysis without pre-treatment would be desirable for research and development. Therefore, the purpose of this work is to directly and rapidly evaluate the degradation degree of lubricating oil.

Methods: (397/400 characters)
Automobile lubricating oil, virgin and after 1000 or 5000 km driving were used. Thermal desorption and pyrolysis(TDP) combined with DART-MS was composed of Q-TOF mass spec equipped with DART ion source and ionRocket TDP device. The samples were used to ~1uL and put into the sample pot. TDP temperature condition was room temperature to 600 °C, a rate of 100 °C/min. To structurally and compositionally characterize samples, Kendrick Mass Defect(KMD) analysis was used.

Results: (886/900 characters)
The mass spectra were detected for the thermal desorption and pyrolysis products of all samples. In the mass spectra detected at 300 °C, additives such as a phenol-type antioxidant, an amine-type antioxidant, and a salicylic acid-type detergent dispersant were detected in all the samples. There was no significant difference in the ratio of additive compounds at 0 and after 1000 km of driving, but after 5000 km driving, the concentrations of the salicylic acid-type detergent and phenolic antioxidant were remarkably decreased. In the mass spectra recorded at 450 °C the base oil dominated the spectrum at 0 km, while after 1000 km of driving, glycol compounds were also detected, which increased in concentration after 5000 km of driving. Additionally, using KMD plots it differences among the samples were clearly observed. By setting the Kendrick Mass unit based on the mass of the repeating structure, compounds with common repeat units aligned in the horizontal direction on the KMD plot, whereas compounds with different structures shifted.

Conclusions: (288/400 characters)
TDP/DART-MS enables the detection of additives and base oil in lubricating oils by gradient heating without pre-treatment and can used to evaluate the degradation extent of lubricating oils quickly and easily by monitoring the amount of antioxidants as a degradation marker. Moreover, a combination of TDP/DART-MS and KMD analyses enabled clear recognition of slightly differences in the base oils between the virgin and degraded samples. Thus, the method described herein can be used for the evaluation of the degree of degradation of lubricating oils.

Novel Aspect: (87/150 characters)
A combination of TDP/DART-MS and KMD analysis is useful for evaluation of degradation degree of lubricating oils.
Keywords: island, asphaltenes, archipelago, non-distillable, vacuum residues, dissociation

Introduction:

Vacuum residues (VR) can be separated by molecular distillation into distillable fractions and a truly non-distillable fraction with boiling points above 687 °C. In this work, infrared multiphoton dissociation (IRMPD) was coupled to Fourier transform ion cyclotron mass spectrometry (FTICR MS) and used to determine the aromatic cores of compounds found within the vacuum residues, as a function of boiling point.

Methods:

Molecular distillate fractions from two VRs were analysed, with boiling points of: 540-603 °C, 603-645 °C, 645-687 °C and, finally, 687+°C (non-distillable), all in atmospheric equivalent temperature (AET). MS/MS spectra were acquired using atmospheric pressure photoionization (APPI) coupled to a 12 T Bruker solariX FTICR MS (Bruker Daltonik GmbH, Bremen, Germany) and IRMPD (25 W CO2 laser).

Results:

Precursor ions were externally accumulated for a period of 0.5- 2 s prior to being transferred to the ion cyclotron resonance cell. It was found that the number of peaks per dalton detected increases exponentially as the boiling point of the fraction increases. A depletion of the relative intensity of polycyclic aromatic hydrocarbons and low heteroatomic-containing species was also observed as the boiling point increased, as expected using Boduszynski’s model.[1] Species with up to four heteroatoms can be found in the non-distillable sample. The class distribution of the fragments indicates that up to two heteroatoms can be part of the aromatic core of the VR species, indicating that some heteroatoms can be present in the alkyl chains. MS/MS results also indicate that structural motifs comprise mainly island type structures for the distillable samples. In contrast, structures with two or more fused aromatic rings connected with alkyl chains (archipelago) are the main constituents of the non-distillable fractions of VRs.

Conclusions

Evidence of island and archipelago motifs were found in VR constituents. According with the number of double bond equivalents (DBE) of the fragments, structures of a vacuum residue can have aromatic cores with up to 8 fused benzene rings and up to two heteroatoms. Archipelago structures are concentrated at the heavy ends fractions of crude oils, in particular in the truly non-distillable fractions.

Novel Aspect:

Use of MS/MS to predict the size of the aromatic core and observation that distillable fractions contain mainly island structures.
References

THE COMPOSITIONAL AND STRUCTURAL CONTINUUM OF PETROLEUM REVEALED BY FT-ICR MASS SPECTROMETRY

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Keywords: FT-ICR, Petroleum, Petroleomics, Asphaltene, Complex Mixture

Introduction:
Over the past 20 years, advances in modern ultra-high resolution mass spectrometry have forever changed the expectations of petroleum analyses. Facilitated by advances in ionization methods (that enable molecular-level analysis irrespective of initial or final boiling point), ultra-high resolution Fourier Transform Ion Cyclotron Resonance mass spectrometry (FT-ICR MS) routinely resolves and identifies tens-of-thousands of species in individual petroleum samples.

Methods:
Petroleum samples were analyzed by FT-ICR mass spectrometry (operating at 9.4 and 21T) by atmospheric pressure photo-ionization (APPI) and/or (+/-) electrospray ionization (ESI) at concentrations between 100 -300 µg/mL. Data was acquired and processed with Predator and Petro-Org software platforms.

Results:
High field FT-ICR MS has been used to revisit, and ultimately support a decades old theory of the composition of petroleum (the Boduszynski Continuum) and expose the compositional constraints (in class, type, and carbon number) imposed by boiling point. Such compositional information facilitates the accurate boiling point prediction of individual elemental compositions based on mass measurement alone, and is now poised to push forward to the molecular-level modeling of heavy ends (Petroleomics). The structural continuum is completed through the infrared multi-photon dissociation of mass isolated segments, and reveals both island (single aromatic core) and archipelago (multi-aromatic core) molecules. Thus, the current results strongly suggest that the decades-old Boduszynski model, which was largely ignored, is in fact correct, and the Yen-Mullins structural model, which is now largely accepted, is in fact, incorrect. Work supported by NSF Division of Materials Research through DMR-11-57490, State of Florida, Florida State University, and the Future Fuels Institute.

Conclusions:
The current clarity on the compositional and structural continua of petroleum is heavily dependent on chromatography. Thus, current and future prospects in the on-line and off-line analysis of petroleum samples will be discussed. Work supported by NSF Division of Materials Research through DMR-11-57490, State of Florida, Florida State University, and the Future Fuels Institute.

Novel Aspect:
Ultra-high resolution FT-ICR MS analysis of petroleum has revealed a compositional and structural continuum of species that changes predictably as a function of boiling point.
Introduction:
Asphaltene precipitation is affected by factors such as heptane to crude oil ratio (HCOR). This means that for the same crude oil, a different set of molecules would precipitate forming a distinct asphaltene depending on these factors. It is expected that molecules more prone to precipitate are going to be those with the higher aromaticity, higher molecular weight and/or higher polar nature. Effect of solvent power on asphaltene properties HCOR is presented.

Methods:
Asphaltenes were separated from a medium crude oil (API of 30.4). Different HCOR were used to induce precipitation. Asphaltenes were analyzed by MRMS. Samples were analyzed using APPI and LDI in positive ion mode with resolving power of 1,300,000 at m/z 400. Analysis of data including calculation of molecular formulas and relative abundances of compound classes was performed using PetroOrg 10.0 (Florida State University).

Results:
Using LDI it was observed that HC classes as well as classes containing one heteroatom became less abundant as the heptane to crude oil ratio increases (decrease of solvent power). The opposite happened for most classes containing two or more heteroatoms. Similar results were found when positive APPI was used. Based on weighted relative abundances, DBE distributions for the samples were shifted to larger DBEs with higher solvent ratios (lower solvent power) using APPI. In contrast, molecular size distribution did not show a clear tendency. Calculations indicated that while differences in average DBE can be as large as 6 units between different HCOR, the maximum difference in molecular size is around 1.5 carbon atoms. These tendencies were also observed for individual classes for the class HC using LDI. The appearance of molecules with larger DBEs as the solvent power of the solvent decreases leads to an increase in the aromaticity. There results were in agreement with elemental analysis as well as IF-IR and 1H-NMR results.

Conclusions:
Composition of the asphaltenes changes dramatically depending on the solvent power of HCOR. Decreases in solvent power produce asphaltenes that are more aromatic. Asphaltenes become enriched in molecules with larger DBEs and with more heteroatoms per molecule. No significant changes in molecular size were observed as a function of HCOR. FT-IR and 1H-NMR measurements confirm the tendencies evaluated by MRMS.

Novel Aspect:
APPI and LDI MRMS can be used to study aromaticity and class distribution of precipitated asphaltenes at different solvent power.

References
956 - DETAILED MOLECULAR CHARACTERIZATION OF THE UPGRADING PROCESS OF PYROLYSIS OIL BY ULTRAHIGH RESOLUTION MASS SPECTROMETRY

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Keywords: Ultrahigh-resolution mass spectrometry, pyrolysis oil; biofuel upgrading

Introduction:
Pyrolysis oil is from biomass is a potential alternative substitution for fossil fuel. A significant drawback of pyrolysis oil is that high acidity, instability and oxygen content limits its usage as transport fuel. Much work has been done to improve the quality of pyrolysis oil. One effective way is hydrotreating approach which helps to remove oxygen and store hydrogen as well to produce petro-like fuel.

Methods:
Organosolv lignin was used for pyrolysis with the condition of argone atmosphere, 600 oC at the rate of 80 oC min-1. Detailed mass spectrometric measurements of the reactions after hydrotreating were carried out using a prototype-Orbitrap Elite equipped with an APCI source.

Results:
Detailed ultrahigh resolution mass spectrometric analysis shows to be an excellent method to understand the detailed conversion reaction of biomass into a fuel. Here, the detailed molecular transformation can be observed at different steps of the reaction. These steps involve the pyrolysis of lignin into a first product, which then is catalytically transformed into a biofuel under hydrogen atmosphere. The results show that depending on the amount of hydrogen present during the hydrotreating process, different types of reaction products can be observed with varying degree of aromaticity. At the end, the hydrotreating products are composed of mainly hydrocarbon species and some minor oxygen-containing compounds, containing up to only four oxygens per molecule. The formation of lower DBE compositions coincides with disappearance of higher DBE compositions. And this trend can also be indicated by comparing the calculated median DBE/C values.

Conclusions:
Catalytic hydrotreating from lignin derived pyrolysis oil can be fully observed using ultrahigh resolution mass spectrometry. Detailed observation of the different reaction steps allows better understanding of the conversion process.

Novel Aspect:
The catalytic hydrotreating process of pyrolysis oil upgrading can be observed in detail to allow better understanding of chemical process.
Keywords: Tandem mass spectrometry; sequencing; sequence-controlled polymers; molecular design; information-containing macromolecules.

Introduction: MS/MS sequencing was found to be the most efficient methodology to retrieve digital information stored in sequence-defined synthetic polymers [1]. In these macromolecules, two comonomers of different mass are defined as the 0- and 1-bit of the ASCII alphabet and placed at defined location throughout the chain to build binary messages [2]. Since the comonomer structure dictates the dissociation behavior, it can be optimized to achieve best MS/MS readability.

Methods: Poly(alkoxyamine phosphodiester)s were synthesized using an orthogonal strategy that employs successively phosphoramidite and radical-radical coupling steps [3]. Methanolic solutions of polymers were supplemented with ammonium acetate prior to electrospray ionization in the negative ion mode. High resolution MS and MS² experiments were performed on a Synapt G2 Q-TOF mass spectrometer (Waters, Manchester, UK).

Results: Sequence-defined poly(phosphodiester)s can store large amounts of information as they can be produced in an automated manner as long monodisperse chains [4]. However, extensive fragmentation of all phosphate bonds in each monomer leads to very busy MS/MS spectra that do not systematically ensure full sequence coverage. To simplify CID data and hence facilitate this sequencing step, a weak alkoxyamine bond was introduced between all monomers: as expected in such poly(alkoxyamine phosphodiester)s, fragmentations specifically occurred at C–ON bonds, making all phosphate groups MS/MS silent [5]. Additional structural optimizations included tailoring the distance between phosphate moieties in order to promote their simultaneous deprotonation, which allowed production of fragments which charge state increases with their polymerization degree. Finally, storage density of these chains was further increased by introducing two instead of one coding moieties in each monomer without modifying their optimized dissociation behavior [6].

Conclusions: In contrast to biomolecules which structure is set by biology, sequence-defined synthetic polymers can be specifically designed to control their CID behavior and ensure full sequence coverage. So simplified dissociation rules can then be easily implemented in the recently-introduced MS-DECODER software [7], allowing efficient MS/MS data analysis and message decoding in the millisecond scale.

Novel Aspect: MS/MS assisted design of sequence-controlled synthetic polymers to improve readability of molecularly encoded information and increase their storage density.

References

Introduction:
Characterizing proteins and their post-translational modifications remain a major challenge for mass spectrometry. Electron capture dissociation (ECD) produces clean spectra of entire proteins while also preserving PTMs but remains impractical because of the technical difficulties of confining low-energy electrons. We have developed an efficient ECD technology that can be retrofitted into most common mass spectrometers.

Methods:
The electromagnetostatic ECD cell is 30 mm long and consists of two magnets, an electron-emitting hot filament and 6 electrostatic lenses. The magnets provide a magnetic field that forces electrons emitted from the filament to be in the flight path of protein ions. The ECD cell is positioned between the mass resolving quad and the mass analyzers in either Orbitraps or in Q-ToF.

Results:
We have developed an ECD cell that can be installed in one hour and does not affect ion transmission [1]. The cell was first optimized to maximize fragmentation of substance P. These same parameters worked well for fragmenting proteins up to 30 kDa. Sequence coverage exceeds 90% for carbonic anhydrase (29 kDa). In addition to producing c and z fragments, a substantial number of d and w sidechain fragments were present in the same spectrum that could distinguish many isobaric leucines from isoleucines. Labile post-translational modifications are retained. For example, all eight phosphoserines in alpha-casein (23 kDa) were retained with 87% sequence coverage. With superoxide dismutase, copper and zinc cofactors remained bound to c and z ions containing their respective binding sites. Deuterium labeling of ubiquitin enabled top-down hydrogen/deuterium exchange with rates consistent with prior NMR results. Sequence coverage of 80-95% for proteins like ubiquitin and a-synuclein (14 kDa) was obtained during UHPLC separations from peaks lasting 1-3 seconds.

Conclusions:
The ECD cell is easily adapted into both Orbitrap and Q-ToF instruments without reducing their sensitivity or transmission. Fragmentation produces clean spectra that allows nearly complete characterization of proteoforms with preservation of labile PTMs. The presence of numerous d and w ions enhances the success of de novo sequencing by allowing many leucine/isoleucine or lysine/glutamine pairs to be easily distinguished. The ECD cell can help map disulfides in bottom up approaches [2].

Novel Aspect:
High sequence coverage of proteins up to at least 30 kDa can be produced at chromatographic speeds by retrofitting the ECD into current mass spectrometers.

References:
970 - THE ROLE OF H2O IN THE DISSOCIATION CHEMISTRY OF [W6O19]2-: A COMBINATIONAL STUDY BY MASS SPECTROMETRY AND DFT CALCULATION

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The role of H2O in the dissociation chemistry of [W6O19]2-: A combinational study by mass spectrometry and DFT calculation
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Keywords: Polyoxometalate; Tandem mass spectrometry; Dissociation mechanism; DFT calculation

Introduction

Polyoxometalates (POMs) are discrete anionic metal-oxygen clusters which exhibit a great diversity of applications in catalysis, medicine, etc. [1] The fragmentation mechanism of discrete polyanions as well as its protonated/organic ammonium/alkali metal adduct ions are well understood by the contribution of a few scientists [2-4], in contrast, the solvated polyanions (in particular H2O as a solvent) chemistry is scarcely investigated. This sort of research can allow us to shed light on the mechanism of the inverse process of nucleation reaction in a solution, which attracts the most attention of synthetic chemists of POMs. Herein, we report our preliminary result regarding the water-solvated polyanions by taking the Lindqvist-type [W6O19]2- as an example to probe the dissociation chemistry of the water-solvated [W6O19]2- in comparison to the bare [W6O19]2- in order to explicitly define the role of water in the dissociation chemistry of polyanions by tandem mass spectrometry combined with density functional theory (DFT) calculation. Tandem mass spectrometry experiments of [W6O19]2- and [W6O19-H2O]2- were performed on Agilent 6520 Q-TOF mass spectrometer with N2 as the collision gas. The MS/MS results show that these two ions went through completely different dissociation pathways, where the former produces [W3O10]2- and [W4O13]2- fragments whilst the later generates predominately the mono-nuclear fragment [HWO4]-. This result prompts us to search further about the reaction pathways for these two precursor ions by DFT calculation. The DFT results show that the reaction may proceed via two distinctive pathways: “water-free” and “water-assisted”. The water-free pathway starts with the cleavage of W-Ob bond of [W6O19]2- without transition state. Water-free process is calculated to be endothermic by about 39.1 kcal mol-1. However, initially, in the water-assisted process, explicit water coordinates with POM by the intermolecular hydrogen bond, which reduces the energy for about 12 kcal mol-1. Then the cleavage of W-Ob bond in the water-assisted pathway is found to be endothermic by about 30.1 kcal mol-1. Next, in the water-free process, the cleavage of W-O bond is favorable thermodynamically, forming [WO4]2-anion and intermediate W5O15. However, for the water-assisted pathway, the water addition proceeds with an 8.2 kcal mol-1 energy barrier at a transition state TS1. All calculated bond parameters (Fig. 1) and IRC calculation results indicate that this is a synchronous process, that is, the addition of H2O accompanied by a H-transfer to form intermediate [HWO4-W5O15OH]2-. Next the formation of [HWO4]- and [W5O15OH]- proceeds without an energy barrier and is calculated to be exothermic by about 80.1 kcal mol-1. The calculation is on the way to find out the lowest-energy routes to reach the final products, respectively.

Fig. 1. The dissociation mechanism of Lindqvist type POM [W6O19]2- (Energy in kcal mol-1)

Method

Tandem mass spectrometry combined with density functional theory (DFT) calculation.

Results

Tandem mass spectrometry experiments of [W6O19]2- and [W6O19-H2O]2- were performed on Agilent 6520 Q-TOF mass spectrometer with N2 as the collision gas. The MS/MS results show that these two ions went through
completely different dissociation pathways, where the former produces \([\text{W}3\text{O}10]\)\(^{-2}\) and \([\text{W}4\text{O}13]\)\(^{-2}\) fragments whilst the later generates predominately the mono-nuclear fragment \([\text{HWO}\text{4}]^{-}\). This result prompts us to search further about the reaction pathways for these two precursor ions by DFT calculation. The DFT results show that the reaction may proceed via two distinctive pathways: “water-free” and “water-assisted”. The water-free and water-assisted pathway all start with the cleavage of W-Ob bond of \([\text{W}6\text{O}19]\)\(^{-2}\) (1) without transition state. Water-free process is calculated to be endothermic by about 39.1 kcal mol\(^{-1}\). Similarly, the water-assisted pathway is found to be endothermic by about 30.1 kcal mol\(^{-1}\). Next, in the water-free process, the cleavage of W-O bond is favorable thermodynamically, forming \([\text{WO}\text{4}]^{-}\) anion and intermediate \([\text{W}5\text{O}15]\)\(^{-}\). However, for the water-assisted pathway, the water addition proceeds with an 8.2 kcal mol\(^{-1}\) energy barrier at a transition state TS\(^{1}\). All calculated bond parameters (Fig. 1) and IRC calculation results indicate that this is a synchronous process, that is, the addition of \(\text{H}_2\text{O}\) accompanied by a H-transfer to form intermediate \([\text{HWO}\text{4-}-\text{W}5\text{O}15\text{OH}]^{-}\). Next the formation of \([\text{HWO}\text{4}]^{-}\) and \([\text{W}5\text{O}15\text{OH}]^{-}\) proceeds without an energy barrier and is calculated to be exothermic by about 80.1 kcal mol\(^{-1}\). The calculation is on the way to find out the lowest-energy routes to reach the final products, respectively.

Conclusions
The Lindqvist-type bare polyanion \([\text{W}6\text{O}19]\)\(^{-2}\) goes through completely different dissociation pathway from its water-solvated counterpart \([\text{W}6\text{O}19\cdot \text{H}_2\text{O}]^{-2}\), where \([\text{W}6\text{O}19]\)\(^{-2}\) produces \([\text{W}3\text{O}10]\)\(^{-2}\) and \([\text{W}4\text{O}13]\)\(^{-2}\) fragments whilst \([\text{W}6\text{O}19\cdot \text{H}_2\text{O}]^{-2}\) generates predominately the mono-nuclear fragment \([\text{HWO}\text{4}]^{-}\). The preliminary result of DFT calculation shows that \(\text{H}_2\text{O}\) can largely lower the activation energy barrier of the initial steps of fragmentation.

Novel Aspect:
This is the first investigation of the bare and the solvated polyanions with the objective to explicitly define the role of water in the dissociation chemistry of POMs.

References:


Introduction:
The AROMA (Astrochemistry Research of Organics with Molecular Analysis) setup [1] has been designed to analyse the organic molecular content of cosmic dust analogues, as part of the NANOCOSMOS ERC project. In this work, we test the capabilities of AROMA to study the energetics of molecular ion fragmentation using Collision Induced Dissociation (CID) scheme. A motivation is to discriminate structural isomers.

Methods:
AROMA is a hybrid setup, composed of a laser desorption/ionization source, coupled with a Linear Quadrupole Ion Trap (LQIT) and an orthogonal Time Of Flight (oTOF). Controlled CID experiments are performed in the LQIT under various conditions of colliding gas pressure and Dipolar Excitation (DE) conditions. Ion dynamics simulations using the Simion software are performed and combined with a Monte Carlo kinetics program.

Results:
We present results on the CID of the pyrene cation C_{16}H_{10}^+ colliding with Ar gas. We explored the influence of the DE parameters (amplitude, frequency, duration of the DE). This includes the two different modes of excitation (on and off resonance). With these parameters we can tune the collisional heating rate, reaching different regimes in the competition between heating and cooling by dissociation. This competition governs the breakdown curve (loss of the parent cation) and impacts the H over 2H branching ratio.

Using Simion, we performed simulations of the ion motion taking into account collisional cooling by He during transfer to the LQIT and subsequent dipolar excitation in the LQIT. A hard sphere model was used to describe collisions. We obtained the distributions of the energies at the centre of mass (ECM) and of the time between collisions. Both distributions are used as inputs in our Monte Carlo model. Comparison between simulations and experiments allows us to constrain the efficiency of kinetic to internal energy conversion.

Conclusions
We have set a protocol to quantify fragmentation in the lowest energy channels. This can be used to discriminate structural isomers in complex mixtures such as cosmic dust analogues or extraterrestrial materials. In particular we have been able to reevaluate the assignment of pyrene as the carrier of m/z=202.08 in the Murchison meteorite [1]. In the future, we will demonstrate how our heating scheme can also be applied to more strongly bonded systems such as C_{60}^+.

Novel Aspect:
Our combined experimental and simulation approach allows us to better control dissociation at threshold for large aromatic ions submitted to collisions with Ar in a LQIT.

References
Investigation of Hemicryptophane Host-Guest Binding Energies Using Advanced Tandem Mass Spectrometry

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Investigation of Hemicryptophane Host-Guest Binding Energies Using Advanced Tandem Mass Spectrometry

Keywords (maximum 5 keywords defining the topics of the abstract)
Host-Guest Chemistry; cryptophanes; blackbody infrared radiative dissociation; collision induced dissociation; MassKinetics

Introduction: (Limit of 400 characters)
In advancing host-guest (H-G) chemistry, considerable effort is spent to synthesize host molecules with specific properties including selectivity and adjustable affinity. An important step in the process is the characterization of binding strengths of the H-G complexes. Here, we present a mass spectrometry based multimodal approach to obtain bond dissociation energies (BDEs) of two hemicryptophane cages with three biologically-relevant guest molecules.

Methods: (Limit of 400 characters)
Two model hemicryptophane cages have been selected as the host molecules and their binding properties towards three biologically important guests: choline (C), acetylcholine (AC) and betaine (B) have been studied in the gas phase. Quantitative comparison of the binding dissociation energies of different H-G pairs was achieved using a combination of BIRD and high-pressure CID mass spectrometric techniques with Rice–Ramsperger–Kassel–Marcus (RRKM) modeling.

Results: (Limit 900 characters)
Obtained BDE values fall within a rather narrow range for all host-guest complexes. Among the informative trends and differences extracted from the data, we note that complexes of cage 1 exhibit higher BDE’s, and thus are all more stable than those of cage 2. This trend establishes that the linkers (naphthyl for cage 1 and phenyl for cage 2) play important roles in determining the strength of the H-G complexes. Another interesting comparison is the relative stability ranking between various guests for a given host cage. Notably, for both cages 1 and 2, the order of stability of H-G complexes is as follows: choline > acetylcholine > betaine. DFT calculations were performed to gain information concerning possible binding modes and relative stabilities of different non-covalent H-G interactions. The tren and the CTV of the hemicryptophane adopted an approximately C3 symmetry. The arrangement of the phenyl or naphthyl linkers for the six H-G complexes gives compact structures that allow the insertion of the ammonium moiety of the guest inside the cage.

Conclusions (Limit of 400 characters)
The multimodal approach described here, using the BIRD technique in combination with high pressure-CID breakdown curves and RRKM modeling, is useful not only for estimation of bond dissociation energies, but also for accurate surveying of the influence of different functional groups on H-G binding properties. Utilization of this approach can provide a wealth of information pertinent to chemists who seek to design new H-G systems with well-honed properties.

Novel Aspect: (Limit of 150 characters)
Two hemicryptophane host molecules are assessed for their binding properties towards three biologically important guests in the gas phase.
Keywords: UVPD, MALDI – UVPD, EP-MALDI – Orbitrap, Top-Down proteomics

Introduction:
Recent literature reports demonstrate that ultraviolet photodissociation can provide extensive sequence coverage for ESI generated protein ions. UVPD has a much lower charge state dependence than ETD and CID, thus the singly charged protein ions produced by MALDI may exhibit equal propensity to fragment. Here we compare UVPD of MALDI and ESI generated ions using a Q-Exactive equipped with a EP-MALDI/ESI dual source and UVPD.

Methods:
All experiments were performed using a Q-Exactive Plus (Thermo Scientific, Bremen) equipped with a ESI/EP-MALDI dual ion source (Spectroglyph LLC, Kennewick WA, USA). UVPD was performed using a 193 nm ArF laser (Excistar XS 500, Coherent, Santa Clara CA, USA) aligned coaxially with the HCD cell. ESI was performed using 10 µM standard protein solutions infused at 5 µL/min. MALDI samples were prepared using the dried droplet method (CHCA matrix, 1000:1 molar excess).

Results:
UVPD of MALDI generated ions was benchmarked against MALDI-ISD and ESI-UVPD performed on the same accurate mass, high resolution mass spectrometer. ESI was first used with peptide and protein standards to optimize laser trigger timing, number of laser pulses and laser pulse energy. UVPD of ESI generated ions using the Q-Exactive Plus performed identically to those previously reported using hybrid instruments. The optimized parameters were used for UVPD of MALDI generated ions, specifically the small peptide bradykinin and the small protein thymosin β4 (monoisotopic masses 1059.57 and 5205.63, respectively). The UVPD fragments were compared at different laser pulse energies, and with those obtained from HCD and ISD.

Conclusions
The results demonstrate that MALDI UVPD outperformed MALDI HCD for all laser pulse energies. While MALDI ISD also provided extensive sequence coverage, its lack of precursor ion selection limits its application to isolated proteins whereas MALDI UVPD can be used on proteins from complex mixtures.

Novel Aspect: UVPD performed on intact protein ions generated by EP-MALDI on a modified Q-Exactive Plus, demonstrating its ability to further improve MS/MS capabilities for MALDI generated ions.
INTRODUCTION:
Surface-induced dissociation (SID) is a powerful tool both for analytical applications and for studies of gas-phase fragmentation of complex ions. We have developed experimental approaches for studying the energetics, dynamics, and kinetics of large ion dissociation using SID, which in combination with comprehensive modeling enable mechanistic understanding of complex ion fragmentation difficult to obtain using other experimental techniques.

METHODS:
Experiments were performed on a specially designed Fourier transform ion cyclotron resonance mass spectrometer (FT-ICR) configured for time- and collision energy-resolved SID experiments. The experimental results were modeled using RRKM and the analytical form of the internal energy deposition function that adequately describes energy transfer in collisions.

RESULTS:
SID experiments were combined with resonant ejection of selected fragment ions. The approach was first examined using well-characterized peptide systems and subsequently used to explore the kinetics of the gas-phase fragmentation of ligated metal clusters. When combined with RRKM modeling, these experiments facilitate detailed understanding of the competition between different fragmentation pathways.

CONCLUSIONS
SID experiments highlight an important role of entropy in dissociation of complex ions. Resonant ejection experiments identify different populations of stable structures and inform on the kinetics of the competing fragmentation reactions.

NOVEL ASPECT:
SID provided unique insights into the energetics of complex ion fragmentation.
Introduction:
The dye Indocyanine Green (ICG) is widely used for medical purposes and as a fluorescent dye [1]. Investigations on the fluorescence of ICG showed very short fluorescence lifetimes [2] which leads to the requirement of femtosecond laser pulses for photodissociation experiments. The results will be compared to collision-induced dissociation processes to investigate stability and reactions of ICG.

Methods:
The mass spectra have been recorded on an Apex III FT-ICR mass spectrometer (7.05 T) (Bruker Daltonik, Germany) using different fragmentation techniques. The ionization was performed via Electrospray Ionization from different solvent mixtures. For the fragmentation reactions, collision gas (Argon) or a femtosecond laser system (Quantronix, USA) with 790 nm and 150 fs pulse duration were used.

Results:
Depending on the solvent mixture, the sodiated or the protonated ions and their clusters were observed in the mass spectra. Collision-induced dissociation (CID) and photodissociation (PD) were applied to the different ions. The photodissociation process at a wavelength of 790 nm resulted in several intense fragments for the sodium adduct and less intense fragments for the protonated molecule. Most fragments arise from the dissociation of a linkage between two identical aromatic systems. Beside these cleavages, only the bisodiated ICG shows the elimination of some small neutral fragments. In contrast to the PD spectra the CID-spectra showed poor fragmentation for the protonated molecule and no fragmentation for the sodium adduct.

Conclusions:
The poor fragmentation in the CID process leads to the conclusion that the collision energy does not exceed the required energy that is reached by multiphoton absorption of the femtosecond laser pulses. The comparison of the fragments from the protonated and the sodiated molecules gives a hint for the influence of the sodium cation on the molecular stability. [3]

Novel Aspect:
Photodissociation reactions of ICG and its clusters in the gas phase using femtosecond laser irradiation.

References:
Introduction:
Quadrupole ion traps play a central role in the investigation of ion-fragmentation processes due to their capability to conduct MSn experiments. The motion of ions inside quadrupole ion traps is complex but can be described by Mathieu’s equation.[1] Due to the complexity of the consequences of the change in stability parameters the fragmentation has been investigated in the past only by simulation programs. We present a systematical investigation of this process.

Methods:
The analytical solution of Mathieu’s equation has been combined with phase-space analysis. Phase-space ellipses have been implemented in calculations over their ellipse angle and explicitly not the quadratic equation, this way avoiding the common treatment of the ellipses as uniformly populated, thus providing a correct treatment of time.

Results:
The phase-shift between the fundamental ion oscillation and the superimposed micromotion creates an elliptic relationship between the position and the velocity of ions at a specific phase of the applied alternating potential. We can show by appropriate application of trigonometry that the angle (the position on the ellipses in phase-space has regarding the coordinate system axes) is related to the variable time and with that, the probability to find an ion on a certain position in phase-space varies.

By the use of these ellipses we provide a straightforward explanation, why and how the amplitudes of ion trajectories can drastically change, resulting in loss of ions, although their m/z value is above the Low-mass cut-off (LMCO) and to which extent this is expected.[2] By the use of the center-of-mass collision energy, E_{com}, we define effective temperatures and further give information about the likelihood of ions enduring successive excitation that can result in consecutive fragmentation.[3]

Conclusions:
We explain how the variables are connected to each other in the complex fragmentation process and provide clues regarding the origin of certain observations as being caused by instrumental or molecular properties. Further general trends are given and it is also discussed how a change in LMCO settings might help achieving the desired information.

Novel Aspect:
Consequences of the change in stability parameters during fragmentation are presented with special emphasis on the likelihood for successive fragmentation events.

References:
T. S. Neugebauer and T. Drewello (in preparation)
T. S. Neugebauer and T. Drewello (in preparation)
Introduction:
The omnitrap platform is a novel linear ion trap sustaining a diverse ion activation network including collision-induced and electron directed dissociation of ions. A series of experiments involving collision-induced dissociation of radical ions formed by meta-ionization of poly-protonated species using >10eV electrons are performed and new informative transitions in the fragmentation patterns are reported for insulin chain B and ubiquitin ions.

Methods:
Precursor ions selected in the quadrupole of a QExactive+ are transferred into the omnitrap platform. Electron meta-ionization of protonated ions is performed by varying electron energy, adjusting irradiation time and the frequency of the trapping waveform to maximize radical ion abundance. CID MS3 experiments of EI-produced hydrogen-deficient radicals are compared to their protonated counterparts in the same charge state.

Results:
Electron meta-ionization of insulin chain B ions is performed and subsequent CID of the radical ions reveals the formation of three complementary c, z fragment pairs near tyrosine residues. A mechanism is proposed to explain the dissociation pathway involving meta-ionization at or near the tyrosine residue followed by the rapid phenolic proton migration towards a basic site, e.g. N-terminus, forming a protonated group, with a neutral radical remaining at the tyrosine residue and initiating N-Ca backbone cleavage. Electron meta-ionization of 7+ ubiquitin ions produced 8+(.) hydrogen deficient species. Comparison of CID MS/MS data of the 8+ protonated ions with the hydrogen deficient radicals at the same charge state reveal significant differences, especially by the presence and position of a, x and c, z fragmentation patterns, as well as significant similarities in the relative abundances of b, y fragments.

Conclusions
The unique versatility of the ion activation network available in the omnitrap platform is demonstrated using MS3 CID experiments of meta-ionized species. Enhanced sequence information is obtained in CID experiments of the radical ions compared to even electron species at the same charge state. Cleavage position, fragment type and abundance are analyzed in great detail revealing the hitherto unreported fragmentation processes.

Novel Aspect:
Novel ion activation tools for the orbitrap mass analyzer and expanded versatility of the omnitrap platform for top-down mass spectrometry.
Keywords: MALDI, tandem TOF instrumentation, high-energy CID, charge-remote fragmentation

Introduction:

By the end of the millennium high-energy CID were moved from FAB/LSIMS tandem sector to MALDI tandem TOF devices. Surprisingly, this generation of instruments exhibits extremely different instrumental features (lasers, collision energies, reflectrons and flight path lengths). In order to demonstrate similarities/differences among high-energy CID spectral data from peptides, lipids and oligosaccharides it is of great interest for future applications [1-4].

Methods:

MALDI high-energy CID spectra of selected peptides, triacylglycerols, glycerophospholipids and oligosaccharides were acquired on an AB Sciex 4700/4800 (ELAB=1 keV), a Bruker DaltonicsultrafleXtreme (ELAB=8 keV), a Shimadzu Axima TOF2 (ELAB=20 keV), a Shimadzu MALDI 7090 (ELAB=20 keV) and a JEOL SpiralTOF/TOF instrument (ELAB=20 keV). MALDI matrices used included α-cyano-4-hydroxy cinnamic acid (only peptides) or 2,4,6-trihydroxy acetophenone.

Results:

 Whereas tandem TOF instruments fitted with a limited energy acceptance dual stage reflectron (AB Sciex, Bruker) are typically operated with a rather low collision energy (1-8 keV), the ones with a wide energy acceptance curved field or offset parabolic reflectron (Shimadzu, Jeol) allow a collision energy of 20 keV. Protonated peptide molecules only exhibit side chain charge-remote fragmentations (d-, v-, w-ions) at 20 keV collisions allowing distinction between isomeric leucine and isoleucine. Triacylglycerols and glycerophospholipids show - depending on the type of precursor ion (protonated, sodiated) - diagnostic charge-remote fragmentation of the alkyl chains best seen at 20 keV collisions. For sodiated triacylglycerols a clear differentiation between sn1-/sn3- versus sn2-substituents could be made, hardly seen at 1-8 keV collisions. In contrast, sodiated oligosaccharides do not seem to require such high collision energies as there is virtually no difference in the fragmentation pattern in the CID spectra taken at 1, 8 or 20 keV.

Conclusions:

Only instruments fitted with wide energy acceptance reflectrons (curved field and offset paraboloid reflectron) yield true high-energy CID spectra of biomolecules with typical product ions in high abundance (peptide side-chain fragmentation, lipid charge-remote fragmentation). Still a major issue for the observation of unwanted PSD ions in most tandem TOF instruments is a long flight path (~4 m) obscuring CID ions due to their lower intensity (a factor of 10-20).

Novel Aspect:

For the first time different designs of MALDI tandem TOF devices fitted with high-energy CID cells are compared for their usefulness in structural elucidation of biomolecules.
References:

Introduction:
Cryogenic infrared spectra can serve as a gold standard for molecular identification in MS [1]. However, a key failing of action spectroscopy remains its extremely low duty cycle, where only one analyte is probed at one wavelength step at one time. Here, we discuss the first mass-selective cryogenic trap [2], which allows the IR spectra of multiple analytes to be probed in parallel, given the predictable mass increase/decrease of tagging spectroscopy [3].

Methods:
A custom cryogenic 2D linear ion trap (cryoLIT) is composed of rectilinear electrodes equipped with slits in the left/right rf electrodes for radial ejection. Temperature control is enabled via a helium cycle cryostat, cartridge heaters, and temperature sensors. A gas pulse of He (and 5% N2)allows ion cooling and tagging. Ions are mass manipulated using an auxiliary dipolar waveform on the left/right rf electrodes that is resonant with the secular frequency.

Results:
When cooling the cryoLIT to cryogenic temperatures, ions exit the trap at lower rf amplitudes in the mass instability scan, which is due to a shrinking of the trap. The magnitude of this shift is related to the expansion coefficient of the material, 0.33% for 17-PH steel and 0.65% for OFHC copper. Two types of tagged ions will be considered here: 1) solvent-tagged ions (H2O and CH3CN) from the electrospray source, or 2) van der Waals N2-tagged ions generated in the cryogenic trap. Given their extremely low binding energies, the parameters for generating and manipulating N2-tagged ions are critical. Still, with optimized trap temperature (24K), pumpdown delays, and isolation waveforms a background-free mass isolation of N2-tagged ions is feasible. High-quality IR spectra of small analytes (tryptophan, MDMA, Leu-enkephalin, etc.) can be recorded in this way and are very comparable to literature cryogenic IR spectra [4]. Current efforts focus on neon tagging, which may be the optimal tag in terms of mass increase (+20) and minimal IR spectral distortion.

Conclusions:
The mass-selective cryoLIT is capable of measuring high-resolution IR spectra of multiple analytes in a multiplexed fashion at reasonable speeds (< 30 min), and at relevant concentrations (nM). This sets the stage for applying cryogenic IR spectroscopy as a bioanalytical tool for small molecule identification (e.g. metabolites). Two-trap designs can further improve speed by decoupling the pumpdown delay (100 ms) from mass manipulation and analysis (100 ms).

Novel Aspect:
First mass-selective cryogenic 2D ion trap where extremely fragile van der Waals-tagged ions can be mass isolated for background-free infrared photodissociation.

References
Introduction:
Recently, MS-based proteomics has become a powerful tool in protein identification and characterization. In the bottom-up approach, the choice of collision energy of the peptide MS/MS experiment significantly affects the performance of the method. High-level objectives, e.g., highest identification score, have not been explored in this respect. The present work maps the energy dependence of this proteomics variable and determines the optimal collision energy.

Methods:
Tryptic digests of complex standards (HeLa, E. Coli) were subject to nano-HPLC-MS/MS experiments at 21 different collision energies on a Bruker Maxis II ETD QTOF instrument. Spectra were matched against the SwissProt database using the Mascot search engine. Further analysis, including comparison of LC-MS/MS runs and fitting of the Mascot score vs. collision energy curves, were performed with in-house developed software using Mascot output files as inputs.

Results:
A cluster analysis of Mascot score vs. energy curves revealed two types of energy dependent behavior (unimodal and bimodal) in about 50–50% of the investigated peptides. For bimodal peptides, number and summed intensity of b fragment ions show maxima around the first score maximum. y ions are less sensitive to collision energy but more intense and numerous near the second score maximum. The optimal collision energy, obtained as the peak positions from one or two fitted Gaussians, follow linear trends with respect to m/z, but the three groups have different m/z dependence. The presence of mobile proton decreases the optimum significantly (1–5 eV), but other factors also seem important indicated by the large residual variance even of the separate linear fits for peptides with mobile and partially mobile protons. Where 2 maxima exist, their difference is surprisingly m/z independent and it is ~17 eV on average for both +2 and +3 peptides.

Conclusions:
An unprecedented bimodal behavior of the score vs. energy curves, linked to the different behavior of y- and b-type ions, was identified for about half of the peptides. For these, the average score increases by ~15% upon energy optimization. On the other hand, for unimodal peptides, the original default collision energy seemed quite good, only a slight increase of ~4% could be achieved. The optimal energy has linear m/z dependence with marked effect of proton mobility.

Novel Aspect:
This is the first study to address collision energy dependence of a peptide identification score and to quantify the role of less abundant b-ions in peptide identification [1].

References
SPECIFIC RADICAL-DRIVEN DISSOCIATION OF HYDROGEN-EXCESS AND -DEFICIENT RADICAL PEPTIDES PRODUCED BY MALDI IN-SOURCE DECAY MASS SPECTROMETRY

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Keywords: Radical-Driven Dissociation, MALDI-ISD, Hydrogen-Excess/Deficient Peptides, Mechanism

Introduction: (Limit of 400 characters)
Radical-driven dissociation (RDD) using ultraviolet, electron transfer, high-energy collision and redox matrix provides us practical information for structure elucidation of organic chemicals, peptides and proteins. Using MALDI MS with redox matrix here we report detailed mechanisms of the formation of hydrogen-excess/deficient radical peptides, \([\text{M+H}]^+\) and \([\text{M – H}]^+\), and specific RDD reactions.

Methods: (Limit of 400 characters)
MALDI-ISD experiments were performed with a TOF MS AXIMA-CFR (Shimadzu, Kyoto, Japan). Redox matrices used were 2,5-dihydroxybenzoic acid (2,5-DHB) and 5-amino-1-naphthol (5,1-ANL) for H-donation and 5-nitrosalicylic acid (5-NSA) and 4-nitro-1-naphthol (4,1-NNL) for H-abstraction [2]. The analytes used were synthetic peptides RGV and EGV having Arg at N-terminus (RGVKVDYGPAMAFADLAAEF, RGV) and C-terminal side (EGVKVDYGPAMAFAPADLAARF, EGV).

Results: (Limit 900 characters)
ISD spectrum of RGV with H-donating matrix 5,1-ANL merely gave \([c+2H]^+\) ions originated from cleavage at N-Ca bond [1], while the use of 2,5-DHB resulted in \([c+2H]^+\) and \([a]^+\) ions and some \([b]^+\) and \([d]^+\) ions. The ISD spectrum of EGV with 5,1-ANL showed preferential \([z+2H]^+\) ions and minor \([y+2H]^+\) and \([c+2H]^+\) ions, while the spectrum with 2,5-DHB gave preference \([y+2H]^+\) ions and minor \([z+2H]^+\), \([x]^+\) and \([c+2H]^+\) ions. The use of 5,1-ANL resulted in only H-excess radical peptide ions \([M + 2H]^+\)-having the radical (unpaired electron) located on carbonyl carbon of the backbone, and the radical ions \([M + 2H]^+\)-can lead to \([c + 2H]^+\) ion and radical intermediate ion \([z + H]^+\), although the intermediate never be observed in ISD spectra. On the other hand, the use of H-abstracting matrix 4,1-NNL resulted in preferential \([a]^+\) ions and some \([b]^+\) and \([d]^+\) ions for RGV and preferential \([y]^+\) ion for EGV.

Conclusions (Limit of 400 characters)
The use of naphthalene skeletal redox matrices 5,1-ANL and 4,1-NNL gave simple and very useful information for sequencing peptides and proteins in the MALDI-ISD experiments [2]. The observable fragment ions such as \([c+2H]^+, [z+2H]^+, [y+2H]^+, [a]^+, [x]^+ and [y]^+\) ions were exactly controlled by the sites of radical (unpaired electron) and Arg residue, and the RDD reactions obeyed simple and successive radical-initiative process.

Novel Aspect: (Limit of 150 characters)
It was indicated that \([a]^+\) and \([ym+2H]^+\) ions were originated from beta-carbon-centered radical ions \([M-H+H]^+\) and radical intermediates \([zm+1+H]^+\), respectively.

References
Introduction:

Comprehensive structural elucidation of glycan mixtures is challenging. Here, glycans are reduced and permethylated or labeled with a reducing-end fixed charge to increase sensitivity, avoid gas-phase structural rearrangement and facilitate spectral interpretation. A PGC column was used to maximize isomer separation [1]. EED was utilized due to its ability to generate informative MS/MS spectra to allow accurate determination of both the topology and linkages.

Methods:

Glycan standards, LNT, LNnT, LNFP I, II, III, V and VI, were either reduced and permethylated [1], or labeled with a reducing-end fixed charge via reductive amination with 3-aminopyridine followed by methylation using iodomethane in ACN [2]. Separation was carried out on a Waters nanoACQUITY UPLC, with a nanoPGC column held at 50 °C. EED-MS/MS analyses were performed on a Bruker 12-T solariX FTICR MS with the cathode bias set between 16 V and 18 V [3-4].

Results:

PGC-LC can often achieve better isomer resolution than RPLC. For example, reduced and permethylated linkage isomers, LNFP II and III, are barely separated by RPLC, yet they are baseline resolved by PGC. Meanwhile, glycans with a reducing-end fixed charge are not retained by RPLC, but form sharp peaks in PGC.

EED can generate more informative spectra than CID or HCD. Here, for glycans with a reducing-end fixed charge, EED produced complete series of Z-, Y- and 1,5X-ions, forming triplets with characteristic spacing that could be easily recognized to facilitate accurate determinations of the glycan topology. Additional radical-driven dissociation pathways were also identified, generating linkage-specific cross-ring, secondary or internal fragments throughout the glycan backbone. The presence and location of the branching site(s) and the linkage configuration at HexNAc residues could also be determined based on characteristic fragmentation patterns occurring only at those sites.

Conclusions:

Isomer resolution was achieved on PGC-LC for glycans in two different derivatized forms. EED of permethylated glycans and glycans with a reducing-end fixed charge produced rich structural information with unique spectral
features for reliable determination of the glycan sequence, branching pattern and linkage configurations. Online PGC-LC-EED-MS/MS should become a powerful method for comprehensive glycome characterization.

Novel Aspect:

Separation and detailed structural characterization of isomeric glycan mixtures are achieved in a single LC-EED-MS/MS analysis.

References:


For information please contact: scientific@imsc2018.it
INTRODUCTION:
Analysis of aldehydes and ketones has been achieved by formation of hydrazone derivatives and negative-ion electrospray tandem mass spectrometry [ESI(-)MS/MS] [1,2]. Upon collision induced dissociation (CID), major product ions were formed by losses of NO and NO2 [3]. In our current ESI(-)MS/MS studies on hydrazones of 2-oxo carboxylic acids, fragmentations are directed by the carboxylate group.

METHODS:
Mass spectra were obtained using ESI(-)MS/MS with MeOH solvent. A triple quadrupole spectrometer gave both product- and precursor-ion spectra, while an ion trap spectrometer gave complementary product-ion spectra. Quantum mechanical computations of gas-phase ions were done using Gaussian 09 software at the MP2/6-311++G(2d,p)//B3LYP6-31++G(2d,p) level of theory.

RESULTS:
Phenyl, 4-nitrophenyl and 2,4-dinitrophenylhydrazone derivatives of glyoxylic and phenylglyoxylic acid were prepared. Upon CID, the deprotonated glyoxylate and phenylglyoxylate hydrazones primarily yielded anilide ions; decarboxylation was a minor process for the glyoxylate derivatives. The major pathway was consistent with decarboxylative elimination [4], in which an anilide ion, a nitrile and CO2 are formed by N–N and C–C bond cleavage. Barriers of less than 100 kJ/mol were determined by computations and breakdown curves showed the influence of nitro and phenyl substituents on the energetics of decarboxylation. ESI of samples in acetonitrile gave reduced abundance of the minor decarboxylated product ion. The minor pathway was attributed to solvent-assisted ionization on nitrogen, rearrangement to Ar-N=N-CH2CO2– (barrier = 130 kJ/mol) and lastly decarboxylation (negligible barrier).

CONCLUSIONS:
The fragmentation behavior of the 2-oxo carboxylate hydrazones resulted from reactions of the carboxyl group. The major fragmentation pathway proceeded by decarboxylative elimination, whereas the minor pathway observed for the glyoxylate hydrazones involved ionization on nitrogen, rearrangement and subsequent decarboxylation.

NOVEL ASPECT:
Different pathways of deprotonated hydrazone derivatives highlight the challenges of predicting the fragmentation behavior of multifunctional ions.

REFERENCES:
Introduction: LILBID is an ion desorption method for native mass spectrometry in which droplets of aqueous solution are irradiated with pulsed IR laser light, leading to explosive expansion of the droplets and release of sample molecules as ions. LILBID-MS is primarily used to assess the stoichiometry of noncovalent biomolecular complexes. Here we outline a new method to quantitatively determine binding affinities with LILBID-MS and present preliminary results from this method.

Methods: In LILBID, harsher or softer laser energy transfer leads to different amounts of dissociation of noncovalently bound biomolecular complexes. In this new method, the sample is measured while purposely varying the energy transfer from the desorption laser to the droplet in the LILBID ion source. The amount of dissociation in the resulting spectra is then plotted against a measure of energy input, resulting in a dissociation curve analogous to a melting curve.

Results: The amount of energy transferred to the sample during desorption can be changed by varying instrument parameters such as laser power and droplet position within the laser beam. However the relationship between these parameters and the amount of energy transferred to the sample is not straightforward. We monitor energy input using the shape of the droplet’s explosive expansion. Certain parameters of the shape of this expansion are closely correlated with both energy input and spectral results and can be used as robust measures of laser energy transfer. During a binding affinity measurement, one of the instrumental parameters for energy transfer is purposely varied as droplets are emitted. For each droplet, an explosion image and a mass spectrum are recorded. The shapes of the explosive expansions are analyzed, and the amounts of dissociation in the spectra are calculated and plotted against the corresponding laser energy transfer, which we derive from the explosion shape. The resulting curve is a plot of dissociation with increasing energy input.

Conclusions: Dissociation curves for different short dsDNA reflect the binding strengths of the samples, demonstrating that this method allows conclusions about the binding strength of biomolecules in solution. Since data for each dissociation plot can be collected within minutes and with a fraction of the sample necessary for a titration or a melting curve with LILBID, this method represents an attractive alternative to titration-based binding affinity experiments.

Novel Aspect: This method constitutes a new way to perform fast top-down analysis of binding affinities of biomolecular complexes.
INTRODUCTION:
Lipidomics, ultraviolet photon dissociation, ozonolysis, double-bond position

Methods:
Lipids were extracted from human blood plasma [1] and analyzed using both shotgun and LC-MS approaches. For the latter, lipids were separated using a Thermo Scientific™ Accucore™ C30 column (2.1 x 150 mm, 2.6 μm) and a Thermo Scientific™ UltiMate 3000™ UHPLC system operated at 50°C, and a flow rate of 325 μL/min. ESI-MS analysis was performed in positive ion mode on a modified Q Exactive HF mass spectrometer with ozone (approximately 11% O3 in oxygen O2) replacing nitrogen as the HCD collision gas. Photodissociation was implemented on a Thermo Scientific™ Orbitrap Fusion™ Lumos™ Tribrid™ MS equipped with a 213 nm UVPD laser.

Results:
Preliminary experiments were performed incorporating OzID on a Thermo Scientific™ Q Exactive™ HF MS to identify the position of acyl chain position and double-bonds in phospholipids and triacylglycerols. By combining HCD activation with OzID on selected [M + Na]+ lipid ions on the Thermo Scientific™ Q Exactive™ HF MS, the relative position of acyl chains on the glycerol backbone could be determined without compromising in scan time. In a shotgun phospholipidome analysis of plasma it was possible to cover the mass range from m/z 650 to 950 in only 14 minutes. Importantly, a single phospholipid HCD/OzID spectrum provides information about the phospholipid class, the number of carbons and double bonds and their sn-position, therefore highlighting the presence of isomeric lipids.

Additionally, reaction of selected lipid ions with ozone inside the mass spectrometer can produce two characteristic product ions: an aldehyde and a Criegee ion. These ions are diagnostic for the double bond position and can be obtained by OzID increasing the HCD cell fill time and decreasing the collision energy. With this technique, double bond position of the analyzed lipids can be readily obtained. We found the triacylglycerol and phospholipid fractions within plasma samples to be rich in monounsaturated fatty acids containing a double bond in either the n-7 or n-9 position. OzID experiments were compared with lipid fragmentation by UVPD on an Orbitrap Fusion Lumos MS equipped with a 213 nm laser. Experiments conducted by Williams et al. [2] and Ryan et al. [3] report this new tool as a promising technique to complete structural elucidation of lipid structures.
For Research Use Only.

Conclusions
In our study we compared ozonolysis and ultraviolet photon-dissociation for comprehensive lipid structure elucidations. Insaturation in cholesterol ester, phospholipids and triacylglycerols can be identified with both techniques due to the generation of structural diagnostic product ions: the aldehyde and a Criegee ion for the Ozonolysis and a pair of ions formed by the cleaving in specific site of the double bond upon photon uptake. Linoleic acid (18:2n-6, n-9) was observed as the major polyunsaturated fatty acid in the lipidome with both techniques.

Novel Aspect:
Successful implementation of OzID and UVPD as complementary techniques for comprehensive global lipid structure elucidation and resolving isomeric lipids.

References:
Introduction:
Ambient mass spectrometry (AMS) has become more and more attractive because of its simple sample pretreatment process, the ability to operate in the open air, and capability to probe the surface of samples of any size and shape. The development of AMS opens a new insight for realizing high-throughput, nondestructive, in situ sample analysis from complex sample[1-3].
The pursuit of high-throughput sample analysis from complex matrix makes the development of multiple ionization techniques with the complementary specialties on demand. Compared with single ambient ionization, combined or hybrid ambient ion source have been attempted to achieve complementary and high-throughput analysis, the reduction of sample complexity, and allowing the simultaneous detection of a broader range of analytes.

Methods:
A versatile integrated ambient ionization source (iAmIS) platform was proposed in this work, based on the ideas of integrating multiple functions, enhancing the efficiency of current ionization techniques, extending the application and decreasing the cost of the instrument. The design of the iAmIS platform combines 5 different ionization mode. All individual and combined ionization mode can be easily realized by modulating parameters.

Results:
AMS and their combination with other fast sample pretreatment approaches (for example, SPE and SPME etc) and the coupling with different instrument have been developed in our lab [2-4]. By using this iAmIS platform, coconut essential oil and perfume sample has been successfully analyzed. On the other hand, together with the immunalrecognition, DESI mode and signal amplification strategy,ultrasensitive detection of proteins in attomolarorzeptomolar were realized.

Conclusions
A versatile integrated ambient ionization source (iAmIS) platform was developed. Complex sample can be analyzed by using this platform, while the sensitive detection of protein at attomolar level can be realized using DESI-MS mode.

Novel Aspect:
A versatile integrated ambient ionization source (iAmIS) platform was developed for diverse applications.

References

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756 - THE ROLE OF NEW MATERIALS AS SAMPLING SUBSTRATES FOR THE DESI-HRMS DETERMINATION OF NEW PSYCHOACTIVE SUBSTANCES IN SALIVA

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Keywords: Desorption electrospray ionization-high resolution mass spectrometry, sampling substrates, new psychoactive substances, screening method

Introduction:
New psychoactive substances (NPS) are a large group of drugs of abuse: being considered as a major threat to public health, the development of rapid methods for their determination is in great demand. DESI-MS enables the in situ analysis of specimens in their native ambient state with minimal sample pretreatment [1,2]. In this context, the role of new materials as sampling substrates is of paramount importance to increase the instrumental response of the analytes.

Methods:
Different sampling substrates, i.e. commercial PTFE, poly(lactic acid) (PLA)- and silica-based films were synthesized and tested for the DESI-HRMS analysis of NPS (synthetic cannabinoids, synthetic cathinones, ketamine) in saliva. Oral fluid samples from volunteers outside clubs and during roadside patrol control on drivers were collected. A Micro Extraction by Packed Sorbent (MEPS)-DESI-HRMS method was optimized and validated for NPS determination.

Results:
The first phase of the study was devoted to the optimization of the DESI parameters affecting the ionization yield and the ion recovery in terms of: i) spray composition; ii) spray flow; iii) acquisition mode; iv) geometrical parameters. With the aim of achieving high sensitivity, different sampling substrates, i.e. unfunctionalized PLA, carbon/PLA-based films and a silica-based coating were synthesized and tested. Both unfunctionalized PLA and the silica-based coating proved to be the best choice for NPS detection. A full factorial design followed by the multi-criteria method of desirability functions was applied for the optimization of the MEPS extraction in terms of both loading and eluting cycles. The method was validated obtaining detection limits in the µg/kg level, good linearity, precision and accuracy. Finally, the method was applied for NPS determination in saliva samples: achieving results in agreement with those obtained by a MEPS-GC-MS method used for confirmatory purposes.

Conclusions:
The synthesized support materials showed enhanced performances in improving the DESI-HRMS responses of the investigated compounds. Both the MEPS-DESI-HRMS and MEPS-GC-MS methods represent suitable tools for NPS analysis in oral fluids, combining screening and confirmatory purposes. In particular, the DESI-HRMS method allowed high throughput analyses characterized by both high sensitivity and selectivity, very short analysis times and low costs.

Novel Aspect:
Pivotal role of new supporting materials in enhancing the MS responses of the investigated compounds, thus allowing their rapid detection at trace levels in complex matrices

References
Introduction:
In recent years, various ionization techniques for mass spectrometry (MS) have been published that allow fast and simple analysis of samples under ambient conditions. Plasma-based methods in particular aroused the interest of many researchers because they offer great potential for use in mobile analytical devices. However, systematic investigations of signal response with this technique, such as the influence of the matrix on signal intensity, are still rare.

Methods:
In a first approach, six anilines each in nine different solvents were spotted on a paper target and analyzed by low temperature plasma ionization (LTPI) MS under optimized conditions [1]. In a second approach, 1 µL of a 1 mM chlorpyrifos solution, a common pesticide, was analyzed from peels of different fruits, vegetables and mushrooms. The samples were analyzed using a plasma source coupled to a Bruker 3000+ ion trap.

Results:
Our results show that the impact of compound characteristics strongly outperforms the influence of simple sample matrices such as different organic solvents and water, with the trend that volatile solvents tend to decrease the signal responsiveness of the analytes. However, several specific solvent-analyte interactions occurred, which have to be considered in targeted applications of this method. When choosing a single compound to assess matrix effects, the pesticide chlorpyrifos was detected on all tested different fruits, vegetables and mushrooms. However, we found a strong influence of the matrix on the signal response. For example, citrus fruits showed a particularly strong signal suppression.

Conclusions:
For the influence of the solvents, with the exception of water, most solvents seemed to be almost equally well suited for mass spectrometry with LTPI. However, a better signal intensity of the analyte with higher boiling solvents was achieved indicating that a high boiling matrix may be optimal with this ionization technique. In contrast, when investigating the influence of biological matrices such as peels of different fruits, vegetables and fungi, the response of chlorpyrifos was particularly decreased when analysis was done from the waxy surfaces of citrus fruits. To find the reasons for this behavior is an ongoing project in our lab.

Novel Aspect:
We present new insights on matrix effects in low temperature plasma ionization for mass spectrometry.

References
APPLICATION OF LDTD/TQMS-HRMS METHODS FOR QUANTIFICATION OF PSYCHOACTIVE COMPOUNDS IN TISSUE MICROSAMPLES.

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Keywords: LDTD, psychoactive compounds, microsamples, brain, juvenile fish

Introduction
Psychoactive pharmaceuticals exhibit continuously increased consumption trends[1]. Although a concentration reached only residual levels a negative effect on aquatic organism have been observed[2]. Laser diode thermal desorption (LDTD) is direct sample introduction technique without previous analyte separation. The new analytical method with this instrumentation have been developed for psychoactive compound analysis in tissue microsamples.

Methods:
The method has been evaluated for low (LDTD-QqQ) and high-resolution (LDTD-HRPS) mass spectrometersthen compared with previously developed method employed liquid chromatography (LC-HRPS). The exclusion of separation technique brings some benefits such as dramatic reduction of analysis time requirement and exclusion of sample filtration as pre-treatment step which can be beneficial specially when total sample amount is limited.

Results:
The LDTD parameters were carefully evaluated to achieve sufficient number of data points, the highest and the most stable response.
Linear of calibration curve (0.1-50 ng·g⁻¹) were confirmed for all mass spectrometers and compounds by R2 coefficient (no lower than 0.9950).
Developed method was validated to brain tissue samples fortified at four concentration levels. For example, average recovery obtained for citalopram at concentration of 5 ng·g⁻¹ was 102±4 % (LDTD-HRPS) and 136±9 % (LDTD-QqQ), respectively. Similar results were obtained for tramadol in a case of QqQ.

Conclusions:
Validated method was used for analysis of brain tissue samples of juvenile fish exposed to psychoactive compounds at environmentally relevant concentrations.Preliminary obtained correspond with results from LC-HRPS and show potential of LDTD-QqQ and LDTD-HRPS method for analysis of complex biological samples in very short run time.

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CENAKVA (No. CZ.1.05/2.1.00/01.0024) and CENAKVA II (No. LO1205 under the NPU I program), Czech Science Foundation (No. 16-06498S).

Novel Aspect:
The newly developed analytical method is suitable for biological microsamples analysis for environmental research purpose.


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1257 - IMPROVEMENT OF NANOELECTROSPRAY AND DESORPTION NANOELECTROSPRAY SIGNAL BY REGULATION OF PRESSURE IN AN INLET OF TRIPLE QUADRUPOLE MASS SPECTROMETER

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Keywords: mass spectrometry, nanoelectrospray ionization, desorption nanoelectrospray

Introduction:
Both nanoelectrospray (nanoESI) and desorption nanoelectrospray (nanoDESI) [1,2] use a narrow spray tip and consume small amounts of samples. We modified a commercial ion source to switch between both ionization modes easily by the change of direction of a spray but sensitivity of nanoDESI was not sufficient. To improve ion signal intensity we have tested regulation of pressure drop between an atmospheric and evacuated region of a mass spectrometer.

Methods:
Experiments were carried out using a Xevo TQD triple quadrupole mass spectrometer (Waters, Manchester, UK). The commercial inlet was replaced by a custom-made inlet with a sample cone and a heated capillary allowing for the pressure regulation. Nanoelectrospray tips (2 ± 1 µm I.D., PicoTips, New Objective, Woburn, USA) were used for nanoESI as well as nanoDESI. Compounds in the wide mass range (m/z from 111 to 1922) were ionized to test the performance of ion sources.

Results:
Mass spectra were acquired for different values of pressure drop between an atmospheric and evacuated region and absolute as well as relative ion signal intensities were compared. Repeatability of the intensities was also evaluated. The relative intensities of ions of analytes were not influenced significantly but absolute signal was changed by pressure regulation in the whole studied mass range. Using nanoESI in positive ion mode, absolute ion signal of oxidized ultramark 443 (m/z 459) and ultramark 1621 (m/z 1222) increased 5 times for pressure drop 0.4 bar. For nanoDESI and the same pressure drop, signal of ion at m/z 459 and m/z 1222 was enhanced 7 and 9 times, respectively. Similar behavior was also observed in negative ion mode, e.g. for benzoic acid, sorbic acid or acetylsalicylic acid. Relative standard deviations of signal intensities did not change significantly for different values of pressure drop.

Conclusions:
New design of the mass spectrometer inlet allowed to increase yield of ions. For nanoDESI some ions were not detected without the pressure regulation. The pressure drop between 0.4-0.7 bar was suitable for the whole studied m/z range. The source modification needed an additional rough pump but sensitivity was improved dramatically. The effect can be influenced by a pumping system of a mass spectrometer as the pressure in an evacuated region depends on its capacity.

Novel Aspect:
New design of an ion source of a triple quadrupole mass spectrometer allowing pressure drop regulation to increase ion signal intensity.

Acknowledgment:
The authors gratefully acknowledge the support from the Czech Science Foundation (Grant 16-20229S).

References:
Introduction: (Limit of 400 characters without spaces)
This is the first study to assess real-time "breath-prints" of children with SESI-HRMS (1, 2, 3) in the clinical environment. In the context of cystic fibrosis (CF), major contributors to morbidity are early and often subclinical pulmonary infection and pronounced neutrophilic inflammation. Early detection of disease and disease associated complications is crucial for implementing timely therapeutic measures to reduce disease burden and improve prognosis.

Methods: (Limit of 400 characters without spaces)
A novel SESI ion source was used with highly inert surfaces. We present data of 41 children (23 CF and 17 healthy controls, HC), 4 - 16 years old, from an ongoing cross-sectional study. Carefully pre-processed MS data was used for biomarker detection and classification. Mann-Whitney U tests together with q-values were applied to isolate m/z values. Subsequent variable reduction through principal component analysis was used to perform binary logistic regression.

Results: (Limit 900 characters without spaces)
Breath analysis with SESI-MS in the clinical environment requires careful control of temperatures, gas flows, contaminations (e.g. fatty acids from skin), cleaning procedures (e.g. cluster formation) and polarity switching. CF and HC had comparable BMI (17.7 ± 3.0 vs. 16.9 ± 2.3, p = 0.17) and FEV1 (z-scores -0.45 ± 1.18 vs. -0.27 ± 1.13, p = 0.37). Four children were Pseudomonas aeruginosa positive. We were able to isolate 43 m/z peaks with elevated intensity signals in CF vs. HC below an FDR control level of q = 0.15. Classification was applied to the first three principal components and resulted in significant outcomes (CF vs. HC) with an average accuracy of 81.3 % and a Cohen’s Kappa value of 0.61. We could annotate the detected m/z peaks with molecular formulae and identify several homologues series of compounds. Structure elucidation is on-going based on comparison of fragment spectra with reference compounds for unequivocal confirmation of the involved molecular markers.

Conclusions (Limit of 400 characters without spaces)
Non-invasive breath analysis with SESI-HRMS identifies CF specific compounds in children. This will allow further phenotyping of CF lung disease, with the potential for early detection of airway infection.

Novel Aspect: (Limit of 150 characters without spaces)
This is the first study to assess real-time "breath-prints" of children with SESI-MS in the clinical environment. We could identify CF specific volatile compounds in children.

References
Keywords: ambient, ionization, DART, paper spray, flame

Introduction:
Each of the ambient ion sources has specific strengths and weaknesses. No one method is universal. The application of several ambient ionization sources (DART, paper spray, inlet ionization and thermal ionization) to widely varying analytical problems will be presented from the perspective of the analytical chemist where no two sample types are the same.

Methods:
Samples were analyzed using a high-resolution time-of-flight mass spectrometer with an exposed atmospheric pressure interface. A Direct Analysis in Real Time (DART) ion source was coupled with a thermal ionization and pyrolysis device. Ambient ionization included a prototype paper spray source, a butane torch, and inlet ionization with 3-nitrobenzonitrile matrix.

Results:
A surprising application is the use of DART analysis for trace quantitation of D2O in water. DART ionization with the thermal desorption source was applied to material analysis such as forensic identification of automotive paint chips and failure analysis, yet direct analysis of natural rubber gave better results than thermal desorption. The latter observation supports the hypothesis that the DART mechanism involves more than just thermal desorption. Other applications could not be analyzed by DART. Thermal ionization was used to detect trace iodide in milk. Inlet ionization was found to be the best method for rapid quantitation of polyphenols in tea infusions, while paper spray was the best method for copolymer analysis and detection of anthocyanins in berry extracts.

Conclusions
Although the DART mechanism involves ionization of atmospheric water, DART can still be used for trace D2O quantitation. Temperature-programmed thermal desorption/DART is useful for material analysis, but it is not always the best approach. The DART mechanism may involve more than simple thermal desorption. While paper spray and inlet ionization produce similar ion types and similar mass spectra, their applications can be complementary.

Novel Aspect:
Contrasting different ambient ionization methods for unusual applications and observing some mechanistic clues.
144 - ENHANCED COUPLING OF ACOUSTIC LEVITATION SAIL MASS SPECTROMETRY FOR DIRECT IONIZATION AND REACTION MONITORING

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Keywords: Containerless reactions, ambient mass spectrometry, acoustic levitation, reaction miniaturization and acceleration

Introduction:
Acoustic (ultrasonic) levitation has emerged as the most practical method of object levitation. In our previous work using DART-MS [1], the heated gas beam of the DART ionization source could not penetrate the acoustic field of the levitator. In this work, solvent-assisted inlet ionization (SAIL), namely ambient ionization occurring directly in the heated inlet of the mass spectrometer, permitted collection and ionization of samples in a single step.

Methods:
Solvent-assisted inlet ionization (SAIL) was coupled to an ion trap mass spectrometer. Online ionization of microdroplet reactions suspended under ambient conditions in an acoustic levitator was achieved directly at the inlet of the mass spectrometer. Fused silica capillary (ID 100 µm x OD 360 µm) dimensions employed in SAIL were critical for optimal ionization and external resistive heating of the capillary inlet enhanced analyte signal intensities.

Results:
Solvent-assisted inlet ionization (SAIL) greatly improved the direct sampling capabilities for the coupling of liquid samples suspended in an acoustic levitator with online mass spectrometric detection. The fused silica tubing employed in SAIL proved optimal for directly extracting droplets from the levitator, simultaneously acting as the sample transfer tube and ionization source. Positioning of the fused silica capillary within the inlet also played a key role and the optimized ionization conditions were achieved by placing the ionizing end of the SAIL capillary less than a millimeter into the inlet of the mass spectrometer. A derivatization reaction of cholest-4-en-3-one with Gerard’s Reagent T consisting of a 6 µL total reaction volume was compared with bulk (600 µL) reactions. Reactions carried out in the reduced reaction volume (in stillo) were approx. 10-fold faster. It was shown that external heating of the inlet of the mass spectrometer provided a 10-fold increase in signal intensity.

Conclusions:
Miniaturizing the reaction scheme to the microliter scale reduced the materials needed to carry out the reaction. Performing reactions in an acoustic field allowed all of the materials to react without interferences from container walls, therefore also enhancing reaction miniaturization. Coupling the micro-reactions with SAIL-MS enabled online real time mass spectrometric monitoring.

Novel Aspect:
Coupling of acoustically levitated droplets (in stillo) with SAIL mass spectrometry enabled enhanced online containerless micro-reaction monitoring.

References
602 - DIRECT INFUSION RESONANCE-ENHANCED MULTIPHOTON IONIZATION MASS SPECTROMETRY OF LIQUID SAMPLES UNDER VACUUM CONDITIONS

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University of Rostock, Chair of Analytical Chemistry, Rostock (1) - Photonion GmbH, Research & Development, Schwerin (2) - University of Urbino, LC-MS Laboratory, Urbino (3)

Keywords: mass spectrometry, photoionization, atmospheric pressure, petroleum, PAH

Introduction:
The generation of ions with ambient ionization techniques are affected by the sample matrix leading to concurrent ion yields of molecular ions, quasi-molecular ions formed by adducts and fragments. In this study, we present direct-infusion resonance-enhanced multi-photon ionization (DI-REMPI) at 266 nm combined with a direct liquid interface [1] for soft and matrix-independent analysis of aromatics in complex oil samples.

Methods:
The instrumental setup basically consists of a nano-flow UPLC for sample injection, a quadrupole mass spectrometer [2] and an Nd.YAG laser. A polycyclic aromatic hydrocarbon (PAH) solution, five diluted crude oils and four diesel samples were injected by a manual injection valve of 20 nl in a continuous solvent flow (400 nl/min) of acetonitrile. Only the tip of the capillary is heated to 280°C and ends 1 mm in front of the repeller inside the ion source.

Results:
The total eluent enters the ion source where it becomes promptly nebulized followed by desolvation, which is supported by the heater enclosing the repeller. REMPI under vacuum conditions and moderate laser intensities refers to a soft, selective and sensitive ionization technique for PAHs [3], so molecular ions of six PAHs were detected from the standard solution with good linear relation between intensity and concentration. Moreover, the addition of 1, 3, 10 and 30% toluene to one crude oil did not affect the mass spectrum except its molecular ion.

In the next step, crude oils were analyzed and compared to REMPI mass spectra of evolved gases released from a thermal balance [4]. Due to the total transport of sample into the ion source by DI-REMPI, the m/z range is extended that compounds which decompose in the thermal balance can still be detected. However, homologue series of odd m/z between 200 and 300 dominated the mass spectra of the crude oils which are assigned to ionization fragments of species with high molecular weight and low ionization energies.

Conclusions
DI-REMPI with direct liquid interface enables to enhance the m/z range of analytes by introducing the entire sample into the ion source while maintaining all advantages of REMPI under vacuum conditions, including no influence of matrix, selectivity for aromatics and the generation of molecular ions. Therefore, DI-REMPI is an alternative ionization technique for liquid chromatography or direct mass spectrometry.

Novel Aspect:
DI-REMPI is a novel soft ionization technique for liquid chromatography or direct mass spectrometry to investigate aromatics in liquid samples without matrix effects.

References
1308 - ACOUSTIC MIST IONISATION PLATFORM FOR HIGH-THROUGHPUT MS-BASED DRUG DISCOVERY

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Keywords: acoustic loading; mass spectrometry; high-throughput screening;

Introduction:
Mass spectrometry (MS) is a widely utilized label-free technology capable of measuring a broad range of analytes but lacks the throughput to support large HTS campaigns. Current commercial ESI MS-based systems using solid-phase extraction (SPE) are capable of running medium-sized screens. We have implemented a platform utilizing acoustics to load samples into a time-of-flight mass spectrometer capable of analysing more than 100,000 samples per day.

Methods:
An acoustic transducer emits sound waves into a sample in a 384-well plate located on a moving XY-stage. High voltage is applied to a charging cone above the well, inducing charge separation in the sample. The acoustic wave ejects a mist of droplets which are drawn through a heated transfer interface into the MS, during transit droplets dry and an ion beam is generated. The extracted ion peak for substrate and product from each well are used to calculate enzyme activity.

Results:
AMI-MS was used to build an enzyme assay to identify inhibitors from the AZ compound collection. Having established the kinetic parameters for this enzyme target, validation sets were tested to understand the variability within the assay. Once the robust nature of both the assay and this new MS technology were proven to meet the requirements of a high throughput screen the assay was moved into production. In total over 2 million samples were tested using a single automated AMI-MS platform over a 5 week period. Typical throughput of 100,000 samples per day were achieved. From the collection, 6745 compounds were identified in the first round of screening as actives - a hit rate of 0.3%. From this population of compounds, 4872 were followed up in concentration response format to confirm the activity, ~75% were confirmed as actives. The AMI-MS platform was further utilised during triaging of compounds to identify false positive samples which were likely to be contaminated with zinc.

Conclusions
AMI-MS proved a robust technology capable of screening a large compound collection (>2 million samples) and identifying chemical equity capable of inhibiting the enzyme target. Typically, this single instrument was capable of processing samples at a rate of 100,000 data points per day. The final hit rate in this assay was ~0.3%, of these the majority confirmed as active in follow up testing (75%).

Novel Aspect:
This is the first demonstration of AMI-MS technology successfully supporting a full collection screen against a pharmaceutically relevant target.
References

For information please contact: scientific@imsc2018.it
Introduction:
SESI-MS is a highly sensitive technique for the analyses of trace gaseous analytes in air and breath. It has been previously applied to several metabolomic studies, including lung infection in a murine model and identification of volatile metabolites with molecular weights up to 1000 Da in human breath. However, a major weakness of this method is a lack of accurate quantification of gaseous analytes, which hinders its application in the biological and medical areas.

Methods:
The physical and chemical processes, which lead to gas-phase ionization of VOC in electrospray and influence of transfer line on the ion composition, are studied. Sequences of ion-molecular reactions involved in the formation of product ions are described and the chemical kinetics is assessed for equilibrium and non-equilibrium situations. Chemical kinetics modeling is exploited combining analytical and numerical methods based on experimental data.

Results:
Chemical kinetics of hydrate ion formation is described as a function of temperature and partial pressure of water vapor in the gas present in the ionization chamber. The influence of transfer from the atmospheric pressure ionization region to the vacuum of the mass spectrometer via heated capillary on the ion composition is characterized. Representative mass spectral data are shown combining the SESI-MS and the selected ion flow tube mass spectrometry, SIFT-MS, experiments. Factors influencing the quantitative sensitivity of SESI-MS are summarized.

Conclusions:
The step-by-step understanding of chemical and physical processes affecting the composition of ions on their journey from the ESI droplets, via the atmospheric pressure gas containing traces of analyte, through the ion transfer line into the low pressure regions of the mass spectrometer will lead into robust methods of quantification of VOCs from the SESI-MS data.

Novel Aspect:
Description of sequence of processes involved in the formation of product ions in SESI will lead into a practical method of absolute quantification of trace gaseous analytes.

References

For information please contact: scientific@imsc2018.it
SPIDERMASS A NOVEL TOOL TO TACKLE IN-VIVO MASS SPECTROMETRY

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Keywords: in-vivo, real-time, Laser, diagnostic, surgery

Introduction:
Over the past 20 years, Mass Spectrometry has been translating progressively from analyzing extracts to raw surfaces. This went with the developments of technologies operating under ambient conditions all grouped within the field of Ambient Ionization Mass Spectrometry (AIMS) [1]. AIMS enable the analysis of raw material without sample preparation but are not suited for in-vivo analysis and at least not with minimal invasiveness. We have developed a novel system for in-vivo MS analysis and in-man use.

Methods:
SpiderMass [2] is composed of an OPO laser system tuned to 2.94 µm to access the most intense absorption band of water molecules. The laser irradiation promotes the excitation of water present within the tissues leading to a phase explosion. The plume of desorbed material is transferred to the MS instrument by aspiration through a tubing of several meters length connected to the MS instrument through a dedicated interface which replaces the conventional source.

Results:
We have studied and optimized the performances of our instrument for different applications. In particular, we have developed a fibered laser terminated by a handpiece allowing free scanning of all surface types. At the laser energy requested for ion production, this system is of low invasiveness only leaving a temporary white trace, which disappears within minutes after the experiment, at the surface of scan tissues. We have then assessed the system for different applications. In oncology, we demonstrate that SpiderMass molecular signatures have the requested specificity and sensitivity for classification purposes of cancer types and grades as exemplified with sarcoma. Further experiments performed in-vivo on model mice developing breast cancer and then intraoperatively at the veterinary surgery room confirm the performances of SpiderMass for cancer research. But SpiderMass reveals also to be well suited for fast diagnostic from FFPE tissue at the pathologist bench, bacteria typing or dermatology with real-time in-man DMPK.

Conclusions
The novel developed SpiderMass system with its fibered laser demonstrates to be an easy and robust tool for MS analysis in-vivo with a minimal invasiveness. This system opens the door to many applications in various fields including clinics for surgeons and pathologists. The capacities of the system to operate in both positive and negative ion modes and the possible analysis of proteins makes it as well as very interesting tool for biological research.

Novel Aspect:
Novel system enabling in-vivo MS analysis

References:
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MOLECULAR SURGERY NAVIGATION BY THE COMBINATION OF MASS SPECTROMETRY WITH MOLECULAR RESONANCE PROBE DESORPTION

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Keywords: Surgery navigation, Cancer, Molecular resonance, EESI

Introduction:
Quantum Molecular Resonance (QMR) technology was recently developed as a surgical tool for tissue dissection [1]. In QMR a well-defined spectrum of high-frequency electromagnetic field is produced to selectively break molecular bonds without inducing a significant rise in local temperature. In general purposes QMR was designed for the surgery. Here we demonstrate QMR technology in combination with MS as a novel Surgery Navigation technique. We show that this technique allows on-line ambient molecular analysis of biological tissues with high chemical sensitivity.

Methods:
The quantum molecular resonance generator was used as a desorption probe for breast cancer tissues chemical scanning. The probe was coupled with a jet pump which delivered desorbed molecules into the home-made EESI source coupled with qTOF mass spectrometer (Maxis, Bruker Daltonics, Germany). Tissue samples were obtained in the Research Center for Obstetrics, Gynecology and Perinatology and stored under 193 K until measurements. All data were processed with PCA and OPLS-DA methods to determine the most significant differences between tissues.

Results:
The method utilized in the study allowed obtaining highly intensive, stable and reproducible signal from tissues in both positive and negative ion modes. Molecular composition differences in real time were shown for different cancer tissues. Multivariate analysis was used to classify the mass spectrometric data. Score plots from OPLS-DA revealed good clasterization of data points with accordance to tissue source. Real-time feedback to the surgeon about margin status in patients with breast cancer was shown. High accuracy tumor boundary detection is also presented.

Conclusions:
A novel on-line ambient platform for biological tissue analysis is introduced

Novel Aspect:
Molecular Surgery Navigation system based on the combination of mass spectrometry with molecular resonance probe desorption was developed.

References:
This work was supported by Ministry of Education and Science of the Russian Federation grant (agreement No. 14.613.21.0059, RFMEFI61316X0059).
Keywords: ionization, cluster ion beam, polymer, SIMS

Introduction:
Secondary Ion Mass Spectrometry (SIMS) has been widely used for the analysis of semiconductors and electronic devices in past decades. Recently, the development of novel ion beam, cluster ion beam, enabled us to detect large organic molecule in SIMS analysis. However, the sensitivity of molecular ion derived from large organic molecules are still insufficient for high-resolution imaging mass spectrometry with SIMS.

Methods:
To increase the sensitivity of organic molecules in SIMS, some alkali metals and organic acids were added to the sample. This is because molecular ions are generally detected as alkali metal-adducted molecular ion or proton-adducted molecular ions. Therefore, by adding alkali metal ion or proton, the molecular ion yields can be thought to be enhanced.

Results:
The model polymer samples used in this study were Polyethylene Glycol (PEG) of average molecular weight of 1000, 2000 and 3000. Lithium, Sodium, Potassium and Cesium were added to PEG sample to investigate the molecular ion yield enhancement by cationization agent. Citric acid and some other organic acids were added to investigate the molecular ion yield enhancement by protonation agent. The concentration of each additive was optimized in the preliminary study. The ion beam used in this study was Argon gas cluster ion beam (Ar-GCIB) of acceleration energy of 10 keV originally developed in Kyoto University. The ion dose density was around 1 E+12 ions/cm² in each measurement. The molecular ion yield of PEG1000, 2000 and 3000 were calculated and compared each other in order to discuss the degree of the molecular ion yield enhancement depends on the molecular weight.

Conclusions:
The molecular ion yield enhancement could be confirmed in all additives in the comparison with without additive sample. In addition, the degree of the molecular ion yield enhancement depended on the chemical structure of targeted organic molecule besides molecular weight.

Novel Aspect:
The molecular ion yield with cationization or protonation agents was investigated. It will help understanding the ionization mechanism under ion beam irradiation.
A COMPARISON BETWEEN DIATHERMIC KNIFE AND CO2-LASER COUPLED TO REIMS-TOF FOR INSTANTANEOUS TISSUE CLASSIFICATION

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Keywords: CO2-Laser, MSI, DESI, REIMS, TOF

Introduction: (Limit of 400 characters)
Lately, numerous studies have shown the benefit of coupling rapid evaporative ionization mass spectrometry (REIMS) to a diathermic knife for rapid tissue sampling and characterization. In addition, Co2 laser can also be used for the same purpose but in a less invasive manner. Herein, we assess the opportunities and limitations of the laser and diathermic knife coupled with REIMS based on quality of data generated and invasiveness of both sampling techniques.

Methods: (Limit of 400 characters)
Fresh tissue (i.e. muscle, liver, bone, bone marrow, cartilage, skin, fat) from different animals were analysed using the two sampling tools. Generated vapors were directly analyzed by a REIMS-TOF system, and tissue/species-specific molecular profiles were recorded. These profiles were integrated in a database and multivariate statistical analysis was employed to build a classification/prediction model which would function as real-time recognition software.

Results: (Limit 900 characters)
It was possible to generate tissue vapors rich in lipids with both sampling tools. Each tissue-specific mass spectrum (= molecular fingerprint) can be recorded within 3 seconds after ablation and cauterization. The laser system allows controlling penetration power and wave type, which results in cleaner spectra with better overall signal, higher S/N ratio and higher reproducibility when compared to the diathermic knife. A statistical model employing principal component analysis and linear discriminant analysis (PCA-LDA) was built based on the characterization of different molecular profiles such as tissue type and animal species. When using one or the other technique on the same tissues, similar molecular profiles are displayed, which demonstrates the possibility to build a common database from both sampling tools.

After analysis, samples were frozen and cut into 10 µm sections. Further investigations via DESI-TOF imaging were conducted to the different lipids composition and distribution in the tissues around the burnt area.

Conclusions (Limit of 400 characters)
Combining REIMS with different sampling interface has shown promises for several applications such as food analysis. We demonstrate laser-REIMS to produce clean cut and more reproducible signal, and the possibility to sample a wider variety of samples including hard tissues, which is not possible with the diathermic knife. The possibility to build a common database for both tools renders REIMS more versatile and extends its field of possible applications.

Novel Aspect: (Limit of 150 characters)
Novel versatile and robust applications of REIMS technology with the possibility to build a common database between sampling techniques for instant sample recognition.

References


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Introduction

Atmospheric Pressure Chemical Ionization (APCI) is a sensitive and selective method to ionize the sample for mass spectrometric analysis. Different ionization chemistries ionize different compounds, and using several ionization schemes will give a comprehensive view on the composition of the sample, without losing the selectivity of the chemical ionization method.

Methods

We introduce a multi-scheme chemical ionization inlet (MION) capable of combining multiple ionization schemes in one instrument. The MION consists one or more ion sources, from which the reagent ions are mixed in the sample without contaminating the sample with the neutral reagents. The ion sources can also be turned off to measure the naturally occurring ambient ions. The MION can also be equipped with a filter desorber/calibrator unit allowing the analysis of filter samples and liquid microinjections.

Results

We studied the performance of the MION with two reagent ions, nitrate (NO3-) and bromide (Br-). The MION is capable of switching between the ionization schemes within seconds, and turning the ionization sources off and switching into the ambient ion mode is as fast. The neutral reagents are prevented from mixing with the sample, leaving the sample as unperturbed as possible.

We determined the sensitivity of the MION with gaseous sulfuric acid in NO3- mode. Sulfuric acid represents the compounds that get ionized at (or close to) the collisional limit. The sensitivity of the MION was found to be on par with the current state of the art NO3--chemical ionization inlet at the low ppq/s level [1].

We also explored the alpha pinene ozonolysis. The reaction results in, among others, a multitude of compounds having a formula C10HxOy, where the amount of oxygen (y) varies over a wide range [2]. The Br- chemistry is selective towards the less oxidized (y = 2 - 10) species, including the hydroperoxyl radical, whereas the NO3--chemistry is towards the more oxidized species (y = 4 - 11).

Conclusions

Our new MION inlet greatly expands the possibilities what can be done in the field of APCI-mass spectrometry with one mass spectrometer. The fast switching and superb sensitivity allows for as many ionization schemes as the user finds practical, without losing much of the time resolution.

Novel Aspect

We have produced a new atmospheric pressure chemical ionization inlet, MION, capable of switching from one ionization scheme to another within seconds.

References

Introduction:
Many applications used in mass spectrometers are highly charge state dependent processes. This includes CID (collision induced dissociation), CIU (collision induced unfolding) as well as IM-MS (ion mobility MS). Different ionization methods result in different detectable charge state distributions. Depending on the application highly and lowly charged species both have their advantages and disadvantages respectively.

Methods:
In LILBID[1] droplets of the analyt solution are transferred into vacuum and irradiated by IR laser pulses. This leads to an explosive expansion of the droplet and thus release of analyte ions into gas phase. The ions are collected using an ion funnel and transferred into a modified qToF[2] (QTOF I; MS Vision) mass spectrometer. Different mechanisms of dissociation (laser power and collision cell) can be combined. For comparison a commercially available nanoESI-MS instrument (Synapt G2s; Waters) was used.

Results:
To investigate the observable charge state distributions samples of both native and chemically modified[3,4] lysozyme were analyzed. Modifying lysosome was achieved by acetylation (alternatively: by propylation) of amino groups by varying excess of acetic (propionic) anhydride at basic pH value. According to the protein’s isoelectric point a change in charge distribution could be observed either with varying pH or with different degree of acetylation in either ion mode. Typically the actively ionizing nanoESI results in highly charged species which is in contrast to the non-actively ionizing / desorbing LILBID ion source where mostly lowly charged species are detected. In addition, with increasing hydrophobicity due to increasing modifications signal intensity issues also occurred.

Conclusions:
When analyzing the average per-species charge states a decrease depending on the degree of modification could be observed as expected. Both ion sources behave very similar in this respect. Interestingly the actual decrease in charge state is significantly lower than expected indicating more complex underlying mechanisms.

Novel Aspect:
LILBID coupled to a qToF enabled the investigation of small modifications to biomolecules used here to study the observable charge states of different ion sources.

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Keywords: Instrumentation; in-the-field and miniature MS; LIT; Ion/buffer gas collision

Introduction:
A LIT, composed of hyperbolic electrodes and disk endcaps, operates a Single Ion Monitoring mode consisting in a series of injection, cooling, isolation and ejection steps, realized by means of only DC switched voltages, to simplify conception and guarantee stability[1,2].
The collisional process of ions with buffer gas is simulated with the aim of improving mass analysis performances depending on: peak width, analysis duration and relative number of ions involved.

Methods:
The design of the electrodes, then ion trajectories have been computed using CPO software[3]. An additional subroutine has been implemented for modeling ion/buffer gas colliding as follows: random drawing according to Poisson law to define collision times (event-driven simulation); modifying ion velocity according to elastic collision law with random uniform scattering angle after collision.

Results:
Collisional process does not induce a significant modification of ion state (distributions in positions and velocities) due to short duration-time of trap filling with ions at injection, with He pressures less than 10-3 torr. For cooling stage, the trap is operated in RF only mode. The collisional process improves the relative number of confined ions. The cooling duration-time, to reach equilibrium ion state, has been estimated at 4.5 ms, involving almost 70 collisions, with He pressure equal to 10-3 torr. At equilibrium ion state, the distributions tend to Gaussian distributions, except for the distribution of axial ion positions. For ion isolation stage, the trap is operated close to the apex. In these conditions, the cooling process induces fast-ion loss. A set of values has been found: 150 µs for isolation step time-duration and 50% of injected ions involved in the mass spectra, with mass separation of 1u and He pressure equal to 10-3 torr.

Conclusions
To reduce ion loss observed during isolation step, the He buffer gas pressure could be reduced, however the duration of cooling time will be increase by the same factor. Dividing by two the pressure, the number of confined ions during isolation step has been found up to 70% and the duration of the sequence is about 10 ms. Mass separation could be improved by a specific ramp to move the trap operating point from the center toward the apex of the stability diagram.

Novel Aspect:
Same performances have been obtained with a small-scale LIT than with a mass filter with larger length for in-the-field application.

References
CHARACTERIZATION OF COLD ELECTRON IONIZATION FOR PORTABLE MASS SPECTROMETERS

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Keywords: Cold Electron Ionization Source, Microchannel Plate, UV LED, Portable Mass Spectrometer

Introduction
Cold electron ionization (CEI) has been developed using a microchannel plate (MCP) electron multiplier initiated by UV photons from light-emitting diode (LED) [1]. Pulsed mode CEI consumed only 5 μW in average and can be controlled in nanosecond scales and is expected to replace the hot cathode electron ionization in portable mass spectrometers with the advantages such as low power consumption, low temperature, and precise control of pulse width and electron density.

Methods
A microchannel plate is used to generate the secondary electrons by illumination of an UV photons generated by various LEDs (240, 260, 280, and 300 nm wavelength) and then sequential current amplification of the electrons was performed with various combinations of electron multipliers such as microchannel plates and channeltrons. A high voltage power supply has delivered up to 2400 V. The emitted electron beam current signal is picked up by an impedance-matched anode.

Results
The property of the electron beam pulse emitted from the MCP illuminated by photon pulse from an UV LED was studied. With UV LED operated with 10 V input in 3 ns, extremely short electron beam pulse was generated which can be used as an ionization pulse in TOF MS. The measured electron beam current was more than ~10^7 electrons within 2 ns. Zubarev et al. has reported 10^7-10^8 electrons were generated by an incident nuclear fission fragments from 252Cf and recorded mass spectra of various samples with a time-of-flight mass spectrometer (TOF MS) [2]. Similar ions were observed with CEI as previous reported in electron-avalanche-desorption (EAD) ionization MS. EAD ionization required sufficient electrons to desorb and ionize the molecules and record the mass spectra. For the portable TOF MS, the duration of the electron beam pulse from CEI can be precisely controlled in nanosecond scales and also provide the sufficient current, so that CEI generated enough electrons to be applicable to the portable TOF MS as well as ion trap MS.

Conclusions
Cold electron ionization provided more than ~10^7 electrons within 2 ns. CEI provided high enough current to ionize the gas analyte in ion trap and TOF MS. In addition, 2 nanosecond cold electron beam pulse had been demonstrated to be an extremely short pulse for portable TOF MS. This CEI is expected to be applicable for the portable MS with low power consumption and precise control of pulse width and electron density.

Novel Aspect
Extremely short and high current cold electron beam pulse was generated by operating a UV-LED and electron multipliers and applied for the portable ion trap and TOF MS.

References
Introduction:
This paper discusses a novel approach to charging ions at atmospheric pressure, suitable for both polar and nonpolar compounds, as well as extracting ions from opposite polarity droplets. Typically ESI is used with the majority of polar compounds and APCI with less polar. Here we discuss a single approach capable of producing an optimum signal for the full range of analyte polarity and to extract ions of opposite polarity.

Methods:
Experiments were performed using a QSight 210 triple quadrupole mass spectrometer with a modified ion source probe that permits adjustable regions of turbulence and laminarization at the exit of the ion formation region. Analytes including myoglobin, reserpine and chlordane were studied. MRM sensitivity was taken at a 500ul/m, as a function of droplet charging. The same single probe physical position and sample tube position was maintained.

Results:
Results are reported for a prototype ion source probe that embodies the characteristics of a turbulence and ion entrainment as well as the ability to optimally charge droplets, including the selection of an ion polarity opposite to the droplet polarity. Single probe S/N at high flow is compared with typical individual APCI and ESI probe results, using compounds which respond to both ESI and APCI, as well to those which respond predominantly to ESI or APCI. For example, although conventionally myoglobin is ESI favourable, reserpine is responsive to both ESI and APCI, and chlordane is APCI favourable, the new probe demonstrates suitable performance for all types. Single probe MRM data was taken of the opposite polarity of the charged droplets for all of the chosen analytes. For example, it was observed that the reserpine droplets can have a variable negative charge while still observing positive reserpine ions. A graph of the reserpine sensitivity vs voltage difference between the probe nebulizer tip and desolvation region illustrates this phenomenon.

Conclusions
The data from the MRM of the chosen analytes show that single probe sensitivity with optimized droplet charging is at least comparable to the individual APCI or ESI probes. In addition, the single probe shows that significant MRM sensitivity is possible for APCI favourable compound chlordane using oppositely charged droplets. The effect of oppositely charged droplets upon chemical interferences is unexplored at this juncture.

Novel Aspect:
A single atmospheric pressure ion probe produces comparable sensitivity to individual ESI and APCI probes as well as producing ions from oppositely charged droplets.
Introduction:
We are demonstrating a new method that can facilitate the laser desorption process without gold nanoparticle treatment step. The use of graphene layer substrate is excellent platform to induce the desorption process using 532 nm-continuous wave (CW) laser. By using this method, high spatial resolution atmospheric pressure mass spectrometry (AP-MS) imaging of humid tissues can be obtained.

Methods:
We used the previously developed AP-nanoPALDI MS system with a change in laser source [1]. One of the most widely used lasers, a 532nm CW laser, was used as the desorption source. A graphene layer substrate was prepared by transferring commercial CVD graphene on Cu foil to slide glass. The specimen was placed on graphene layer substrate and positioned on the scanning stage for MS imaging.

Results:
Many previous studies reported using graphene layer as a substrate for absorbing laser energy have shown that the laser source was located above the specimen and the laser light first met the specimen rather than the graphene layer[2-4]. Thus, in order for the graphene layer to role as a light absorbing layer, the thickness of the specimen had to be thin. In our proposed AP-MS system, since the laser light was focused on the specimen by the objective lens of the inverted type optical microscope, the laser source was placed under the specimen, so that the laser light was irradiated to the graphene layer first and the light energy was converted to heat from graphene layer and transferred to the tissue specimen. Therefore, micrometer-resolution ion images for mouse hippocampal tissue slices were obtained under ambient conditions.

Conclusions:
Compared to the preparation of the nanoparticles treated biological specimen, while the nanoparticle treatment of the specimen was omitted, the process of transferring the graphene layer was added to the slide glass substrate. The graphene layer substrates can be prepared and stored in advance, resulting in a simplified specimen preparation and a great advantage in preparing living tissue faster.

Novel Aspect:
The efficient laser desorption of bio-molecules on the wet-state hippocampal tissue is possible by use of graphene layer substrate.

References
Portable Time-of-flight Mass Spectrometer using a Cold Electron Beam Pulse

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keywords ; Time-of-Flight, cold electron ionization, portable mass spectrometer, nanosecond electron pulse

Introduction
A time-of-flight mass spectrometer (TOF MS) is widely used due to fast data acquisition, wide mass range and high sensitivity. Since a very short ionization pulse is essential for high mass resolving power, a fast laser pulse is commonly used in a TOF MS. However, the size and power requirements of the laser device limit the miniaturization of a TOF MS. To solve this problem, a tiny cold electron ionization (CEI) source was suggested to generate the short ionization pulse.

Methods
The cold electrons were generated with 3 microchannel plates (MCPs) and one of MCPs was illuminated by UV photons to generate the secondary electrons [1]. They are accelerated to a metal surface where sample molecules are adsorbed. The desorbed ions are analyzed through a 18 cm ion flight tube and recorded with an oscilloscope with 2.5 GHz bandwidth. The electronic circuit for nano second electron pulse was operated with 10 V, 200 mA and 3 nanosecond.

Results
The cold electron trajectory was calculated with SIMION® Version 8.1 and analytical Methods: And the calculated trajectory was confirmed with the luminescence of phosphors materials pasted on the sample plate. The cold electron ionization source recently developed in this laboratory has shown a current of up to 9 mA in 2 nanoseconds duration. This indicates the number of electrons up to 3x10^7 that would be enough for recording an usual mass spectrum. The size of cold electron ionization source is less than 2 cm^3 and the power consumption is 10 mA with a voltage of 10 V, which means 0.5 nW in average power with a 5 nanosecond per second. With the integrated CEI TOF MS, volatile gas molecules, benzene, toluene and xylene, were injected separately and detected molecular ions with H+ and CnH2n-1+ ions as previous reported in EAD (electron-avalanche-desorption) MS [2].

Conclusions
The experimental results of CEI TOF MS using a small cold electron source that generates more than 10^7 electrons in a few nanoseconds indicate that a portable CEI TOF MS with a suitable high mass resolution can detect the gas molecules. Further instrumental optimization is under development and higher electron currents and improved operating parameters will provide a useful TOF MS applicable in the field.

Novel Aspect
A portable TOF-MS was developed using an extremely short ionization electron beam pulse generated with cold electron ionization method.

References
Introduction: Ionization Sources in Mass Spectrometry
Electron Ionization is one of the most frequently used methods in MS. While being efficient, reproducible, and non-selective, it is still a “hard ionization” method that results in high fragmentation and loss of molecular ions. A dual mode EI (DMEI) source that allows both “soft” and “hard” ionization has been developed and coupled to a GC. Such combination allows both qualitative and quantitative analyses for various volatile and semi-volatile mixtures.

Methods:
Dual ion source was coupled to a modified PerkinElmer Qsight220 triple quadrupole MS and a PerkinElmer Clarus 680 GC. Gaseous pure compounds and their mixtures were injected into the capillary column of the GC. Once separated compounds were then carried by the carrier gas (He) into the DMEI source where they were ionized; resulting ions were then analyzed by the MS/MS. System performance (reproducibility, linearity and detection limits) will be presented and discussed.

Results:
Pure hexane, heptane, toluene, ethylbenzene, p-xylene, o-xylene and their mixtures were analyzed using GC-DMEI-MS/MS system. Compounds were successfully separated using GC, and then individually ionized in the DMEI. Depending on the ionization source settings two fragmentation patterns were achieved. One with minimal fragmentation and substantial molecular ion peak (“soft” ionization) and another with extensive fragmentation and almost no molecular ion peak (“hard” ionization). Different electronic settings of the ionization source allowed changing fragmentation patterns and signal intensities. Various tests showed that both modes are valuable and efficient; hence one source (DMEI) can be used for both compound identification (hard ionization) and compound quantification (soft ionization) with no change in the hardware. And since switching between hard/soft modes is almost instantaneous, both modes can be used during one sample analysis.

Conclusions
It was shown that dual mode electron ionization source can be used to perform soft and hard ionization of volatile compounds with no changes in the hardware. And while these are preliminary results and further investigations are still required, current system performance is promising and its future development is justified.

Novel Aspect:
Introduction of an EI source that can be used in “traditional” and “soft” ionization modes. Use of GC-DMEI-MS/MS system for analysis of gaseous analytes and their mixture.
Keywords: Ion source, ESI, APPI, Isotopic exchange, Proteins, Orbitrap.

Introduction:
The high variety of species that need to be investigated by a mass spectrometry determined the development and use of different ion sources. For example, ESI is one of the most suitable ion sources for investigation of peptides, proteins or natural organic substances, hormones can be investigated using APCI and APPI etc. With the introduction of the ion funnel, that can effectively gather ions, it became possible to develop a multiple inlet systems in which ions from different sources are transported into the fore vacuum region through different inlet capillaries. Here we present the simple approach for the combination of different ion sources on a single mass spectrometer and simultaneous performing different gas phase reactions without any interference between them [1-3].

Methods:
All experiments were performed on an orbitrap QExactive mass spectrometer (Thermo) with installed ion funnel and fore vacuum MALDI (Spectroglyph). The front panel with the MALDI translational stage was replaced with specially developed plate with several inlet capillaries. For the experiment with multiple ion sources copper tubes were connected to inlet capillaries. Valves enabled switching channels on and off. For experiments with thermal dissociation stainless steel tubes were connected to inlet capillaries. One of the tubes was wrapped in a special heat resistant cable with a nichrome conductor in the magnesium oxide and steel braid. This cable allowed heating of the extended capillary to 600 oC. To perform H/D exchange a droplet of D2O was placed below the capillary inlet.

Results:
By using separate inlet capillaries and relatively long transfer tubes with valves we have succeeded in combining 4 different ion sources: APPI, radioactive ion source, native ESI and regular ESI. Switching between channels takes less then 1s and no interference between channels was observed. We were able to observe simultaneously native tetramers of streptavidin, unfolded ubiquitin, crude oil and residue organic present in air. The developed source was used for internal calibration of humic substances by infusing clusters of phosphorus acid trough another channel. Using the independence of different channels it is possible to develop a system in which ions (produced by the same or different ion sources) would be subjected to the ion-molecular reactions, such as thermal fragmentation or H/D exchange in such a way that trough one channel always pass a non-modified parent ions.

We have developed a novel concept of MS-SWATH in which parent ions are observed simultaneously with the fragment ions in the same mass spectrum. Peptides of Cytochrome C digest were separated by liquid chromatography, ionized by ESI and delivered to mass spectrometer through 2 different capillaries one of which was heated to induce thermal dissociation. Another channel remains cold. By regulating flow in channels it is possible to regulate intensities of parent and fragment ions which are present in spectrum simultaneously.

Instead of thermal dissociation other gas phase reactions can be performed in different channels independently. We demonstrated this for the gas phase H/D exchange reaction. Using multiplexed ion source it is possible to rapidly switch between the channel in which H/D reaction takes place and the reference channel. It was found that cannels do not affect each other.

Conclusions
We have developed a novel approach to the combination of different ion sources on a single mass spectrometer. By using separate inlet capillaries and relatively long transfer tubes with valves we have succeeded in combining 4
different ion sources: APPI, radioactive ion source, native ESI and regular ESI. Switching between channels takes less than 1s and no interference between channels was observed. Such different objects as crude oil and streptavidin tetramers can be infused and recorded simultaneously. The possibility to transport ions through a 1.5 long tube makes it possible to assemble different ion sources on different working places that would not interfere with each other.

Use of our approach considerably expands the capability of gas phase chemistry to the investigation of structure of unknown sample. Indeed, ions produced from the same ion source, possibly, preselected by Filed Asymmetric Ion Mobility Spectrometry (FAIMS), could be split into different transfer tubes in which different gas phase reactions occur. One of the channels may be remained as reference. We have demonstrated such idea for thermal dissociation and gas phase H/D exchange reactions. Using valves it is possible to rapidly switch between different channels and it was proved that channels do not affect one another. Comparison of mass spectra recorded when different channels are open, will provide structural and chemical information about unknown species.

Novel Aspect:
A multiplexed ion source allowing simultaneous combination of various ion sources and performing gas phase reactions was developed.

References


For information please contact: scientific@imsc2018.it
IDENTIFICATION OF CHEMICALS IN DIFFERENT OBJECTS BY DIRECT ANALYSIS IN REAL TIME MASS SPECTROMETRY

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Keywords: direct analysis in real time mass spectrometry (DART-MS), semi-quantitative analysis, textile articles, healthcare products, polyurethane

Introduction:
The manufacture of textile, healthcare and polyurethane (PU) products employs a diverse range of processes using a large number of chemicals. Residues of these substances may remain in the products. The investigation of chemicals in these clothing and hygiene and PU articles (e.g. socks, shirts, sanitary articles, sterile gauze bandages, sponges, foam toys) is important since they can get into direct contact with the human skin.

Methods:
Measurements were performed with a MicroTOF-Q type Qq-TOF MS instrument (Bruker Daltoniks, Bremen, Germany) equipped with a DART SVP ion-source (IonSense, Saugus, MA, USA). The solid samples were manually introduced into the DART gas stream without any sample pretreatment. The results were confirmed by GC-MS and HPLC-MS.

Results:
The presence of more than 100 chemicals have been revealed in 50 different commercially available clothing, personal care, healthcare, and PU items [1,2]. The identification was confirmed by DART MS/MS experiments for 42 compounds by combining the MS/MS spectra with findings from the literature and databases of spectra. In the clothing and healthcare items the most notable hazardous substances were nonylphenol ethoxylates (NPEs), phthalic acid esters, amines released by azo dyes, quinoline derivates, tributyl phosphate, tris(2,4-di-tert-butylphenyl) phosphate, erucamide. Residues of catalysts, stabilizers, antioxidants, flame retardants, plasticizers, chain extenders, chain terminators, polyols, solvents, degradation products and contaminants, a few of them presumably toxic, were detected and identified in the PU items. Automated data acquisition and processing method and an efficient semi-quantitative DART-MS analysis were developed for the recognition, and quantitation of NPE residues, respectively.

Conclusions:
The large number and diversity of the detected compounds proves unambiguously the effectiveness of DART-MS and DART-MS/MS for the rapid identification of compounds in clothing, personal care, healthcare, and PU items without any sample preparation. Our experiments might have drawn attention to the presence of many harmful substances on the surface of commonly used products that can come into direct contact with human skin, or even blood.

Novel Aspect:
Our work has explored a new application field of the DART-MS technique. A semi-quantitative DART-MS analysis was developed to determine the concentration of NPE.

Acknowledgement:
The work was supported by the GINOP-2.3.2-15-2016-00041 project and by the grant K-116465.

References
Developing Mass Spectrometry-Based Explosives Trace Detectors for Secure City

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Keywords: Trace explosives detection, mass spectrometry, high-throughput, TNT, TATP

Introduction:
The threats of terrorism using explosives have been a serious problem around the world [1]. In this study, we developed two types of mass spectrometry-based explosive trace detectors (ETDs), namely, a high-throughput ETD which detects explosive particles using non-contact sampling, and a compact ETD which detects explosive particles and their vapor by one sampling unit.

Methods:
Based on the prototype of high-throughput ETD [2, 3], the non-contact sampling system was improved using a fluid dynamic simulation for stable high sampling efficiency. A mass spectrometer which operated as a sensor in that ETD was downsized, leading to the downsizing of overall system. As the other type of ETD, we combined such mass spectrometer with both a vapor introduction port and swab sampling function.

Results:
The newly developed high-throughput ETD has an ID card insertion port where several air nozzles are located. The position of such nozzles were decided based on the fluid dynamic simulation. An air jet from the nozzles efficiently dislodge explosive particles from the ID card and explosive molecules are then detected by mass spectrometry. We confirmed that the high-throughput ETD detected one of representative explosive molecule, TNT within 3 sec after the insertion of the ID card and its sensitivity was ng level. This throughput enables that ETD to operate as a security gate inspecting all people passing the gate. The compact ETD with a weight of around 60 kg has a function of continuous monitoring of surrounding gas and swab sampling. Experimental results suggested that the compact ETD detected vapor of TATP, which is popular improvised explosive device, at a concentration of below 0.1 ppm and TNT particle at ng level. The compact ETD can be interspersed in several locations to increase the security level in a wide area.

Conclusions:
High-throughput ETD and compact ETD were developed based on the same mass spectrometer. Use of those ETD as a security gate and an area monitoring system is considered to increase the security level in a wide area of the city.

Novel Aspect:
Based on the mass spectrometer as a core technology, changes in a sampling system resulted in two types of ETD used for several purposes.

References
Direct Detection of Small N-alkanes at Sub-ppbv Level in Breath Air by Photoelectron Induced O2+ Cation Chemical Ionization Mass Spectrometry at kPa Pressure

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Keywords: small n-alkanes, high-pressure photoionization induced O2+ cation chemical ionization, kPa pressure, direct detection, time-of-flight mass spectrometry

Introduction:
Alkanes are important target analytes in the fields of petrochemical industry, environmental detection, as well as being a significant product in human metabolic processes.[1] However, online and real-time measurement of saturated hydrocarbons, especially small n-alkanes, using direct mass spectrometry, remains a great challenge due to their low basicity and fewer ionizable functional groups.

Methods:
A high-pressure photoionization induced O2+ cation chemical ionization (HPPI-OCI) source was developed and coupled with a time-of-flight mass spectrometer. An efficient online pretreatment system was developed for eliminating the interference of humidity and carbonyl compounds. High-intensity O2+ reactant ion could be generated by photoelectron ionization for efficient ionization of n-alkanes.[2]

Results:
The HPPI-OCI mass spectrum pattern of C3-C6 n-alkanes differed a lot at different ion source pressure. The quasi-molecular ions [M-H]+ were gradually dominating and the fragmentation of n-alkanes by O2+-CI was depressed while the ion source pressure was elevated from 88 to 1080 Pa, with more than three orders of magnitude improvement in signal intensity. This was attributed to collisional deactivation effect, by which [M-H]+ tend to be more stable other than fragmentation, as the excess energy in the quasi-molecule ions could be more efficiently quenched at higher pressures. The achieved LODs (S/N=3) were down to 0.07-0.14 ppbv for C3-C6 n-alkanes in one minute. The HPPI-OCI TOFMS was successfully used in the analysis of exhaled small n-alkanes of healthy smokers and nonsmokers as well as the concentration variation of exhaled small n-alkanes after alcohol consumption.

Conclusions:
This work not only provides new insights for controlling the chemical ionization process by adjusting the ion source pressures but also prompts an innovative and high-performance direct MS technique for small n-alkanes analysis. It is likely that the HPPI-OCI MS will be a powerful tool in various research fields such as on-site environmental analysis and high-throughput medical diagnostics.

Novel Aspect:
Small n-alkanes can be soft ionized with O2+ cation at kPa pressure and direct detected at sub-ppbv level.

References:
EXPLOSIVES DETECTION WITH A MOBILE THERMAL DESORPTION DIRECT ANALYSIS IN REAL TIME MASS SPECTROMETRY SYSTEM (TD- DART-MS)

Frederick Li (1) - Brittany Laramee (1) - Paul Liang (1) - Brian Musselman (1)

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Keywords: DART-MS, Thermal Desorption, Explosives Detection, Swipe Sampling, Mobile MS System

Introduction (400 Characters without spaces)

A thermal desorber is configured to a DART-MS system on a mobile platform to permit the detection of explosives from swipe sampling. The mobility aspect of this configuration can permit mass spectrometry based detection of explosives in the field. This work presents the capability of the system and serves as a means of determining the readiness level of the system as an explosive trace detector.

Methods (400 Characters without spaces)

A thermal desorber unit is coupled to a DART equipped single quadrupole mass spectrometer, which is configured to a mobile cart. Standards of explosives were deposited onto PTFE-coated fiber glass swipes for analysis. A design of experiment was employed to evaluate conditions that affect detection and sensitivity. Limit of detection was determined for each explosive.

Results (900 Characters without spaces)

Explosives were deposited directly onto PTFE-coated fiberglass swipes and analyzed using the thermal desorber operated at three temperatures: 100°C, 200°C and 300°C. Preliminary results showed the response was at the highest at 200°C for more than half of the explosives. Desorption is near instantaneous for all 10 explosives, typically requiring 5 seconds after insertion of the swipe. Complete desorption of the explosive from the swipe can be achieved in 6-12 seconds, which is dependent on the vapor pressure of the explosive and quantity on the swipe. Thermal desorber temperature, the DART grid voltage and flow rate of the pump used for DART had a significant effect on sensitivity. Sensitivities between 5 ng and 50 ng for both organic explosives such as TNT, RDX, HMTD and TATP and inorganic explosives such as potassium perchlorate were consistently achieved.

Conclusions (400 Characters without spaces)

Detection of all explosives was consistently achieved at levels between 5 ng and 50 ng. TD-DART-MS provides a means for rapid detection of explosives from swipe sampling. The mobility aspect of this system is attractive for field detection of explosives. Future work will include determining the sensitivity of additional explosives such as smokeless powder, C-4 and homemade explosives (HMEs).

Novel Aspect (150 Characters without spaces)

TD-DART-MS on a mobile platform can enable field detection of a wide range of explosives from swipe sampling.
1352 - USING AUTOMATED LOW VOLUME PIPETTING TO ENABLE HIGH THROUGHPUT DART-MS DETECTION OF DRUGS DIRECTLY FROM BIOLOGICAL MATRICES

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Keywords: DART-MS, Drugs of abuse, Automation, High-throughput Experimentation, Toxicology

Introduction:
Direct Analysis in Real Time Mass Spectrometry (DART-MS) is a technique capable of directly analyzing biological samples without sample preparation. Utilizing a 384 format nanoliter pipettor to spot nanoliter droplets on a wire mesh, matrix effects can be reduced up to a factor of 100 times, thus enhancing analyte detection. The 384 sample mesh can then be analyzed with DART-MS at the rate of one sample per second, achieving complete analysis in less than 10 minutes.

Methods:
Urine was spiked with various drugs and internal standard (IS) and serially diluted to simulate real-world urine samples submitted for drug tests. Nanoliter droplets of the spiked urine were spotted on a mesh screen using a 384 nanoliter pipettor. A DART ion-source with a high throughput (HT) module was interfaced to a quadrupole mass spectrometer to enable rapid analysis of the mesh screen samples.

Results:
Various sample deposition volumes, 50 – 1000 nL, and sample concentrations, 50 ng mL−1 – 1000 ng mL−1, were evaluated to determine the level of matrix effect reduction as well as the lowest droplet volume that provided sufficient response for detection. A constant final amount of material was deposited using different size droplets of varying concentration; Preliminary results showed that the low droplet volume provided comparable signal intensity and a significant reduction of matrix effects. A five-point calibration curve was constructed using 200 nL droplets, and quantification with IS correction showed that the data was reproducible, with a RSD% less than 1%. The drug concentrations were accurately quantified between 50 – 1000 ng mL−1 within a 10% error.

Conclusions
Using nanoliter volume quantities of sample reduced the matrix interference and enabled direct analysis of the samples with DART-MS without any prior sample pre-treatment. The high throughput DART-MS configuration can enable the analysis of 384 samples in approximately 7 minutes and is capable of providing accurate and reproducible quantitation. Future work will include a wider drug panel and different biological matrices.

Novel Aspect:
Nanoliter volumes can reduce the matrix effects of biological samples, therefore enabling rapid analysis of biological samples using DART-MS without sample manipulation.
Introduction:
Supercharging is the ability to increase analytes’ average charge state by supplementing electrospray solvents with a low volatility compound. The supercharging methodology is useful for MS and MS/MS, but the mechanism(s) responsible for increasing charge are contentious. We describe a “supercharging” mechanism that applies to native and denaturing solutions and demonstrate its utility to predict agents that sub- and supercharge.

Methods:
ESI mass spectra were acquired on hybrid quadrupole time-of-flight (QTOF; Waters Synapt G2-Si), ESI-TOF (Agilent), and 15-Tesla Fourier transform-ion cyclotron resonance (FT-ICR; Bruker solariX) spectrometers. NanoESI sources used borosilicate glass capillaries with metal coatings (Proxeon Biosystems) operated at low analyte flow (< 50 nL/min). Final protein concentrations for ESI-MS (non-denaturing) were ~ 5 μM in 20 mM ammonium acetate, pH 6.8.

Results:
Multiple mechanisms have been proposed to explain how low-volatility additives increase charge in ESI, but they typically explain only subsets of experimental conditions or analytes; e.g., surface tensions exceeding those of CH3CN or CH3OH were credited with some additives’ abilities to increase protein charge from denaturing solutions, while increases delivered by the same compounds from near-native solutions were attributed to protein denaturation, driven by the additives’ increasing concentration within the evaporating droplet. The former proposal was subsequently countered by evaporation rate arguments addressing surface tension changes in evolving droplets, while the latter proposal was countered by many experimental observations.

Many factors impact charge state distributions (CSDs), but we think they can be unified into a single mechanism, applicable to a broad range of molecules from both denaturing and non-denaturing solutions. A correct mechanism will predict how new reagents affect charging in ESI.

Conclusions:
The hypothesis that supercharging and sub-charging molecules have specific Bronsted acidity and basicity properties (ideally neutral molecules that are both very weak bases and very weak acids compared to H2O), was tested by examining how adding related compounds of known pKa/pKHB (pyrazole, 1-nitropyrazole, 3-nitropyrazole, pyrrole, 1-ethyl pyrrole, 2-methyl pyrrole, and others) to ESI solvents altered CSDs. Analyte charging shifted in accordance with predictions.

Novel Aspect: (Limit of 150 characters without spaces)
Supercharging and sub-charging additives for positive ion mode can be predicted based on the solution phase basicities of low-volatility compounds.

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93 - DIRECT MASS SPECTROMETRY ANALYSIS USING IN-CAPILLARY IONIC LIQUIDS-BASED DISPERSIVE LIQUID-LIQUID MICROEXTRACTION AND SONIC-SPRAY IONIZATION

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Direct Mass Spectrometry Analysis Using In-Capillary Ionic Liquids-Based Dispersive Liquid-Liquid Microextraction and Sonic-Spray Ionization

Keywords: In-Capillary Ionic Liquids-Based Dispersive Liquid-Liquid Microextraction; Orbitrap Mass Spectrometry; Sonic-Spray Ionization; Direct Mass Spectrometry Analysis

Introduction: (Limit of 400 characters)
Public concern on the environmental and human impact of chemical approaches has encouraged analytical chemists to introduce safer and cleaner alternatives. Ionic liquids (ILs) are showing increasingly promising perspectives as green solvents in a diversity of fields. The unique physicochemical properties make ILs favorable alternative media to replace organic solvents for extraction, separation and detection techniques. In this study, a novel ILs-based dispersive liquid-liquid microextraction (DLLME) method coupled with sonic-spray ionization (SSI) has been developed for direct mass spectrometry analysis of environmental water samples. An in-situ formed dicaticionic IL was introduced to not only act as an effective extraction medium, but also create a positively charged complex with target analytes, allowing a significant increase in sensitivity.

Methods: (Limit of 400 characters)
A disposable borosilicate glass capillary (1.5 mm o.d., 0.86 mm i.d., 8 cm length) with a pulled tip (5 µm i.d.) was prepared using a P-1000 micropipette puller (Warner Instrument, Hamden, CT, USA). Four liquid plugs were sequentially injected into the capillary from the open end: 2 µL of aqueous Li[NTf2] solution at 30 µM, 20 µL of environmental water sample, 2 µL of aqueous [C4(MIM)2]Br2 solution at 20 µM, and 10 µL of dichloromethane. An in-capillary liquid-liquid microextraction was facilitated by the simple action of tilting the capillary up and down. The dichloromethane phase migrated to the tip end of the capillary due to higher density. The process does not need high voltage and was performed at ambient temperature.

Results: (Limit 900 characters)
Perfluorinated compounds (PFCs), an exemplary class of chemical substances of very high concern for regulatory control due to their persistent, bioaccumulative and toxic properties, were selected for method development and validation. The environmental water plug was sandwiched between the two miscible, hydrophilic IL plugs of Li[NTf2] and [C4(MIM)2]Br2. Within the combined aqueous phase, Li[NTf2] acted as an ion-exchange reagent and underwent an in situ metathesis reaction with [C4(MIM)2]Br2 to form a new hydrophobic IL of ([C4(MIM)2][NTf2]2). The exchange process generated a cloudy, homogeneous solution with fine microdroplets of the resulting extractant phase which greatly increased the contact surface area of the IL extraction solvent resulting in enhanced extraction efficiencies for perfluorooctanoic acid (PFOA) and perfluorooctanesulfonic acid (PFOS). After hand swing, the 10 µL dichloromethane liquid slug migrated to the tip end of capillary, extracting the hydrophobic compounds. The Venturi effect occurs when the high-velocity nebulizing gas flows through the capillary tip, causing a reduction in fluid pressure and self-pumping effect. The signal profile proved to be stable monitored by means of base peak chromatogram (BPC) and extracted ion chromatogram (EIC). The dichloromethane phase gives a clear mass spectrum with only two characteristic ions for m/z 719.1 (combination of [C2++PFOS-]+) and m/z 500.1 (combination of [C2++Tf2N-]+). This method detect the positively charged adduct which composed of dications and analyte anions. Especially when determining the concentration of PFCs which can hardly undergo fragmentation under negative MS/MS mode. The adduct lost either a proton or a methyl imidazolium group of dications, resulting in a singly
charged fragment for detection. Quantitative analysis was performed with internal standard calibration curve method, and the limits of detection (LODs) and limits of quantitation (LOQs) were 0.03 and 0.15 ng/mL for PFOS, and 0.05 and 0.21 ng/mL for PFOA.

Figure 1 a) The schematic diagram of direct in-situ microextraction and sonic-spray ionization analysis of water samples; b) MS/MS spectra recorded for 10 μL water solution containing 10 ng/mL PFOS; c) Quantitative analysis of PFOS and PFOA spiked water solution (0.5-100 ng/mL) with 13C4-PFOS and 13C4-PFOA (20 ng/mL) as IS compounds.

Novel Aspect: (Limit of 150 characters)
An ILs-based dispersive liquid-liquid microextraction (DLLME) method coupled with sonic-spray ionization (SSI) has been developed for direct mass spectrometry analysis.

References:
Substrate Modification for the Analysis of Highly Volatile Chemical Warfare Agents by Paper Spray Mass Spectrometry

Phillip Mach (1) - Elizabeth Dhummakupt (2) - Paul Demond (1) - Daniel Carmany (1) - Michael Busch (1) - Trenton Tovar (2) - Ann Ploskonka (3) - Jared DeCoste (2) - Trevor Glaros (2)

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Substrate Modification for the Analysis of Highly Volatile Chemical Warfare Agents by Paper Spray Mass Spectrometry

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Keywords: Paper spray, chemical warfare agents, volatility, metal-organic framework, derivitization

Introduction:

Paper spray mass spectrometry (PS-MS) has been shown successful analyze chemical warfare agent (CWA) simulants [1,2]. However, transitioning to actual G-series CWAs (i.e. sarin, soman) for analysis via PS-MS proves challenging due to volatility differences between the simulants and actual CWAs. In order to address this challenge, methods to fix these chemistries utilizing 2-[(dimethylamino)methyl]phenol (2-DMAMP) and metal-organic frameworks (MOFs) to extend their analytical lifetime were explored.

Methods:

Two separate methods of modification were utilized: 1) MOFs were weighed out in 1:1 ratio with cornstarch and dissolved in 5 mL of water. An aliquot was spotted onto cut glass fiber filters and allowed to dry. 2) Two microliters of a 2-DMAMP solution in methanol were spotted onto paper and allowed to dry.

For each method, quantities of CWAs were applied in solution or vaporized to the treated substrates. Time courses and calibration curves were subsequently carried out.

Results:

Attempts to analyze G-series CWAs on paper substrates were not successful, as the agents volatilize off the paper in less than five minutes. Analysis on glass substrates only extends the analytical lifetime for up to 15 minutes. The addition of the MOFs HKUST-1 and UiO-66 to the glass substrate was found to extend the analytical lifetime of GB, GD, and GF for up to at least 60 minutes.

Paper substrates treated with 2-DMAMP were spotted with either GB or GF and analyzed over time. The G-DMAMP complexes formed were persistent out to 96 hours (the last time point tested). Additionally, calibration curves of the two G-DMAMP complexes were generated, and the limit of detection (LOD) for GB-DMAMP was 0.941 ng/mL and GF-DMAMP was 5.14 ng/mL.

Conclusions:

Substrate modifications have proven useful to (1) prolong the analytical lifetime of highly volatile native G agents and (2) complex with the G agents to reduce their volatility. The MOF approach allowed for a four-fold extension in time, while preserving the ability to analyze the native molecule. The 2-DMAMP allowed for long term capture through derivitization. Sub-1 ng/mL limits of detection were achieved with G-DMAMP complexes, and the complexes remained stable for up to 96 hours.

Novel Aspect:

First on-substrate modification and reaction of highly volatile chemical warfare agents.
References:
Funding for this project is provided by the Defense Threat Reduction Agency – Joint Science and Technology Office for Chemical and Biological Defense

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LASER-ABLATION RAPID EVAPORATIVE IONIZATION MASS SPECTROMETRY (LA-REIMS) FOR LIPIDOMICS ANALYSIS OF LIVING CHO CELLS DIRECTLY FROM THE CELL CULTURE

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Imperial College London, Surgery and Cancer, London (1) - GSK, Biopharm Upstream Process Research, London (2)

Keywords: Mass Spectrometry, Lipidomics, REIMS, Cells

Introduction
Lipidomics analysis of living cells is still a difficult task, mainly because of the low abundance lipids that demand the use of time-consuming lipid extraction Methods: In the present study we report the application of LA-REIMS directly in the cell culture, in ambient environment.

Methods
LA-REIMS was performed in CHO-S cell culture liquid aliquots using the OPO (optical parametric oscillator) laser, which was connected through a REIMS source interface to a Waters G2-XS Q-ToF. The advantage of the OPO laser in contrast to others is the ability to vary the wavelength in the mid- and far-infrared region, which allows selective excitation of different functional groups.

Results
LA-REIMS connected to a Q-ToF and operated in full scan mode was able to detect most of the lipids related to CHO-S cell line, as reported in the literature [1,2], with good sensitivity.

The culture medium was used as control sample in order to distinguish the peaks coming from the cells. CHO-S cell pellets were also tested for comparison purposes. Different laser settings were tested for optimization, and the best signal was obtained with a mid-range laser power and 2900 nm wavelength.

The background “noise” was consistently low, allowing detection of low abundant lipids.

The majority of the m/z values detected in the cell culture and the cell pellet but not in the control sample were matched to putative lipids of all classes using accurate mass annotation and statistical analysis showed clear differences between the control and the sample.

Conclusions
The results of this study indicate that LA-REIMS can be used for fast and high-throughput lipidomics analysis in cell cultures without requiring any sample preparation. It is a fast and user-friendly method that is applicable in both the industry and academia for cell analysis at different levels of development.

Novel Aspect
LA-REIMS can be used for fast and high-throughput analysis of living cells as a biological snapshot of cell cultures in their native state.

References
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273 - OPTICAL CHARACTERISTICS OF MATRIX MATERIALS FOR MATRIX-ASSISTED LASER DESORPTION/IONIZATION

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Keywords: matrix-assisted laser desorption/ionization, photo-dissociation, photosensitization, excited triplet state

Introduction:
Finding new matrix materials for matrix-assisted laser desorption/ionization (MALDI) is an analytical demand for biological study to analyze wider variety of molecular species and to improve ion yield. Matrix materials have been opted conventionally based on acid–base neutralization mechanism [1]. In the present study, we aimed to characterize matrix materials from optical aspects.

Methods:
Absorption spectra of matrix materials dissolved in MeOH or sprayed on quartz slide were measured with U-3500 spectrometer (Hitachi). Photo-dissociation of matrix materials was evaluated with time course of absorption spectra of matrix solutions after various irradiation periods of Nd:YAG laser. Photosensitization effect of matrix materials was detected as 1270 nm emission from singlet oxygen [2].

Results:
α-Cyano-4-hydroxycinnamic acid, sinapic acid, 2,5-Dihydroxybenzoic acid, 2,5-Dihydroxyacetophenone, NorHarmane, 4-Nitroaniline, 2′,4′,6′-Trihydroxyacetophenone monohydrate, 9-Aminoacridine, and 1,5-Diaminonaphthalene were analyzed as typical matrices. Absorption profile obtained in a film state was more suited for wavelength at 337 nm or 355 nm than in a solution state. Absorption profile after irradiation of 355 nm Nd:YAG laser showed decrease of peaks characteristic to matrix materials reflecting photo-dissociation. As the photo-dissociation was reduced by bubbling with nitrogen gas, it dues to dissolved oxygen. In addition, photosensitized singlet oxygen was detected as 1270 nm emission from most of typical matrix materials against 355 nm Nd:YAG laser irradiation. These suggest that the laser excited matrix materials to a triplet state through intersystem crossing.

Conclusions
Photo-dissociation and/or photosensitization effect were found in all the typical matrix materials used in this study. Both photo-dissociation and photosensitization are related to production of singlet oxygen by 355 nm Nd:YAG laser irradiation. Therefore, matrix material for MALDI can be characterized as excitation to triplet state by MALDI laser or photosensitization ability.

Novel Aspect:
Excitation to a triplet state with 355 nm laser was revealed as a common optical characteristic to matrix materials for MALDI.

References
Introduction:
Pluripotent stem cells are a valuable source for cell production. Biomaterials have been discovered that assist in pluripotency maintenance, but the response mechanism is not well understood. Pre-adsorption of proteins is crucial in this process [1]. LESA-MS has shown potential to be used for analysis of adsorbed proteins [2]. Here, we describe the development of a high throughput LESA-MS strategy to analyze strongly attached proteins in a complex matrix.

Methods:
Essential 8™ medium (E8) was incubated for 1 hour onto the Droplet Microarray which has shown to allow robust automatic surface extraction [3]. Removal of interfering compounds was optimized using ammonium formate solutions and a 10-minute washing. Next, optimization of in situ trypsin digestion was done using organic-aqueous systems and MS-compatible detergents which have shown to enhance in-solution digestion [4]. Tryptic digests were then analyzed by LESA-MS.

Results:
Surface washing was optimized by varying the NH4HCOO concentration. To determine the washing efficiency and reproducibility, the total sodium intensity was calculated using an in-house MATLAB script. One washing round significantly reduced the amount of sodium independent of the NH4HCOO concentration. More washing rounds as well as the NH4HCOO concentration were not found to have a significant effect on the washing efficiency. The most reproducible washing was obtained using deionized water. Performing trypsin digestion with a mixed organic-aqueous system (DMF/50 mM NH4HCO3 (30:70 v/v) increased the number of identified peptides as well as the MASCOT scores for BSA compared with trypsin digestion using an ammonium bicarbonate solution solely. Addition of a MS-compatible detergent seems to interfere with in situ digestion or MS analysis due to insufficient removal of the breakdown products.

Conclusions:
We showed that with discrete sample areas the extraction repeatability and detection limits of LESA-MS for peptides can be improved. Further, using an optimized washing protocol inorganic salts present in E8 could effectively be removed prior to in situ digestion enhancing the peptide signal. In addition, the use of organic-aqueous trypsin solution significantly enhances the in situ digestion efficiency and protein identification.

Novel Aspect:
This is work is the first to show a strategy directing towards high throughput analysis of surface-adsorbed proteins for media- and cell-derived proteins.

References:

EXTENDING THE POLARITY RANGE FOR DIRECT SPME-MS ANALYSES BY USING NOVEL CAPILLARY DBDI AND APPI SOURCES

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Keywords: SPME-MS, DBDI, APPI, reactivity, electrodes

Introduction:
Although direct interfaces of solid-phase microextraction (SPME) with mass spectrometry (MS) are rapidly spreading and proved themselves as a fast and convenient way to analyze complex sample matrices, some limitations still need to be addressed: the limited range of compound polarity achieved by most ambient ionization sources, the sometimes severe matrix effects, and the lack of proper sample introduction strategies for a simple and fast analytical workflow.

Methods:
The capillary atmospheric pressure photoionization (cAPPI) and dielectric barrier discharge ionization (DBDI) sources used in this study were built in-house, in a capillary format directly connected to the MS using a leak-tight connection. For both sources, dopants were used to enhance ionization efficiency. In addition, the ionization efficiency for nonpolar compounds was increased for DBDI by using customized electrode geometry (spring- and ring-shaped).

Results:
Most ambient ionization sources used for direct SPME-MS analyses are mainly sensitive towards polar compounds. Capillary DBDI and APPI allowed to efficiently ionize nonpolar compounds (e.g., PAHs), introduced into the sources by thermally desorbing SPME fibers. In most cases, more polar compounds could be ionized efficiently as well. The results show a sensitivity in the pg/mL concentration range, even in the presence of relatively complex sample matrices like lake water or food samples. The analytical workflow was completely automated with a commercial PAL RTC autosampler (CTC Analytics), with a throughput of 1 sample every 1.5 minutes. For cAPPI, best results were obtained with chlorobenzene, fluorobenzene and acetone as dopants. For DBDI, the use of dopants affected the ionization mechanism, resulting in the preferential formation of radical versus protonated cations. The energy involved in the ionization processes was quantified by using substituted benzylamines thermometer ions.

Conclusions
The proposed approach allowed to quantify polar and nonpolar compounds from relatively complex matrices within minutes. The range of compound polarity accessible to SPME-MS direct analyses was broadened. The selectivity of the ionization process could be tuned by using different dopants. In addition, specific DBD electrode configurations allowed to extend or contract the plasma generated inside the source, promoting chemical reactions inside the plasma.

Novel Aspect:
Nonchromatographic analysis of complex samples by efficient capillary plasma- and photoionization sources. Reactivity tuned by dopants and custom electrode configuration.
Introduction:

We are using combined mid-infrared and deep-ultraviolet two-laser ablation coupled with electrospray ionization for ambient mass spectrometry of biomolecules in tissue. The goal is to increase nanoparticle production and improve sensitivity by using the UV laser to disrupt the tissue structure followed by IR ablation and ionization.

Methods:

In this work, we are using a 193 nm ArF excimer laser to disrupt the tissue prior to irradiating with a 3000 nm IR optical parametric oscillator. Both lasers are focused onto the same target spot and separated in time by an adjustable delay. A nanospray needle is directed at the inlet on-axis of a modified quadrupole time-of-flight mass spectrometer.

Results:

The two-laser ablation system has been constructed and initial studies carried out for optimization of the system with peptide and protein standards. The lasers are mounted on an aluminum breadboard adjacent to the ion source and are focused onto the target with a single calcium fluoride lens for each beam. The dual-laser configuration can be operated either with the UV firing first to disrupt the covalent bonding in the tissue or with the IR firing first to heat the tissue. Initial studies with 193 nm laser ablation electrospray ionization demonstrate that the deep-UV is a much softer ionization method than might be anticipated and can produce ions from peptides and proteins without a matrix and with little fragmentation. Based on this interesting new result, initial experiments are aimed at improving the efficiency of the deep-UV ablation using IR laser pre-heating.

Conclusions:

We have demonstrated that deep-UV and IR ablation coupled with electrospray ionization is a promising soft ionization method for large molecules with applications to tissue imaging. Continuing experiments are aimed at optimizing the UV and IR laser pulse energies and time delay to improve the sensitivity as well as improving the UV laser focus to improve spatial resolution.

Novel Aspect:

Combined mid-infrared and deep-ultraviolet laser ablation on the same spot for laser ablation electrospray ionization imaging.
RAPID QUALITATIVE ANALYSIS OF TWO FLAVONOIDS, RUTIN AND SILYBIN, IN MEDICAL PILLS BY DIRECT ANALYSIS IN REAL TIME MASS SPECTROMETRY (DART-MS) COMBINED WITH IN SITU DERIVATIZATION

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Keywords: direct analysis in real time mass spectrometry (DART-MS), silylation, in situ derivatization, flavonoid derivatives, silybin, rutin

Introduction:
Flavonoids belong to the family of benzopyrones having a wide range of biological and pharmacological effects, including anti-allergic, anti-inflammatory, antioxidant, anti-cancer activities. The conventionally used mass spectrometric and chromatographic methods are quite time consuming and labor intensive. In contrast, direct analysis in real time mass spectrometry (DART-MS), enable the examination of solid samples without sample preparation.

Methods:
Measurements were performed with a MicroTOF-Q type Qq-TOF MS instrument (Bruker Daltoniks, Bremen, Germany) equipped with a DART SVP ion-source (IonSense, Saugus, MA, USA). Derivatization of the standard samples was carried out on DART sampling rods by adding 2 μL of the silylation reagent to the standard powder. These samples were manually inserted into the helium gas stream.

Results:
Direct analysis in real time mass spectrometry (DART-MS) with in situ silylation was used for the rapid analysis of the flavonoids (silybin, rutin) [1]. Three commercially available silylation reagents were applied. The flavonoids were detected with various degrees of silylation, and the formation of dimers was also observed. HMDS/TMCS/pyridine was the best choice for the DART-MS analysis of silybin, and BSA/TMCS/TMSI was the most effective for the detection of rutin. The effects of the DART source temperature on desorption, ionization, in-source fragmentation, dimer formation, and hydrolysis of the trimethylsilyl groups were also studied. In addition, the collision-induced dissociation (CID) properties of the derivatized silybin and rutin were explored. With our in situ silylation method, the derivatized bioactive compounds in intact medical pills could also be detected by DART-MS.

Conclusions:
Our experiments showed that in situ silylation enables the use of DART-MS for the analysis of compounds that have low volatilities and/or are thermally unstable, such as silybin and rutin. Three silylating reagents were tested, and significant differences were found in their efficiencies of silylation. HMDS/TMCS/pyridine was the best choice for the DART-MS analysis of silybin, whereas BSA/TMCS/TMSI was the most effective for the detection of rutin.

Novel Aspect:
Our work has explored a new application field of the DART-MS technique. A method was developed for the rapid analysis of silybin and rutin by DART-MS using in situ silylation.

Acknowledgement:
The work was supported by the GINOP-2.3.2-15-2016-00041 project and by the grant K-116465 and supported through the New National Excellence Program of the Ministry of Human Capacities, ÚNKP-16-3 (T. Nagy).

Reference
Introduction:
Ambient sampling and ionization (ASI) technique has been attracted attention due to its capability of rapid chemical analysis and/or chemical imaging. We have developed the technique named as tapping-mode scanning probe electrospray ionization (t-SPESI) [1-4], in which the unique fusion of the key techniques used in atomic force microscopy (AFM) and mass spectrometry (MS) is achieved, to realize ASI with a single capillary probe.

Methods:
For the advanced SPESI, we developed the feedback control system. The optical lever method is based on the technology used in AFM. The laser light is irradiated to the capillary probe and the displacement of the reflected light is detected with the photodiode to obtain the oscillation amplitude of the probe. The position of Z-stage is controlled to maintain the oscillation amplitude during ASI with proportional-integral-derivative (PID) algorithm.

Results:
The optical lever method developed in this study enables us to measure the oscillation amplitude of capillary probe. The analog to digital converter, field programmable gate array, real-time OS and PC are controlled by the homemade software coded in LabVIEW. We confirmed the oscillation amplitude is proportional to the input voltage to the piezo actuator which is attached to the capillary probe for the stable oscillation at resonant frequency. The feedback control system maintains the oscillation amplitude at the desired set point by adjusting the Z position of sample during the ASI by t-SPESI. This system can be utilized not only to stabilize the probe scanning on the sample surface with micro asperity but also to measure the surface roughness. The measurements of the mass spectra and the output signal to the z-stage is executed simultaneously. The ion images and the topographic image are reconstructed.

Conclusions:
The information of the probe oscillation is utilized to improve the reproducibility of ASI with SPESI. We demonstrated the simultaneous visualization of the chemical distribution and the surface shape. This simple optical technique is expected to provide the mechanical properties of the nano-litter-volume liquid bridge containing extracted molecules from the sample.

Novel Aspect:
The stability improvement of ASI and the multimodal imaging are realized by the optical lever method with t-SPESI.

References
Development of a Multimodal Imaging Platform based on Photoinduced Thermal Desorption and Analysis by Atmospheric Pressure Chemical Ionization Mass Spectrometry

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The key to advancing materials is to understand and control their structure and chemistry. Thorough chemical characterization can be challenging since many existing techniques analyze only a few properties of the specimen, thereby requiring multiple measurement platforms to acquire the necessary information. The multimodal combination of atomic force microscopy (AFM) and mass spectrometry (MS) transcends existing analytical capabilities for nanometer scale spatially resolved correlation of the chemical and physical properties of a sample surface. We recently introduced the utilization of a photoinduced cantilever heating technology developed by Oxford Instruments for the localized thermal desorption and analysis and presented a closed cell design for sampling on an Oxford Instruments Cypher ES microscope to interface with a Thermo Orbitrap Velos Pro mass spectrometer using inline atmospheric pressure chemical ionization (APCI). The photoinduced cantilever heating technology works with standard AFM probes that are compatible with advanced AFM modes for the nanomechanical and electromechanical characterization of samples. We previously demonstrated below 500 nm spatial resolution for the spot-sampling by thermal desorption from thin layers and the chemical analysis of small organic molecules in full scan MS mode.

We demonstrate the application of multiple and advanced AFM measurement modes such as AMFM nanomechanical characterization on a single AFM cantilever, combined with photoinduced thermal desorption and analysis by mass spectrometry to link chemical composition with material functionality. We show the chemical analysis by mass spectrometry of gas phase species evolving from polymeric material in contact with a heated AFM probe, spatially resolved with nanometer resolution, and identify small organic molecules and characteristic fragments and pyrolysis products from the polymer. We present results from systematic studies of factors limiting the efficiency of transport and ionization of material evolving from the sample surface, with the objective to enhance the achievable spatial resolution and compound coverage of the multimodal imaging platform. Parameters studied include the timing of the photothermal cantilever heating and analysis by mass spectrometry, transport conditions for the gaseous material from the closed desorption cell to the mass spectrometer, and the operating conditions of the inline ionization stage. Computational fluid dynamics (CFD) simulations were used to study the uptake and transport of material. Experimental studies on prototype cell models prepared by additive manufacturing provide support for the modeling data.
A novel modular and versatile TOF platform combinable with various ion sources

B. Instrumentation and Methods (Ambient and new ionization methods)

Keywords: TOF, TOF-MS, mass spectrometry, API, PTR-MS

Introduction
Time-Of-Flight (TOF) mass spectrometers are state-of-the-art analyzers not only in Proton-Transfer-Reaction – Mass Spectrometry (PTR-MS) [1], but many other fields. Depending on the application the TOF instruments range from bulky high-resolution to compact low-resolution models. Here we present a novel modular TOF design, which can be adjusted in dimensions and mass resolution and used in combination with various ion sources.

Methods
The newly developed TOF recipient consists of an orthogonal injection "base unit" equipped with an ion mirror and microchannel plate detector. The flight path of ions can be extended by installing one or two recipient extension modules, respectively. An ion funnel and a hexapole ion guide can be integrated for ion focusing, either in combination or separately. We tested the setup with a PTR source and a novel Atmospheric Pressure interface (API) front-end.

Results
Utilizing a well established PTR ion source [2] we evaluated the performance of the modular TOF platform (0, 1 and 2 flight path extension modules, respectively) in combination with an RF ion funnel and hexapole ion guide, while analyzing a certified gas standard (TO-14A). The mass resolution of the compact base unit without any RF focusing device is above 1500 m/Δm and the sensitivity is between 40 (below m/z 100) and more than 100 cps/ppbv (above m/z 100). By the implementation of an ion funnel and a hexapole ion guide the sensitivity gets significantly improved, i.e. to more than 500 cps/ppbv. After installation of 2 extension modules, an ion funnel and a hexapole ion guide a mass resolution of >6000 m/Δm and sensitivities of >1000 cps/ppbv can be achieved. The latter means that after an integration time of one minute the detection limit of the instrument is below 1 pptv.

Exchanging the PTR source with an API front-end, consisting of differential pump stages and an additional hexapole ion guide, we are able to investigate ions in the atmosphere over broad m/z range.

Conclusions
The results obtained with the modular TOF platform in combination with a PTR ion source indicate an excellent performance, which surpasses known PTR-TOFMS instruments [3]. The design is highly versatile, so that e.g. using two TOF base units in opposite polarity with API front-ends, creates a revolutionary API-TOF instrument for simultaneously detecting positive and negative ions over a broad m/z range.

Novel Aspect
A modular TOF design enables maximum flexibility utilizing extension modules while performing at high mass resolution and sensitivity.

References
WHAT DETERMINES THE POSTIONISATION EFFICIENCY IN MALDI-2: A COMBINED SOFT-/HARDWARE-BASED SET-UP TO SEMI-AUTOMATICALLY CHARACTERIZE THE ROLE OF FIVE RELEVANT INPUT PARAMETERS

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Keywords: MALDI-2, Post-ionisation, Laser fluence, wavelength

Introduction:
MALDI with laser-induced post-ionization (PI), also named MALDI-2, was recently introduced by us to increase ion yields for numerous classes of lipids, metabolites and carbohydrates [1]. Here we present a semi-automatic protocol to investigate and optimize the efficiency of the PI process in dependence of five relevant input parameters: pulse energies of the two lasers, PI laser wavelength, delay between the laser pulses, and buffer gas pressure in the ion source.

Methods:
All experiments were conducted with a modified Synapt G2-S mass spectrometer (Waters) [1]. A N2 laser (337 nm) served for desorption/ablation of material and a wavelength-tunable optical parametric oscillator (OPO) laser (relevant range, 220 – 350 nm) for PI. 2,5-Dihydroxybenzoic acid (DHB) and a mixture of polar phospholipids were investigated as matrix/analyte system. Samples were prepared by spraying the prepared mixture onto a glass slide.

Results:
At the core of our study stands a newly developed setup that combines hardware components and LabVIEW VI-based software for control and read-out of all relevant input parameters. In this way, during a MALDI imaging run on a homogenous sample, up to four (out of the overall five studied) input parameters were systematically varied and mass spectra recorded simultaneously. For example, by use of motor-driven laser energy attenuators (neutral gradient filters) and solenoid gas valves, laser fluences and buffer gas pressure in the ion source were precisely adjusted in predefined steps. The complex obtained MS data set including total ion counts and abundances of molecular ion signals of matrix, analytes and fragments in dependence of the respective input parameters was used to identify optimal MALDI-2 conditions for the tested DHB/lipid system. As will also be discussed in our poster, the so collected multidimensional data cube provides valuable insight into the mechanisms underlying the MALDI and MALDI-2 processes [2].

Conclusions:
Our new software/hardware setup allowed for (rapid) characterisation of the MALDI-2 process in dependence of all relevant input parameters. Our approach could not only support further optimization of MALDI-2 for different matrix systems for lipids, but also allow for a screening of new analytes (e.g., peptides and proteins) which under current conditions show no significant PI. Furthermore the approach is a valuable tool to study the MALDI and PI processes.

Novel Aspect:
Combined hard-/software setup for optimization and characterization of the MALDI-2 process in dependence of five input parameters and preliminary data for a DHB/lipid system.

References:
Introduction:
Previous studies [1;2] have shown signs of terpenes forming large clusters in low temperature plasma (LTP) ion mobility spectrometry hyphenations. In this work we present further studies of terpene systems using a Bruker fourier transform ion cyclotron resonance mass spectrometer (FT-ICR-MS).

Methods:
Mass analysis were performed on a 7.05 T FT-ICR system, provided by Bruker Daltonik. A LTP torch built according to the design of Nørgaard et al. [3] with slight variations was used as an ion source. Plasma gas and sample gas were Argon (99.999 %) by Air Liquid Deutschland GmbH. As a test substances a great variety of diterpenes and sesquiterpenes were used. All purchased from Sigma Aldrich (Taufkirchen).

Results:
Mass spectra of LTP FT-ICR hyphenations mostly show a great variety of ions. Predominantly Dehydrated species, oxides and fragments can be detected. Furthermore clusters of all these can be found. Depending on the analyte species clusters can reach a size of up to eight times the molecule. All mass spectra indicate only ions formed via Penning ionization induced by excited Argon molecules. As a result the most intense signal belongs to the \([\text{M-H}]^+\) molecule. To get further information about the oxidation process also the presence of water in the sample was analyzed. Depending on the amount of water the cluster size and the oxidation rate varies. Also the presence of atmospheric Oxygen and Nitrogen was investigated.

Conclusions:
Measurements show that the main ionization product are the \([\text{M-H}]^+\) as well as \([\text{M-2H-H}]^+\). It is most likely that the corresponding mechanism is a radical reactions followed by a dissociation into a cation and one electron. This is also underlined by the formation of dehydrated species and \(\text{C}x\text{H}y\) losses. The cluster formation is highly dependent on structure of the analyte molecule.

Novel Aspect:
Investigation of the clustering and fragmentation patterns of LTP ionization products under atmospheric conditions via FT-ICR-MS.

References:
341 - DEVELOPMENT AND APPLICATION OF A NOVEL PLASMA ION SOURCE WITH GC-MS/MS

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Keywords: Tandem quadrupole mass spectrometry, Gas chromatography, Glow discharge plasma

Introduction:
Developing an ion source for GC-MS/MS system capable of generating enhanced signal for molecular ions is very important for the analysis of unknown compounds. Traditional GC-MS with EI source often results in extensive fragmentation and the sample identification is mainly through a library search. Although library identification is a valuable tool, the reliability of the results cannot be ensured in many cases due to the absence of molecular ions.

Methods:
All experiments were performed with a plasma ionization source integrated to a modified Qsight™ 220 triple quadrupole mass spectrometer equipped with a Clarus 680 GC. Several commercially available classes of compounds such as hydrocarbons, pesticides and terpenes were injected into the GC, which then were introduced to the plasma source by a heated transfer line, ionized and subsequently transmitted into the triple quad mass analyzer and detected.

Results:
Our results indicated that the plasma source could efficiently ionize a wide range of compounds with a significant sensitivity enhancement of the precursor ions through soft ionization. The plasma source results in a very clean full mass spectrum with minimal fragments dominated by the precursor ions. This is in contrast to results of standard GC-EI systems in which the spectrum is dominated by fragments, while the precursor ion is absent or low in abundance. This allows for accurate identification of unknown compounds as well as direct quantitation by selection of precursor ions. Additionally, acquired fragmentation at various collision energies in MS/MS revealed very similar fragmentation patterns as those of electron ionization, which offers an opportunity for library identification.

Conclusions
Plasma source is a very simple, inexpensive and robust ion source which requires very low maintenance. GC-MS/MS with the plasma source offers improved performance and wider application over the conventional GC-MS or GC-MS/MS systems with the EI source by efficiently enhancing the sensitivity of GC-MS/MS analyses, with the unique advantage of producing high yield of intact molecular ions without extensive fragmentation due to the softer ionization.

Novel Aspect:
Development of a low-cost and efficient plasma ionization source for a GC-MS/MS system which enhances molecular ions yield

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285 - A NOVEL VOLTAGE-ASSISTED PAPER SPRAY-APPI-HRMS METHOD FOR THE SCREENING AND QUANTIFICATION OF SEMI-VOLATILE FLORINATED COMPOUNDS

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Keywords: ambient mass spectrometry, paper spray, non-polar solvents, photoionization, semi-volatile fluorinated compounds

Introduction:

Paper spray (PS) mass spectrometry is a fast ionization technique widely used for the detection of polar compounds mainly based in ESI ionization mechanisms. To improve the ionization of non-polar or low polar compounds, the traditional PS technique has been modified for the direct analysis of semi-volatile fluorinated pollutants by ambient-HRMS [1] in negative ionization mode using atmospheric pressure photoionization (APPI)[2].

Methods:

Standards and samples were loaded into chromatography paper triangles and were placed in front of a Q-Orbitrap with the APPI-UV lamp set orthogonally between the sharp tip and the MS inlet. Toluene/cyclohexane was used for the extraction/transport of analytes to the paper tip and for dopant-assisted APPI. By applying 1.5 kV, a spray was generated to desorb the non-polar analytes to be further photoionized in gas phase using negative ionization mode for HRMS analysis.

Results:

Several working parameters, including dopant, solvent mixture, spray voltage and paper and APPI-UV lamp positions, which may affect the ionization, extraction and transport efficiency of the analytes, were carefully optimized for the analysis of the two most challenging families of fluorinated compounds by PS, zero-voltage PS-APPI and voltage-assisted PS-APPI. Under PS-HRMS and zero-voltage PS-APPI-HRMS conditions, most analytes were not or poorly ionized, while using voltage-assisted PS-APPI-HRMS, useful [M-H]- and/or [M+O2]- ions were detected. High resolution MS/MS experiments were also performed for further confirmatory purposes. Thus, major improvements in terms of sensitivity were obtained by using voltage-assisted PS-APPI ionization and method quality parameters demonstrated good repeatability, reproducibility and linearity with negligible matrix effects for the analysis of waterproof liquid matrices. Besides, internal standard method allowed the quantification of these families of compounds at pg mL-1 levels.

Conclusions:

The use of APPI as an alternative ionization for PS-based techniques allowed the screening and quantification of non-polar and/or low polar compounds by PS. The voltage-assisted PS-APPI-HRMS method was applied for the detection of semi-volatile fluorinated compounds in real samples without any sample manipulation at µg mL-1 levels demonstrating its potential as a fast and useful technique for quality control laboratories.

Novel Aspect:

To the best of our knowledge, this is the first time that PS is successfully combined with APPI for the determination of semi-volatile fluorinated compounds.

References
ADVANCES IN SELECTED ION FLOW-DRIFT TUBE MASS SPECTROMETRY, SIFDT-MS, FOR AMBIENT QUANTIFICATION OF TRACE VOLATILE COMPOUNDS IN AIR.

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Czech Academy of Sciences, J. Heyrovský Institute of Physical Chemistry, Praha (1)

Keywords: SIFT-MS, SIFDT-MS, VOCs, quantification, chemical ionization

Introduction:
The Selected Ion Flow-Drift Tube Mass Spectrometry, SIFDT-MS, technique represents an important development of the Selected Ion Flow Tube Mass Spectrometry, SIFT-MS, technique building on its strengths (rapid switching between reagent ions and near thermal ion chemistry) and removing some of its weaknesses (lower sensitivity in comparison with proton transfer reaction, PTR, MS). It has a range of applications in biological, medical and environmental fields.

Methods:
The selected reagent ions (either H3O+, NO+ or O2+) for selective chemical ionization of volatile compounds present in the air matrix are created in an external ion source and injected into the flow-drift tube reactor with well-defined reaction time and reagent ion-analyte molecule interaction energy, \( E_r \). The absolute concentrations of the analytes can thus be accurately quantified using the chemical kinetics principles.

Results:
Accuracy of quantification is shown to be determined by the accuracy of the reaction time measurement and by the uncertainties in the values of the ion-molecule reaction rate constants. The reactions of H3O+, NO+ and O2+ ions have thus been studied with a range of compounds, including ketones, terpenes, isoprene isoflurane, sevoflurane and acetonitrile in order to reveal their dependencies on ion–molecule interaction energies, \( E_r \), whilst also gaining insight into their mechanisms. The rate constants, \( k \), for the reactions of H3O+ proceeding via exothermic proton transfer are seen to reduce by about one third over the \( E_r \) range from 0.05 eV up to 0.5 eV, in agreement with the collisional theory. The NO+/ketones reactions often proceed via the formation of excited (NO+M)* adduct ions that partially fragment, and \( k \) reduce with \( E_r \) sometimes more dramatically. The \( k \) for the O2++ charge transfer reactions are observed to be greater than their calculated \( k_c \) implying that long distance electron transfer occurs.

Conclusions
Whilst several advantages of SIFDT-MS over the established SIFT-MS are becoming apparent (including better sensitivity, improved accuracy and feasibility of design of portable instrument), it is very important to assess the potential complications due to increased \( E_r \), including fragmentation and changes of \( k \). This technique thus has a potential for accurate analyses in matrices such as environmental air, food flavours or exhaled breath.

Novel Aspect:
New experimental data are presented and interpreted on the kinetics of ion-molecule reactions allowing a new approach to soft chemical ionization of trace compounds in air.

References
Introduction
Selected Ion Flow-Drift Tube Mass Spectrometry, SIFDT-MS, was developed to quantify volatile organic compounds in air. The original instrument had two quadrupole mass filters: to select the reagent ions from the microwave discharge into the drift tube reactor and to analyze the product ions. To increase the sensitivity, the first quadrupole and the microwave discharge were replaced by a triple source creating the reagent ions by sequences of ion-molecule reactions.

Methods
The SIFDT-MS apparatus and the basic principles of the technique were previously described [1, 2]. For the present work a newly designed ion source was constructed consisting of three hollow cathode discharges arranged parallel to the drift tube axis. Different gas (water vapor, NO or O2) is used in each discharge creating reagent ions H3O+, NO+ or O2+ rapidly switchable by drift lens voltages.

Results
It was demonstrated that the ion source allows simultaneous use of three different gases to produce reagent ions of interest that can be selected by applying optimized sets of DC electric potentials to drift lenses. Increase in the analytical sensitivity is described that was achieved by greater reagent ion intensity increased several times due to the absence of the orifice between the ion source and the drift tube, which are now pumped by a common vacuum line and have similar pressures of about 2 mbar. Ion-molecule reactions in the drift tube are governed by the voltage applied across the drift tube. It is shown how the relative electric field strength E/N affects the reaction time, the ion interaction energy and the diffusion losses. Illustrative results include enhanced product ion fragmentation [3] and suppression of unwanted reactions, for example formation of hydrated ions.

Conclusions
The presented enhancement of SIFDT-MS makes this technique more sensitive and allows construction of robust and small instruments. It thus represents a viable addition to the family of soft chemical ionization mass spectrometric techniques such as SIFT-MS and PTR-MS that are becoming widely used in the area of real time quantification of the VOCs in the medical, food and environmental fields.

Novel Aspect
Three reagent ions H3O+, NO+ or O2+ are produced in the triple ion source simultaneously and can be selected instantly at greater intensities than possible using a mass filter.

References
DIRECT COUPLING OF MAGNETIC-PARTICLE-BASED SAMPLE PREPARATION STRATEGIES TO MASS SPECTROMETRY VIA MICROFLUIDIC OPEN INTERFACE

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Keywords: Dispersive droplet extraction, Fast analysis, Magnetic particles, Microfluidic Open Interface, SPME-MS

Introduction:

Magnetic micro- and nanoparticles are an emerging technology in the field of solid-phase extractions.[1] However, current analysis strategies are limited to off-line desorption where just a fraction of the solution is detected. Hence, we developed a new design of the microfluidic open interface (MOI) which acts as an interface for the direct coupling of the magnetic-particles-based sample preparation to mass spectrometry resulting in increased sensitivity while reducing the time of analysis and sample size.[2]

Methods: (Limit of 400 characters without spaces)

Dispersive extractions of six model small molecules from droplets of PBS and urine using C18 magnetic nanoparticles in droplets for 30 s were performed. On the other side, IMAC magnetic microparticles were dispersed in droplets of β-casein digests and in-droplet β-casein trypsin digestions for extraction of phosphopeptides. In both approaches, the nanoparticles were collected by applying a magnetic field followed by direct desorption via MOI and MS detection.

Results:

The methodology developed for the direct coupling of magnetic particles to MS via MOI was evaluated using nano and microparticles. The desorption consisted in the generation of an isolated droplet (= five µL) that wets the particles, these are held by the magnetic field applied by a 3D-printed holder. Then, the enriched droplet is rapidly drained to the ESI source where the compounds are ionized. The dispersive extraction using nanoparticles from droplets (20-40 µL) was completed after 10 seconds. Limits of quantitation in the range 1-5 ng/mL were achieved in PBS and urine for all the compounds with an overall analysis time below 2 min. On the other hand, IMAC microparticles allowed efficient phosphopeptide extraction in less than 5 minutes from protein digests and in-droplet digest as a screening tool. Because of the high sensitivity achieved in both cases, the amount of particles used was within 30-100 µg per sample, which is about ten times less than conventional exhaustive extraction Methods:

Conclusions

A technology to efficiently couple magnetic-particle-based extractions to MS has been developed. The combination of fast desorption kinetics, low desorption volume, and no further dilutions made MOI-MS a rapid and very sensitive strategy. To proof the concept, droplet extractions using about 50 µg of particles were tested for small and medium-size molecules. The results demonstrated good sensitivity using the minimum amount of sample and particles in a short analysis time.

Novel Aspect

A novel interface to directly and efficiently couple magnetic-particle based extractions to MS via a microfluidic open interface was developed for fast and rapid analysis.

References
Introduction:
The development and improvement of new ionization techniques for mass spectrometry often requires dedicated, specific sampling approaches. Recently, a novel ionization scheme for ambient MS has been introduced based on a quasi-continuous laser-induced plasma, which was ignited directly before the MS inlet. This setup combines the general advantages of ambient ionization, provides electro neutrality, sufficient duty cycle and a ubiquitous plasma medium. [1, 2]

Methods:
A high repetition rate DPSS laser (Conqueror 3-LAMBDA, Nd:YVO4, 1 - 500 kHz, average output power: 12 W at 50 kHz, Compact Laser Solutions GmbH, Germany) and the corresponding optomechanical system were installed on an optical breadboard above the inlet of a LCQ DecaXP ion trap mass spectrometer. The quasi-continuous airborne plasma was ignited inside the sprayed sample in front of the inlet via focused laser irradiation.

Results:
The introduction of liquid samples into laser-induced plasmas requires higher plasma power during solvent evaporation as compared to gaseous samples. This increased demand was approached via a two-fold strategy: Firstly, an alternative, more powerful, laser plasma driven by the fundamental instead of the second harmonic wavelength was implemented, which provided a 10-fold increase of signal intensity, while maintaining the same reagent ion pattern as the previous plasma. Protonated water clusters [(H2O)nH]+, NH4+ as well as charge transfer promoting ion O2+, dominated the reagent ion mass spectrum. Secondly, a miniaturized nebulizer was used to minimize the size of the plasma quenching solvent droplets. The result of these improvements was a new and very stable ion source for direct microfluidic coupling. A variety of samples demonstrated the performance of the ion source.

Conclusions:
A laser-driven plasma was shown to be a powerful ion source for gaseous and solid samples. For the first time, liquid samples were examined using the novel source. In addition to demonstrating an improved strategy for igniting the laser plasma, this contribution also covers the miniaturization of the spray source for enhanced ionization, while minimizing sample consumption via a microfluidic spray systems.

Novel Aspect:
The first laser plasma ionization spectrum was recorded for liquid sample

References
Development and the application of a 96 high throughput solid phase microextraction (SPME) transmission mode (TM) system for direct analysis in real time (DART) mass spectrometry (MS)

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Development and the application of a 96 high throughput solid phase microextraction (SPME) transmission mode (TM) system for direct analysis in real time (DART) mass spectrometry (MS)

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Keywords: High throughput; Ambient mass spectrometry (AMS); Direct analysis in real time (DART); Solid phase microextraction (SPME); Drugs

Introduction

Development of a high throughput system capable of delivering the two “Rs” (rapidity and reliability) is one of the most sought-after research fields in the area of ambient ionization mass spectrometry (AIMS). In this research, we have taken the single SPME-TM mesh and transformed it into a 12 mesh format for rapid high throughput detection/quantification of opioids in biomatrices using direct analysis in real time mass spectrometry (MS).

Methods

SPME-TM meshes were manufactured to a 12 mesh format using pre-made photochemically milled stainless steel substrates, which were etched and adequately coated with C18 particles. Urine and plasma samples were spiked with a set of opioids, followed by a 10 minute extraction step and desorption at 450 °C of DART source with tandem MS analysis. A set of 96 samples was extracted, analyzed and interpreted in less than 1.5 hours.

Results

The SPME-TM 12 mesh strip were created utilizing a careful fabrication protocol with respect to etching and coating process, while their performance was maximized using a robust holder made at University of Waterloo (UW) capable of analyzing all 12 meshes at the same time. Positive preliminary assessment of mesh performance (i.e. coating stability, extraction efficiency) led us to apply the meshes for a high throughput quantification of selected opioids in urine and plasma. Experimental procedure was optimized with respect to extraction time, DART source temperature (TEM), rail speed and pH of samples ultimately resulting in low limits of quantification (LOQ) (i.e. 0.5 ng mL-1 fentanyl for PBS and urine and 1 ng mL-1 for plasma), acceptable accuracy (73.4-109.5 %) and repeatability (1.6-11.3%). Indeed, these results take a firm step toward establishing DART SPME-TM as potential tool for detection of varying substances in scenarios where high throughput is required.

Conclusions

Novel Aspect

First reported use of a high throughput SPME 96 system for opioid detection using DART/MS
Introduction
Rapid detection of prohibited substances in biologicals is important in forensic science. Herein, we report on results obtained for detection and quantification of drugs of abuse in urine and oral fluid (OF) by using biocompatible single use plastic SPME-TM meshes made of polyetheretherketone (PEEK) coupled to direct analysis in real time mass spectrometry (DART-MS).

Methods
PEEK meshes were coated with hydrophilic lipophilic (HLB) particles. The biofluids were spiked with a mix of drugs and internal standards. The meshes were used for extraction of selected drugs in 700 µL of urine and 300 µL of OF. Extraction time was kept at 1 minute, followed by wash in water and drying. Meshes were placed onto a homemade holder and desorbed between the DART source and the MS on a linear rail. Analysis was carried out using a triple quadrupole MS with MRM mode.

Results
The goal behind development of the plastic meshes was to be create a tool which could potentially be used for on-site in vivo analysis. Indeed, the plastic meshes provided us good results for quantification of 13 different drugs in urine and OF, with linearity ranging from 0.5-200 ng/mL and R² values > 0.99. Validation points were tested at 4 different levels and provided acceptable accuracy in the range of 75-127 %. Also, the LOQs obtained for the analytes were below the limits imposed by substance abuse and mental health services administration (SAMSHA). In addition, OF samples were collected from a volunteer for detection of caffeine levels prior to and after coffee consumption. The results obtained at different time points were in accordance with caffeine half-life for healthy adults. In addition, we looked at impact of material on heat transfer in DART. Such findings highlight the fact that ambient ionization techniques such as DART are very useful tools in providing law enforcement with much needed reliable and rapid data.

Conclusions
Results obtained indicate great potential of SPME-TM DART for use in many scenarios where rapid, reliable and quality data is required in a timely fashion. Utilization of alternate materials such as PEEK opens up a great prospect for use in situations where in vivo detection of prohibited substances may be required, in addition to having great prospect for use with portable MS instrumentation.

References
Novel aspect
First use of biocompatible Poly(etheretherketone) as SPME-TM meshes for rapid detection of drugs of abuse and exploration of impact of material on heat transfer in DART
778 - IMPROVEMENTS IN SMALL MOLECULE DETECTION VIA MALDI-MS USING BINARY MIXTURES OF NOVEL IONLESS MATRICES

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Keywords: MALDI-MS, ionless matrices, binary matrices, small molecule detection

Introduction:
Matrix-assisted laser desorption/ionisation mass spectrometry (MALDI-MS) is mainly used for the analyses of larger molecules (m/z > 1000) due to interfering mass signals below m/z 600 generated by conventional matrices. So-called ionless matrices like graphene [1] and the recently introduced DPN [2] do not generate matrix signals and therefore allow detection of small molecules. This study aims to improve their sensitivity by applying mixtures of such matrices.

Methods:
Several established matrix compounds like 1,8-di(piperdinyl)-naphthalene (DPN), 9-aminoacridine [3] and 2-naphthylsulfonic acid [4] were mixed in distinct ratios and their ionization potential was assessed via MALDI-ToF-MS using a variety of analytes e.g. fatty acids and amino acids. Additionally, in several experiments graphene, as well as strong acids or bases were applied together with these matrices to test their influence on the ionization performance.

Results:
When the ionless matrices are used in binary mixtures, a clear increase in signal intensity can be observed. The ionizing properties of these blends are higher than those of the single matrices. Graphene for example was able to improve the performance of DPN in negative ion mode especially for hydrophobic analytes like fatty acids while the spectra remained free of any signals besides the ones from the analytes. Furthermore, the required energy per laser pulse for optimal signal intensity is reduced when graphene is applied alongside DPN.

Conclusions:
The mechanism of ionization in MALDI is a complex interplay between absorption and distribution of laser energy, proton transfer between analyte and matrix, and separation of ion pairs in the gas phase. By employing mixtures of matrices, a possible existing shortcoming of a single matrix in one of these aspects can be mitigated if the other matrix surpasses the first in this particular property.

Novel Aspect:
A substantial improvement of sensitivity for the detection of small molecules via MALDI-MS using mixtures of ionless MALDI-matrices as well as graphene.

References:
Introduction
MAI seems to be a reliable alternative to ESI and MALDI for some applications. Solvent free and laser free aspects of MAI appeal to most mass spectrometrists[1,2]. The primary disadvantage of MAI is rapid evaporation/consumption of the matrix in the vacuum during analysis. There are relatively few literature reports of measuring lipids by MAI-FTMS. Here we show the use of MAI-FTMS lipid profiles in differentiating a variety of cooking oils and bacteria.

Methods
Samples were prepared by dissolving in matrix solutions. MAI analysis was done by FTMS immediately after drying the sample solutions in either positive or negative ion mode. The FT-ICR mass spectrometer is a 9.4 T Apex FT-ICR mass spectrometer (Bruker, Bremen, Germany). After air drying and loading, the plate voltage was set at 300 V. The acquisition size was at least 512 K points for high signal abundance. Twenty acquisitions were averaged to obtain a mass spectrum.

Results
3-nitrobenzonitrile (3-NBN) solubilized in acetonitrile was used as a matrix. Quality MAI-FTMS spectra from TAGs in cooking oil results when prepared with high concentrations of matrix dissolved in pure acetonitrile. Phospholipids from the bacteria only required dilute matrix. Generally, increasing the amount of water in the matrix allowed high quality phospholipid spectra with increases in the lifetime of matrix in vacuum. MS/MS fragmentation yields all important peaks in MAI-FTMS. TAG profiles obtained for all the cooking oils, peanut, soy, sunflower, coconut, cocoa butter, canola, and vegetable, are identical to those obtained with MALDI. MAI had more reproducible intensity variation, allowing statistical determination of small compositional differences[3]. Although most of the MALDI-observed phosphatidylethanolamines (PE) lipids from E. coli are adducts of sodium or potassium, MAI primarily produces protonated.

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Introduction
A wide range of organic molecules from solid, liquid and gaseous samples can be detected with low-temperature plasma (LTP) ionization mass spectrometry (MS) under ambient conditions [1]. LTP probes are relatively easy to build, but there is no commercial system available. Thus, we designed an LTP probe from 3D-printed and standard parts and tested its analytical performance on chemical and biological samples.

Methods
We constructed an LTP probe from standard and 3D-printed components and tested the suitability of the 3D-LTP probe for MS by measuring reference standards and complex samples [2]. For automated sampling and imaging, we built a robot based on RepRap technology. We investigated the biocompatibility of LTP-MS by monitoring the nicotine content in tobacco leaves for 10 days and subsequently evaluating the tissue damages with a microscope.

Results
The 3D-LTP probe was functional and coupled to a standard MS analyzer. The 3D files were published under a Creative Commons (non-commercial) Public License. Compounds of different volatility and polarity and with a molecular weight up to ~500 Da could be detected from simple and complex matrices. The diameter of the plasma beam was about 200 μm. The technical resolution of the sampling robot was 12.5 μm. Therefore, ambient mass imaging of eukaryotic tissues with cellular resolution is possible when combined with laser desorption (LD).

We subjected tobacco plants with modulated nicotine biosynthesis to daily measurements for 11 days. The LTP-MS measurements resembled the expected time course which indicated correct monitoring of the biosynthesis. Staining and microscopic evaluation only showed minor tissue damage and no systemic effect was observed. This evidencedemonstrated the high potential of LTP-MS for in vivo biosynthesis studies [3].

Conclusions
The 3D-LTP probe can be easily built from 3D-printed and commercially available parts. The plasma jet diameter is sufficient for imaging macroscopic samples. Short-time exposure of plant surfaces to LTP only resulted in negligible (microscopic) lesions, thus demonstrating the value of LTP-MS for in vivo measurements. Our license model promotes the free exchange and innovation of LTP probe designs.

Novel Aspect
The Creative Commons (non-commercial) license of the 3D-LTP probe permits the rapid building of custom low-temperature plasma probes.

References
IDENTIFICATION OF MEDULLOBLASTOMA SUBGROUPS ON INTRASURGICAL TIMESCALES WITH PICOSECOND INFRARED LASER DESORPTION MASS SPECTROMETRY

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Keywords: Medulloblastoma, picosecond infrared laser, mass spectrometry, molecular pathology, real time analysis

Introduction: Medulloblastoma (MB) is the most common brain tumor in children that is comprised of four different subgroups (WNT, SHH, Group 3, Group 4) each with distinct prognostic characteristics [1-3]. While risk stratification of MB patients based on molecular features may offer the prospect of personalized therapies, the long analysis times of conventional classification methods prevents subgroup-specific adjustments of the extent or the aggressiveness of the resection.

Methods: We investigated the potential of rapid tumor small molecule profiling with Picosecond Infrared Laser desorption Mass Spectrometry (PIRL-MS) [4,5] as a new method of medulloblastoma subgroup classification. A local tissue bank comprising 113 ex vivo medulloblastoma tumors was subjected to 10-second PIRL-MS data collection, and Principal Component Analysis with Linear Discriminant Analysis (PCA-LDA).

Results: A model for MB subgroups was built based on the laser extracted lipid profiles of 72 independent tumors, comprised of 190, 10-second PIRL-MS data points. Forty one de-identified unknown tumors were subjected to 10-second PIRL-MS sampling and real time PCA-LDA analysis using the model above where the resultant 124 sampling events, after the application of a 95% PCA-LDA prediction probability threshold, yielded a 99% correct classification rate, and only one misclassified data point. This was accompanied with 28 sampling events that were not classifiable into any known MB subgroups. We thus used an extended MB signature library that was comprised of 278 sampling events to better capture the intrinsic biological heterogeneity of MB, through which were able to correctly reclassify 21 out of the said previously unclassifiable sampling events with no misclassification. Detailed post ablation histopathological analysis of MB samples suggests that intratumoral heterogeneity at the site of laser ablation can explain the apparent misclassification.

Conclusions: Our observations suggest that rapid tumor small molecule content profiling with PIRL-MS is effective in medulloblastoma subgroup classification with only 10 seconds of sampling, an analysis time thus opening up the possibility of subgroup specific treatment of medulloblastoma beginning in the operating room during a surgical procedure.


References:


INTRODUCTION: On-line breath analysis is a powerful approach to obtain insights into the metabolism of a person. With ambient ionization methods, this can be achieved rapidly and completely non-invasively, opening up interesting possibilities to diagnosed diseases via exhaled breath, to discern diurnal changes in the metabolism, and to monitor drug use (or drug abuse) by analyzing the chemical composition of exhaled breath.

METHODS: Besides SESI-MS there are other methods in use for breath analysis, notably selected ion flow tube-mass spectrometry (SIFT-MS) and proton transfer reaction-mass spectrometry (PTR-MS). These will be compared in terms of analytical performance (mass resolution, upper mass limit, detection limit, softness of ionization, etc.) and the coverage of biomarkers (polarity, volatility, molecular weight, etc.).

RESULTS: SESI-MS afford sub-ppt limits of detection, and analysis of compounds with molecular weights up to 1000 Da. If coupled to a high-resolution instrument, SESI-MS offer excellent coverage of the breath metabolome and deep insight into the metabolic state of a person. Moreover, the high resolution allows detailed characterization of compounds that can be used as biomarkers [1]. This keynote lecture will present several examples in medical diagnosis, including the detection of novel biomarkers for respiratory diseases such as obstructive sleep apnea (OSA) [2], chronic obstructive pulmonary disease (COPD) [3], pulmonary fibrosis, and asthma. Monitoring of drug compliance and pharmacokinetics [4] via real-time SESI-MS will also be shown.

CONCLUSIONS: A variety of biomarkers that describe diseases have been detected in exhaled breath and identified via SESI-MS, often aided by chromatography of exhaled breath condensate and comparison against commercially available or synthetic reference compounds.

NEW ASPECT: Noninvasive monitoring of the detailed chemical composition of exhaled breath via SESI-HRMS.

REFERENCES
Keywords: imaging mass spectrometry, instrumentation, high spatial resolution

Introduction:
Measurement methods of spatial distribution of molecules at cellular-scale are required in many fields. Recently, scanning type imaging mass spectrometry with matrix-assisted laser desorption/ionization is intensively used for biomolecular analysis. However, the spatial resolution of scanning MALDI-IMS is limited to about 10 - 100 μm and inadequate for cellular-scale observation. Therefore, we are developing a stigmatic MALDI imaging mass spectrometer to achieve spatial resolution of sub-micron.[1]

Methods:
The experimental apparatus for stigmatic imaging consists of a MALDI ion source with focusing ion optics, TOF mass spectrometer and a time and position sensitive ion detector. Sample molecules ionized by laser irradiation are extracted and accelerated by extraction electrode. Ion distributions at the sample plate are magnified and projected with the ion optical lens system onto the detector.

Results:
For a stigmatic MALDI imaging, laser irradiation spot is focused to several hundreds micro-meter diameter with accurate uniformity. This diameter is several times larger than ordinary MALDI, so laser power is required about ten times larger than ordinary one. And for uniform laser irradiation, we develop a laser beam homogenizer system consists of a dynamic rotating diffuser to eliminate laser speckle and a square core optical fiber to form flat-top square profile. We used this system with a high power UV (355 nm) pulse laser (NL202, Ekspla, Lithuania) and evaluated imaging performance by observation of test samples. We observed grid pattern formed by test samples of crystal violet dye covered with fine metal mesh. We verified imaging quality by this experiment and then observed more practical samples of eye tissue of mouse and amoeba cells.

Conclusions:
We developed new stigmatic imaging mass spectrometer with a uniform laser beam system. Our evaluation experiments verified that this apparatus achieved high spatial resolution of 1 micro-meter with a field of view of 500 x 500 micro-meter within observation time of several minutes. This spatial resolution and measurement speed was drastically improved from conventional scanning type imaging mass spectrometers. We also show applications of this apparatus to actual biological samples.

Novel Aspect:
We developed optics of stigmatic MALDI imaging mass spectrometer and observed high spatial resolution image.

References:
Introduction:
Laser ablation electrospray ionization (LAESI) is an ionization method applicable for mass spectrometry imaging (MSI) on biological samples [1]. Making use of indigenous water, LAESI is not dependent on externally applied matrices, reducing the sample preparation component of any experiment considerably. By integrating a profilometer, sample topography becomes measurable and employable during laser ablation, allowing access to ecologically relevant samples.

Methods:
Various parts of *Gossypium hirsutum* plants were used as a model system to evaluate a custom build LAESI source for MSI [2]. Before conducting MSI experiments, the surface topography was measured and subsequently applied to guide the laser ablation process during the actual MS experiment. Sample preparation was unnecessary, except trimming the sample to fit a microscopy slide. Mass spectra were acquired in positive and negative ion mode on a Synapt HDMS (Waters).

Results:
During MSI experiments performed on different ROIs of leaves and stems with our custom-build LAESI source, profilometry was successfully applied to guide the laser ablation. As a result, laser ablation marks were of equal diameter, reproducible and independent of sample surface topography. Specialized metabolites with known insect deterring properties [3], such as gossypol and heliocide isoforms were localized in specialized glands distributed all over *G. hirsutum* plants. By exploiting the measured height profiles, the diameter of laser ablation marks was controllable between 30 µm and ~150 µm in a reproducible manner. Based on experiments on *G. hirsutum*, ecologically relevant interactions between various other plant species and microbial interaction partners were investigated.

Conclusions:
Our custom-build LAESI source allows for the inclusion of a profilometry stepin MSI experiments. The described set-up is able to deal with challenges posed by ecologically relevant samples of delicate plant-microbial interactions.

Novel Aspect:
Analysis of various plant surfaces and plant-microbe interactions without the need for sample preparation or sectioning.

References
Bartels, B. et al., RSC ADVANCES, 7(15), 9045-9050 (2017).
Improved sensitivity and speed of acquisition using quadrupole based mass spectrometer for DESI-MSI

Emmanuelle Claude (1) - Praveen Harapanahalli (2) - Emrys Jones (3)

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Improved sensitivity and speed of acquisition using quadrupole based mass spectrometer for DESI-MSI

Emmanuelle Claude, Praveen Harapanahalli, Emrys Jones

Introduction: (Limit of 400 characters)
DESI-MSI is typically known for the mapping of small molecules directly from tissue sections. DESI MSI has been proven to be successful when used on Time-Of-Flight (TOF) based mass spectrometers in particular for untargeted analysis or mass spectral fingerprinting. Tandem quadrupole mass spectrometers are renowned for their sensitivity and specificity for targeted application using Single Ion Monitoring (SIR) or Multi Reaction Monitoring (MRM) modes of acquisition.

Methods: (Limit of 400 characters)
Experiments were performed on a Xevo TQ-XS (Waters) in SIR or MRM modes of acquisition and a Xevo Q-Tof G2-XS (Waters) in MS or MS/MS modes of acquisition. A 2D DESI stage (Prosolia) DESI was used with spray conditions set 2µL/min, 95:5 MeOH:water and a nebulising gas pressure of 5 bar. DESI imaging datasets were subsequently processed and visualised using in-house software tools and High Definition Imaging (HDI) software.

Results: (Limit 900 characters)
To evaluate the level of sensitivity, dilution series of several drug compounds including Propanolol and Bromofenac were spotted on tissue sections from 0.25ng up to 1 µg. On the TQ system, MRM mode of acquisition was set with a first transition for the drug and second transition for an endogenous lipid. On the oa-TOF system, MS and MS/MS modes of acquisition were used. The limit of detection for Propanolol using the TOF system was 2.5 ng on tissue whereas it was 0.25ng for the DESI TQ. The limit of detection for Bromofenac using the TOF system was 50 ng on tissue in MS and whereas it was 0.25ng on tissue for the DESI TQ. A similar improvement in level detection of drugs was observed from dosed tissue section using the DESI TQ allowing mapping drugs in tissue at therapeutic levels which is not straightforward using TOF mass spectrometer for MSI.

Conclusions (Limit of 400 characters)
With the improved sensitivity, it was possible to increase the speed of analysis using stage rate from 1 to 30 scans/sor the number of MRM, resulting in a decreased dwell time for each pixel.

Conclusions (Limit of 400 characters)
DESI mounted on TQ mass spectrometer has clearly demonstrated some advantages in an imaging setting such as increased sensitivity allowing drug compounds to be mapped at therapeutic level from dosed tissue sections.

Novel aspects: (Limit of 150 characters)
Mapping of drug compounds at therapeutic level using highly sensitive and selective DESI mounted on triple quadrupole mass spectrometer.
1288 - PATERNÒ-BÜCHI PHOTO-DERIVATIZATION PROTOCOL FOR MALDI-MS IMAGING OF CARBON-CARBON DOUBLE BOND POSITIONAL ISOMERS OF PHOSPHO- AND GLYCOLIPIDS IN BIOLOGICAL TISSUES

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Keywords: Paternò-Büchi reaction, Benzaldehyde, MALDI-MS imaging, Double bond positional isomers, MALDI-2

Introduction: Current MALDI-MS imaging (MALDI-MSI) techniques visualize the global distribution of phospho- and glycolipids in tissue sections, but are generally blind to the physiologically relevant carbon-carbon double bond (C=C) position. Here we present a fully MALDI-(2)-MSI compatible “on-tissue” Paternò-Büchi (PB) photo-derivatization protocol that enables mapping the distribution of C=C positional isomers of numerous phospho- and glycolipids in mouse brain.

Methods: Benzaldehyde (BA; mmonoisotopic106.04 Da) vapor was introduced into a reaction chamber and condensed on the sample (sections of murine brain) at ~6 °C. PB reaction was triggered for 1-2 min by use of a 254 nm-UV lamp. Subsequently, BA was allowed to evaporate at ambient temperature. After coating with matrix, samples were analyzed at 25 µm pixel size with a Q Exactive plus Orbitrap that was equipped with a dual MALDI/ESI injector and a postionization laser for MALDI-2 [1,2].

Results: Making use of our combined PB-MALDI-2 approach, numerous phospho- and glycolipids produced clear [M+BA+H]+ ion signals in the positive ion mode. Overall PB photo-derivatization yields were in the 5–30% range (depending on lipid class). Low-energy CID of the protonated PB products generated clear MS/MS spectra and characteristic ion pairs exhibiting a mass difference of 74.05 Da. These unambiguously revealed the C=C positions for numerous singly and poly-unsaturated phospho- and glycosphingolipids in mouse cerebellum. Several C=C positional isomers of phosphatidylcholine and, for the first time, phosphatidylserine species were found to be expressed with highly differential levels in the white and gray matter areas of cerebellum. In contrast, C=C isomers of galactosylceramides were found with equal distributions in the myelin-rich white matter. Importantly, at the applied lateral resolution of 25 µm, no tissue damage or analyte delocalization, which could be associated with our PB protocol, was observed.

Conclusions: Our PB-MALDI-2-MS/MS protocol provides a valuable glimpse into phospholipid organization complexity in tissues with currently unknown biological background. Compared to the also just introduced ozon-based derivatization protocol (OzID) for differentiation of C=C positional isomers [3], our approach has the advantage to widen the class of amenable lipids; moreover, use of any standard MALDI-MSI instrument (also without postionization module) is possible.

Novel Aspect: Paternò-Büchi reaction-based protocol to visualize the distribution of C=C positional isomers of phospho- and glycolipids by MALDI-MS imaging at high lateral resolution

References
Introduction: The interest of scientists for MALDI imaging is growing since the last decade and latest developments has led to a significant impact in the pharmaceutical and cosmetics field, but also in materials research. MALDI imaging is now enabled on high resolution Orbitrap instruments by means of Atmospheric Pressure MALDI sources available on the market.

Methods: An AP-MALDI UHR ion source (Masstech Inc.) is coupled to a LTQ/Orbitrap Elite high resolution mass spectrometer (Thermo Scientific) was used for the development of targeted and untargeted imaging Mass Spectrometry experiments. Samples were coated with matrices using a HTX-TM sprayer.

Results: Several modes of operation of the AP-MALDI Orbitrap setup are presented based on samples from various types and sizes for the detection of targeted and untargeted molecules. The instrument allows for both large and small scale imaging (using Pixel map or Constant speed raster modes). The information extracted from each image pixel may be adjusted to either untargeted (full scan HRMS) or targeted (MS/MS or MSn). These various experiments may performed simultaneously in both analyzers in each location of the sample.

Conclusions: We show the characteristics and applications of a Masstech AP-MALDI UHR ion source to enable targeted and untargeted MALDI imaging capabilities down to 10 micron lateral resolution, when associated to a LTQ/Orbitrap mass spectrometer. This system allows to rapidly switch from the MALDI configuration to LCMS configuration.

Novel Aspect: Easily interchangeable AP-MALDI (MasstechUHR ion source) for targeted and untargeted MALDI-MS or -MSn imaging capabilities down to 10 micron lateral resolution.
Keywords: MALDI imaging mass spectrometry, hyperspectral chemical imaging, electrooptic fluorescent probes, Alzheimer’s disease, plaque polymorphism

Introduction: Amyloidogenic aggregation of beta-amyloid peptides into senile deposits is the major pathological hallmark of Alzheimer’s disease (AD). While the exact mechanisms of AD pathogenesis are not fully understood, plaque pathology has been identified as critical, driving AD pathogenesis. Delineating plaque pathology in situ is however challenging and calls for novel, multimodal chemical imaging tools such as imaging mass spectrometry in combination with other biochemical imaging techniques.

Methods: A hyperspectral chemical imaging paradigm is presented, implementing multimodal MALDI imaging MS of peptides and lipids along with chemical imaging using electrooptic fluorescent probes that recognize structural characteristics of Aβ aggregation dynamics. The method was employed for probing Aβ plaque polymorphism in human AD brain as well as in transgenic AD mouse models.

Results: The results allowed to identify various Aβ and lipid species associated with AD pathogenesis. Here, the data on in human and mouse tissue show both a significant increase of Aβ1-40 in senile, neurotoxic plaques as compared to plaques of diffuse, pre-amyloid morphology, where no Aβ1-40 was observed both in human AD as well as in control patients that exhibit amyloid pathology but no cognitive decline. Additional data in mice show that this increase in Aβ1-40 was associated with plaquematurisation over time, where both senile plaques and cerebrovascular deposits at 18 months contained higher Aβ1-40/1-42 levels than at 12 months. A proportionally higher Aβ1-42 content appears therefore to be characteristic for pre-mature deposits suggesting diffuse plaques to be precursors of senile plaques and that maturation into senile plaques is associated with AD pathogenesis. These data show for the first time what Aβ and lipid profiles are associated with differences in plaque morphology in progressing AD pathology.

Conclusions: Taken together, the here presented hyperspectral chemical imaging paradigm of individual, chemically and structurally delineated Aβ deposits provides insight in the chemical aspects underlying plaque polymorphism observed in Alzheimer’s disease pathology that is not facilitated by other biochemical techniques. The results show that it is possible to attribute structural features of plaques to Aβ profiles and highlight the role of different Aβ isoforms and associated neuronal lipids in formation of distinct biochemical conformation of Aβ aggregates.

Novel Aspect: The data highlight the potential of implanting different MALDI imaging modes with other chemical imaging tools for comprehensively probing molecular disease pathology.

References

Molecular Imaging at the Interface of Health and Disease

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Keywords: Imaging MS, MALDI, SIMS, Clinical diagnostics, Isomers

Introduction:
A comprehensive understanding of molecular patterns of health and disease is needed to pave the way for personalized medicine and tissue regeneration [1]. The best way to capture disease complexity is to chart and connect multilevel molecular information within a tissue, using mass spectrometry. Charting this territory with molecular maps from cells and tissue has become reality through the clinical implementation of imaging mass spectrometry [2]

Methods:
High resolution FTMS technologies (FTICR-MS and Orbitrap MS) combined with smart ion chemistry, stable isotope labeling approaches, ambient ionization, new data acquisition approaches and funnel based MALDI ion sources allows us to address some of the open challenges that still exist in the field of imaging MS. In concert with high throughput MALDI-TOF imaging technologies truly translation clinical studies have become reality.

Results:
We pushed the limit of spatial resolution, molecular resolution and maybe more importantly, structural resolution forward in imaging MS. A novel elevated pressure MALDI imaging ion source is employed to reveal local biochemistry at high throughput. In addition, we have employed OzID in combination with MALDI-MSI to reveal the distinctive regulatory role of lipid isomeric forms in a variety of diseases. These methods are employed for the generation of molecular atlases from cells and tissue and complemented with high throughput “omics” approaches. Multimodal approaches enable the study of clinical samples at a variety of molecular and spatial scales. The combination with tools from structural biology makes it possible to perform mass spectrometric experiments at length scales from cells to patients.

Conclusions
We have demonstrated how new MS based chemical microscopes target biomedical tissue analysis in various diseases as well as other chemically complex surfaces. In concert they elucidate the way in which local environments can influence molecular signaling pathways on various scales. The integration of this pathway information in a surgical setting is imminent, but innovations that push the boundaries of the technology and its application are still needed.

Novel Aspect:
Imaging MS reveals new structural detail in tissue analysis

References
Heat-assisted laser ablation electrospray ionization (HA-LAESI) for MS imaging of animal tissue

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Keywords: HA-LAESI, LAESI, imaging, tissue, laser

Introduction:
Heat-assisted laser ablation electrospray ionization (HA-LAESI) [1] and laser ablation electrospray ionization (LAESI) [2] are ambient MS techniques for the sensitive analysis of compounds with low molecular weight (<1000) from water-rich samples. Previously, HA-LAESI has been showed to be more efficient in ionizing nonpolar and neutral compounds than LAESI, and thus HA-LAESI is expected to show complementary data in the imaging of animal tissue.

Methods:
Imaging measurements were carried out using a Bruker micrOTOF MS. In HA-LAESI measurements, tissue was ablated using an OPOTEK IR Opolette 2940 laser, and a heated capillary extension was used to add heat to electrospray which was emitted with a typical 100 µm I.D. stainless steel needle. The tissue sections were cooled to -4 °C using a Peltier element and they were rastered inside a N2 filled box. Waters Xevo QTOF with a ACQUITY UPLC was used for compound identification.

Results:
Previously, a simple technique for improving the focus of OPO IR lasers has been demonstrated by solely increasing the laser beam travel distance [3]. The new focusing technique was combined with HA-LAESI to show heat maps of tissue sections with <50 µm pixel size. The HA-LAESI imaging technique was applied to several types of whole tissues, such as mouse brain, heart and kidney. Some of the detected analytes were identified using Waters’ UPLC/MS system. E.g. from mouse brain tissue, HA-LAESI showed spectra covered with high intensity phospholipid, cholesterol, ceramide, and cerebroside signals.

Conclusions
HA-LAESI was found to be a suitable technique for high resolution imaging of animal tissues, and it showed to be more efficient in ionizing nonpolar compounds than LAESI.

Novel Aspect:
High resolution HA-LAESI imaging of animal tissues

References
59 - HYBRID SIMS: A NEW SIMS INSTRUMENT FOR HIGH RESOLUTION ORGANIC IMAGING WITH HIGHEST MASS-RESOLVING POWER AND MS/MS

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Keywords: SIMS, Imaging, MS/MS, Orbitrap

Introduction:
SIMS offers the possibility to acquire chemical information from submicron regions. This capability has been especially intriguing for researchers with life science applications. The vision to image molecules has been driving instrumental development. While new ion sources expanded the usability, SIMS analysers lacked the required mass resolution, mass accuracy and MS/MS capabilities.

Methods:
To specifically address the requirements in the life science field a powerful new Hybrid SIMS instrument [1] was developed in a research project by IONTOF and Thermo Fisher ScientificTM, in close cooperation with the National Centre of Excellence in Mass Spectrometry Imaging, GlaxoSmithKline, and the University of Nottingham, following Prof Ian S Gilmore’s original idea.

Results:
In this contribution we will demonstrate the possibilities of the new instrumental design, which combines an OrbitrapTM-based Thermo ScientificTM Q ExactiveTM HF mass analyser with a high-end ToF-SIMS system from IONTOF GmbH. First application data of high resolution SIMS spectrometry, MS/MS analyses, high resolution imaging of tissues and depth profiles of biological samples will be presented. Especially the power of combining the two mass analysers on a single instrument – time-of-flight analyser for fast images with highest spatial resolution and orbital trapping analyser for highest mass resolution and mass accuracy – will be highlighted. Results also cover single beam depth profiles through porcine skin samples, which allow for a clear skin layer elucidation following different molecular ion signals.

Conclusions:
The new Hybrid SIMS instrument combines for the first time highest mass resolution (> 240,000) and highest mass accuracy (< 1 ppm) and full MS/MS capabilities with high resolution cluster SIMS imaging, making SIMS an even more viable technique for the analysis of life science samples.

Novel Aspect:
The combination of the imaging capabilities of a TOF analyser with the unique performance of the OrbitrapTM provides a new level of SIMS information.

References:
Introduction:
Drug discovery aims at finding suitable drug candidates that are being metabolized and distributed to the target site, bind to the biological receptors, and block or stimulate a metabolic reaction.[1] Mass spectrometry imaging has established itself as a powerful tool to provide spatial information on drugs and metabolites but suffers from low sensitivity and irreproducibility due to background ions from biological sample matrix.[2]

Methods:
Our work reports a novel targeted MS imaging approach using desorption electrospray ionization (DESI) combined with multiple reaction monitoring (MRM), neutral loss (NL), precursor ion (PREC), and product ion (PROD) scans for the analysis of drugs and lipids in pre-clinical studies. MRM uses a precursor ion-product ion transition to provide the selectivity required to separate isobaric species based on different product ions.

Results:
For this purpose, a set of experiments is executed on a pre-clinical drug study in which multiple dog liver tissues were analyzed using DESI prior to PROD, NL, and MRM modes to target, map and identify two potential drug candidates. The accumulation of the drugs correlated with histological lesions (already annotated by the pathologist) indicates severe fibrosis which resulted in stopping these compounds from further development. In addition, we further assessed the potential of MRM, NL, and PREC imaging modes by monitoring several endogenous lipid species in consecutive tissue sections, to complement the investigation of the toxic effect. The improved selectivity and sensitivity of this technology was investigated by the comparison of NL and PREC scanning modes with the MRM mode of low abundant lipid species in rat brain.

Conclusions:
Our approach opens doors for the pharmaceutical industry to target and image drugs and metabolites with high selectivity without the need for expensive high mass resolution imaging technologies. The use of a triple quadrupole MS not only offers higher selectivity and sensitivity but also allows to explore a (semi) quantitative strategy due to its high dynamic range.

Novel Aspect:
The selective capability of a triple quadrupole is now combined with mass spectrometry imaging for the first time by using targeted NL, PREC, and MRM imaging.

References
Introduction
Accurate pulse detection is vital in data acquisition systems for mass spectrometry but problems such as component aging and temperature variations typically reduce the accuracy of the detection and may generate misleading results. Drift in baseline level and pattern noise from interleaved analog-to-digital converters (ADCs) may result in missed pulses or incorrect detection of false pulses.

Methods
Data acquisition modules from Teledyne SP Devices utilize on-board real-time signal processing to remove baseline drift and reduce ADC pattern noise. The Digital Baseline Stabilizer (DBS) operate in the background and perform highly accurate baseline adjustment to a user-defined target value. ADC pattern noise reduction is achieved by continuous background calibration that corrects for gain, offset and time skew mismatch errors.

Results
Baseline adjustment and pattern noise reduction results in significantly improved detection accuracy. Continuous background correction allows the user-controlled trigger level to be valid over time without any need for manual calibration procedures. Baseline drift and pattern noise can be significant sources of noise – the latter with mismatch errors ranging up to a hundred ADC codes [1]. DBS reduces the pattern noise to a level corresponding to 22 bits resolution.

Conclusions
High-precision pulse detection requires estimation and correction of imperfections and mismatch errors in analog electronic components. Baseline drift in data acquisition for mass spectrometry can lead to misleading pulse characteristics and peak values as they are determined against an incorrect baseline level and ADC pattern noise can further significantly contribute to false readings.

Novel Aspect
DBS shows how expertise in signal processing, hardware knowledge, and application know-how is combined to create an optimized system solution.

References
OPEN DEVELOPMENT KIT FOR BUILDING AN AMBIENT MASS IMAGING ROBOT WITH MICROMETRIC RESOLUTION

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Keywords: ambient ionization, ambient imaging, 3D printing, low temperature plasma, instrumentation

Introduction
Ambient ionization has revolutionized mass spectrometry, because samples can be analyzed under atmospheric condition without prior preparation, including imaging [1]. Technologies such as 3D printing help to easily share, implement and reproduce ambient ionization probes [2]. Inspired by this technology, we decided to generate an automated sampling platform based on RepRap systems for high throughput analyses and imaging to further enhance the utility of AIMS.

Methods
RepRap technology was used for the ambient sampling platform. The system was designed in our laboratory and builtin by MakerMex (Gto. Mexico). Additional accessories to adjust ionization sources and optical tools were designed using Blender and printed using a MM1 Beta RepRap printer with an acrylonitrilebutadiene styrene (ABS) polymer. For system control and data analysis, the software rmsiGUI was built based on the free statistics software R using shiny package.

Results
We developed a complete kit for an ambient sampling platform, consisting of - robot - software - adapters. The sampling platform can incorporate new tools/accessories depending on the applications. The system can be used for imaging and high throughput analyses. The theoretical resolution of our platform is 12.5 μm. However, the natural diffusion of the compounds generates a practical resolution of 200 μm. We used a PlasmaChip (NovionX, Germany) to automate sample analyses and for ambient imaging we used a 3D-plasma probe alone or in combination with laser desorption (LD). The rmsiGUI software has tabs for different functions, generates a G-code to control the robot movements, creates simzML files for mass spectrometry imaging, uploads a photo from a sample and marks a region of interest (ROI). Data can be analyzed by multiple statistical tools to identify metabolic markers.

Conclusions
We demonstrated the functionality of an open, modular ambient sampling platform for automated sampling and mass imaging. The rmsiGUI is able to perform a complete analysis from MSI data. The design of the sampling robot and the rmsiGUI software are freely available for non-commercial uses.

Novel Aspect
We present an open platform for analysis under ambient conditions based on RepRap technology which enables the user to design new features based on his specific needs.

References

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UNAMBIGUOUS PRECURSOR MASS ASSIGNMENT ENABLES ACCURATE AND SENSITIVE PEPTIDE IDENTIFICATION OF DATA-INDEPENDENT ACQUISITION MASS SPECTROMETRY DATA

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Keywords: Data-independent acquisition, multiplexed MS/MS spectra, precursor mass, multi-stage database search

Introduction:
Data-independent acquisition (DIA) approach has emerged as a promising strategy for robust and large-scale quantitation with the development of advanced mass spectrometer and data analysis tool [1]. However, accurate and sensitive interpretation of highly complex multiplexed MS/MS spectra acquired through DIA experiment still remains a challenge.

Methods:
Novel DIA data analysis strategy are proposed utilizing unambiguously determined precursor mass. Accurate monoisotopic masses of multiple precursor ions corresponding to multiplexed MS/MS spectra were determined through mPE-MMR(multiplexed post-experimental monoisotopic mass refinement) procedure which was previously developed and applied for interpretation of multiplexed MS/MS spectra from DDA data [1].

Results:
MS/MS data with determined precursor mass through mPE-MMR were searched against spectral library using in-house built spectral library search tool (The first stage search) under precursor mass and retention time tolerance. MS/MS data without peptide identification were searched against customized protein database using MS-GF+ search engine (The second stage search) [2]. The remained uninterpreted MS/MS data after the first and second stage search process were subjected to MODplus search engine[3] specified for unrestricted modification detection. Up to 60,000 peptides were identified from 3hr gradient DIA experiments. Comparing to conventional DIA data analysis approach which does not consider precursor mass during database search, our strategy utilizing precursor mass effectively increase accuracy and sensitivity in peptide identification.

Conclusions
Interpreting multiplexed MS/MS spectra of DIA data were interpreted with assigned precursor mass through mPE-MMR and multi-stage database search. It provided increased sensitivity and accuracy in peptide identification.

Novel Aspect
Previously developed DIA data analysis tools considered elution time and fragment ions are considered for targeted extraction or spectral library search. There are few methods providing intuitive accuracy determinant parameter of identified peptides such as mass measurement accuracy.

References

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356 - INFRARED LASER ABLATION MICROSAMPLING COUPLED WITH MALDI IMAGING

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Keywords: MALDI, Imaging, Laser, Ablation

Introduction:
Matrix-assisted laser desorption/ionization (MALDI) mass spectrometry imaging (MSI) is a powerful method for determining the location of biomolecules in tissue; however, protein identification and quantification remains challenging. The goal of this project is to develop an imaging workflow that combines MALDI imaging with laser ablation microsampling for liquid chromatography tandem mass spectrometry.

Methods:
In the combined workflow, MALDI imaging is used to identify regions of interest (ROI) from intact proteins. The ROI are sampled using infrared laser ablation and the captured material is analyzed by LC-MS/MS using data independent acquisition to identify and quantify the proteins. The data are cross-correlated to identify the localized proteins in the MALDI images.

Results:
Development of the combined approach is aimed at creating an automated system for ablation and capture and using it in a coupled workflow of MALDI imaging and LC MS/MS analysis. The infrared laser ablation and capture system uses a mid-infrared optical parametric oscillator laser with a custom reflective objective that has a large working distance and good numerical aperture. We have developed custom positioning software that allows MALDI MSI heat maps to be overlaid on camera images to co-register ROI ablation with the IR laser. Tissue sections are mounted on conductive microscope slides and either consecutive sections or MALDI analyzed sections can be used. Laser ablated proteins are digested with magnetic capture beads and the peptides released for analysis with a Waters nanoAcquity UPLC system coupled to a Synapt G2-HDMS.

Conclusions
MALDI MSI coupled with region-specific laser ablation sampling for LC MS/MS is a fast and versatile approach for spatially resolved tissue proteomics. We have demonstrated that proteins can be identified from spatially localized regions and are developing new methods for correlating the intact proteins observed in MALDI with the proteins identified by tandem mass spectrometry.

Novel Aspect:
Coupled MALDI imaging with high precision infrared laser ablation capture for LC MS/MS for protein identification and quantification.
Introduction
CycloBranch (http://ms.biomed.cas.cz/cyclobranch/) is an open tool for de novo sequencing of non-ribosomal peptides and for dereplication of natural products from MALDI-MS, LC-MS, MSI (mass spectrometry imaging) and MS/MS datasets [1, 2]. Recently, a fusion of MSI data (imzML) with histology images was also reported [3]. Here, we present how CycloBranch can be used for more reliable dereplication of natural products benefiting from isotopic fine structure [4].

Methods
The high-resolution mass spectra of standard bacterial siderophores [5] were collected on 12T SolariX FTICR mass spectrometer (Bruker Daltonics, Billerica, USA) equipped with ESI source in positive ion mode. The results were verified by nuclear magnetic resonance (NMR) spectroscopy by 1H NMR, 1H-13C HSQC, 1H-15N HSQC, 1H-13C HMBC, TOCSY and J-resolved experiments (AVANCE III 700 MHz, Bruker Biospin GmbH, Rheinstetten, Germany).

Results
The fine isotope patterns of pyoverdines D/E - whose structures differ by (de)amidation only - were recorded with 840,000 @ m/z 1334 resolution in a conventional mass spectrum. If the minimum relative intensity threshold was 0.5%, the tool annotated 11 out of 12 theoretical peaks of pyoverdine D (m/z 1334.6) and 8 out of 9 peaks of pyoverdine E (m/z 1333.6). The fragmentation mass spectrum was collected with 550,000 @ m/z 1334 resolution. The complete m/z scale including the sequence-specific b-ion series was generated by CycloBranch in silico. The tool correctly annotated 201 out of 221 experimental peaks exceeding 0.5% relative intensity. Sixteen other peaks with intensities below 2.4% were correctly unannotated artefacts, and 4 remaining peaks with intensities below 1.6% were unknown. We observed 19 peaks with relative intensities 0.5-7.5% (without isotopologs) specific to pyoverdine E and only one peak m/z 601.24 (3%) unique to pyoverdine D. The remaining peaks corresponded to fragment ions of both pyoverdines.

Conclusions
The fine isotope feature of our open-source software called CycloBranch was reported on a showcase example of bacterial siderophores pyoverdines D/E whose masses differed by 1 Da. The quantitative results were confirmed by NMR analyses. Acknowledgement: Ministry of Education, Youth and Sports of the Czech Republic (NPU LO1509) and Czech Science Foundation (16-20229S).

Novel Aspect
A free software utilizing the benefits of isotopic fine structure for dereplication of natural products from MALDI-MS, LC-MS, MSI, and MS/MS datasets.

References
**694 - BIG DATA POST-PROCESSING FOR HIGHER THROUGHPUT MALDI/DESI FTMS IMAGING**

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Keywords: FTMS, MALDI/DESI, imaging, transient, software

**Introduction:**
High mass resolution FTMS MALDI and DESI imaging techniques are widely employed for analyzing biological tissues, plants, and oils. Real-life sample complexity, tiny quantities of analytes, and long experimental times require increased sensitivity, resolution, and throughput. Here, we describe innovations in FTMS transient acquisition and processing algorithms and their integration into big data analysis workflows for leveraging FTMS imaging applications.

**Methods:**
MALDI and DESI imaging data were acquired with commercial ICR and Orbitrap FTMS instruments. Mass spectra and transients were recorded in parallel using original instrument manufacturer and in-house developed data acquisition hardware and software. Data post-processing was performed using custom software tools and algorithms, including absorption mode FT (aFT) and least-squares fitting (LSF), using multicore and graphic card processing capabilities.

**Results:**
We evaluated the key benefits resulting from the use of a novel big data processing workflow. Disabling on-the-fly FTMS data processing (including FT, peak picking, and recording data on a drive) reduced the total time of measurements up to 2-fold per image. Additionally, the scan rate was further increased via an optimized use of overhead times and application of aFT (allows approx. half the transient time compared to magnitude mode processing) or LSF for targeted imaging. The LSF demonstrated superior performance to magnitude and aFT in terms of required minimum transient length. LSF processing, being implemented on graphic cards, reduced data processing times to practically-useful acceptable levels. Increases in sensitivity and identification confidence were achieved by transient or full-profile mass spectra summation over a region of interest and summation across multiple experiments, e.g., 3D MALDI/DESI. Mass accuracy was improved using an internal pixel-by-pixel non-linear mass re-calibration based on binomial averaging.

**Conclusions**
Increasing the rate at which FTMS transients are generated, while storing them for post-processing and re-analysis, appears attractive for higher-throughput MALDI/DESI imaging applications. The aFT and LSF methods allow the use of shorter transients, without compromised accuracy in identification of molecules (e.g., lipids). Increased sensitivity enhances the confidence and the number of lipids identified and quantified per MALDI/DESI imaging experiment.

**Novel Aspect:**
Advanced algorithms of time-domain signal (transient) acquisition and post-processing, and big-data analysis boost FTMS performance for MALDI/DESI imaging applications.

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INTRODUCTION:
In electrospray ionization (ESI) ions are often transferred through a narrow capillary from ambient to a lower pressure stage, where complex ion optics like ion funnels are used to form the ion beam. The particle transport is governed by electric forces, space charge and by the gas flow. Their interplay determines the behavior and in particular the efficiency of the source. However, most simulations include only some of these effects, often neglecting the gas dynamics.

METHODS:
We simulate the gas flow of the capillary and the first pumping stage including an ion funnel. The resulting flow fields are used to simulate the ion transport by a self-written software. It allows to treat the space charge effects and collimation by the rf- and dc-fields of the ion funnel by first principles and to quantify the different effects. It also reveals the conditions the ions experience, like temperature, pressure and electric fields, during transfer.

RESULTS:
We find a strong influence of the gas flow on the ion transmission. High transmission rates recently observed can be reproduced by the simulation and understood, with numerical results comparing quantitatively to the experiment. The key factor to obtain high transmission in a transfer capillary is transport in a laminar flow. High transmission is further obtained for low net currents (low space charge repulsion) and good pre-collimation of an electrospray before entering the transfer capillary, as for instance achieved by recently presented funnel inlets. But also under ideal laminar conditions, space charge effects determine the maximal current transmitted through the capillary. Time resolved simulations with many individual particles allow an in-depth description especially in the ion funnel. When designing an ESI sources all the different aspects need to be taken into account.

CONCLUSIONS:
Gas flows in the high pressure part of an ESI interface are important for ion transport and deserve higher attention due to their large potential for optimizing ion sources. Space charge effects become important for high currents and can partly be mitigated by an improved extraction and collimation at the inlet by the gas flows. Better understanding of the interplay of space charge and gas flow is the key to high current and high transmission ion sources.

NOVEL ASPECT:
A comprehensive simulation of the transfer capillary and the first stage with an ion funnel reveals detailed insight into important aspects of the ion transport.
Introduction:
In MALDI, only a minor fraction of desorbed molecules is concomitantly ionized and, therefore, available for mass spectrometric analysis. To mitigate this inherent shortcoming, we here present a new strategy that utilizes a gas discharge for post ionization (PI) inside an intermediate pressure ion source. The RF-driven dielectric barrier discharge (DBD) is pulsed and timed to directly interact with the MALDI plume, leading to effective PI of matrix and analyte.

Methods:
The DBD unit was integrated into the sample holder assembly of a Spectroglyph dual funnel MALDI/ESI ion source, coupled to an Orbitrap MS. Consisting of an insulated antenna and a metal counter electrode, the DBD produces a spatially confined plasma right above the surface of the MALDI sample. Custom-made electronics were used to drive and trigger the discharge. MALDI-DBD-MS imaging experiments were performed with different matrices and in both polarities.

Results:
In the first part of the presentation, concept and considerations for the design of the plasma region and RF-electronics for pulsed DBD-PI inside the intermediate pressure ion source will be described. This is followed by results from fundamental studies on the influence of relevant input parameters like DBD pulse duration, peak-to-peak voltage, delay between laser and DBD pulses, and the effect of gas pressure and its composition on the plasma. Careful optimization of laser and plasma parameters resulted in PI of phospho- and glycolipids with low degree of fragmentation. Under these optimized conditions, the DBD-MALDI mass spectra largely resemble those as produced by laser-induced PI in a MALDI-2 setting [1,2], i.e., a boost of protonated/deprotonated lipids and further metabolites is obtained. First DBD-MALDI imaging results obtained with mouse brain sections will be presented.

Conclusions:
Pulsed DBD-PI holds great potential for increasing the sensitivity of MALDI-MSI in intermediate pressure ion sources at low costs. Synchronized to laser-generated material ejection, short bursts of DBD, suitably tailored in length and intensity, lead to a strong post ionization of matrix molecules and subsequent charge transfer reactions to neutral analytes within the evolving MALDI-plume.

Novel Aspect:
A low-cost pulsed dielectric barrier discharge assembly for effective post ionization of lipids in an elevated pressure MALDI MS ion source

VALIDATION STUDIES OF PHOSPHOPEPTIDE ENRICHMENT PROCEDURES USING TIO2-ATTACHED MEMBRANE FUNNEL-BASED SPRAY IONIZATION FOR IMAGING MASS SPECTROMETRY

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Keywords: Imaging mass spectrometry, protein digestion, phosphopeptides, titanium dioxide, selective enrichment

Introduction:
Imaging Mass Spectrometry (IMS) displays the distribution of different chemicals across a surface [1]. In this work, the analytical performance of an automated membrane funnel-based spray ion source was validated for phosphopeptides selective imaging mass spectrometry. Array of TiO2-attached membrane funnels was fabricated for in situ clean-up and enrichment of phosphopeptides to alleviate matrix suppression effect in the ionization process(es) [2].

Methods:
In-solution bovine beta-casein digest mixed with two phosphopeptide standards with different loadings were analyzed using funnel spray ionization mass spectrometry. In-funnel digestion of bovine beta-casein with different loadings was also performed. To evaluate the attainable spatial resolution of the IMS based on funnel spray ionization, “flower” figures were inkjet-printed onto Parafilm® membrane and images with different inter-funnel distances were obtained.

Results:
The digestion derived phosphopeptides and the phosphopeptide standards were detected with good reproducibility. The analytical method exhibited reasonable linearity over a concentration range of 2.5 to 10 pmol for phosphoprotein loadings and 0.5 to 4.5 pmol for phosphopeptides loadings, demonstrating the feasibility TiO2-attached membrane funnels in removing interfering non-phosphorylated peptides then selectively enriching phosphopeptides from complex protein digest samples. A mono-phosphorylated peptide was added as internal standard to the in-funnel digest before mass spectrometry analysis. This could reduce the experimental influence and improve the linearity of the analytical method. The best attainable spatial resolution for the IMS experiment was 200 microns and there was no detectable cross-contamination. Attempts to further reduce the inter-funnel distance using the current funnel fabrication method were hampered by the deformation of the membrane surface.

Conclusions:
Phosphopeptides from protein digest were selectively detected with satisfactory detection limit, reproducibility and linearity using our developed workflow. IMS experiment with reasonable spatial resolution was demonstrated on this ion source. It is believed that this in situ single-pixel sample pretreatment method is a promising technique for the image studies of phosphoproteins of biological tissues.

Novel Aspect:
An array of funnels could be used to pixelize a tissue surface for in situ single-pixel protein digestion and selective enrichment of phosphopeptides.

References
B.09 INSTRUMENTATION AND METHODS - INFORMATICS TOOLS AND DATA ANALYSIS

1011 - PHASE-CONSTRAINED SPECTRUM DECONVOLUTION-- REAL TIME SUPER-RESOLUTION PROCESSING OF FTMS DATA

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ThermoFisher Scientific, LSMS, Bremen (1)

Keywords: FTMS, Orbitrap, super-resolution, high-throughput, TMT

Introduction:
The Phase-Constrained Spectrum Deconvolution Method (ΦSDM) [1] is a novel signal analysis approach which enables qualitatively faster FTMS scan rates while preserving the levels of mass resolution and accuracy. This presentation discusses real-time ΦSDM processing of complex experimental spectra that provide the increased depth of analyte coverage.

Methods:
The MS experiments were conducted on the research grade Q Exactive™ family of Orbitrap™ mass spectrometers. The LC separation was done on EASY-Spray™ PepMap™ RSLC C18 column using UltiMate™ 3000 NCS system. Intact proteins were infused directly. An auxiliary computer equipped with four Titan X Nvidia™ GPU cards was used for the full mass range ΦSDM calculations. Spectral windows comprising the TMT labels were processed directly on the instrument’s computer.

Results:
Back to back LCMS runs of 11-plex TMT labeled HeLa digests demonstrated that the ΦSDM facilitates a significant increase in the spectral acquisition rate on a research grade Q Exactive HF which results in 1.74x more MS/MS scans and allowed the 1.55x PSM increase. The numbers of quantified peptides and proteins is boosted by factors of 1.47 and 1.32, respectively. The Q Exactive HF-X data shows the 2.39x MS/MS scan rates and 1.99x PSM’s. The peptide and protein quantification improvement was demonstrated as 1.82x and 1.62x, correspondingly.

Real-time ΦSDM processing of intact protein spectra isotopically resolves the entire charge state envelope of Ubiquitin (8.6 kDa) with MS scan rate in excess of 30 Hz, with the reported deconvolved nominal mass accuracy of ~1ppm. For the carbonic anhydrase (29 kDa), the baseline isotopic resolution of the charge state envelope is achievable at scan rates of ~7.5 Hz, with the reported nominal mass accuracy of ~2ppm.

Conclusions
ΦSDM has been used for real-time LC experiments. Its ability to provide the super-FT resolution allowed higher MS/MS rates and, consequently, boosted up the identification and quantification. Protein isotopic enveloped were completely resolved with ΦSDM even at high repetition rates, which allowed reliable charge state identification and accurate reporting of the nominal mass.

Novel Aspect:
ΦSDM, a new FTMS algorithm, was for the first time implemented for real-time data processing. Quantitation improvement and superior mass resolution are demonstrated.

References
For information please contact: scientific@imsc2018.it
TAMI SOFTWARE FOR THE PROVISION OF ELEMENTAL FORMULA FROM YOUR SINGLE QUADRUPOLE GC-MS OR LC-MS DATA

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TAMI Software for the Provision of Elemental Formula from your Single Quadrupole GC-MS or LC-MS Data

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Introduction

In order to significantly improve sample identification by GC-MS we developed the Tal-Aviv Molecule Identifier (TAMI) software. TAMI inverts the molecular ion isotopomeric pattern into elemental formulae and automatically confirms or rejects NIST library identification for improved confidence in the identification. When NIST identification fails, TAMI provides the sample compound elemental formula from standard centroid data files.

Method

When the quadrupole MS scans in profile mode, TAMI improves the mass accuracy (typically <50 ppm) and combined with isotope distribution analysis it usually provides the correct elemental formula as number one in its hit list.

The TAMI software was extensively explored with Agilent 5975/5977 GC-MS, Aviv Analytical 5977-SMB GC-MS with Cold EI that provides enhanced molecular ions, Thermo ISQ Chromeleon and Advion CMS LC-MS data files with the following main benefits:

Preliminary data

A) Automatic linking with the library. TAMI automatically confirms or rejects the NIST library identification via independent isotope abundance analysis.
B) Independent provision of elemental formula from standard centroid data files. In case of rejection of the library identification such as when the compound is not in the library, TAMI independently provides a list of elemental formulae with declining order of matching to the experimental data.
C) Simple to use. The linking of TAMI with the NIST library is automated while the provision of elemental formula requires only one mouse click.
D) Improved mass accuracy. When the quadrupole MS scans in profile mode TAMI improves the quadrupole mass accuracy via the use of special weighted average algorithm and mass calibration with PFTBA to be typically better than 50 ppm.
E) Special compatibility with Thermo Chromeleon Data Files. The Thermo ISQ in combination with Chromeleon software provides <50 ppm mass accuracy using the TAMI PFTBA based mass tune function with standard centroid data files.

Novel Aspect

Software for the provision of elemental formula from unit resolution quadrupole GC-MS or LC-MS based on isotope abundance analysis and improved mass accuracy

Keywords Elemental formula from quadrupole MS
The database for cross-platform mining biomarkers in the brain tumor tissue

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Keywords: mass-spectrometry, database, biomarkers, brain tumors, high and low resolution

Introduction:
One of the most challenging problems when using highly specialized mass spectrometry instruments and ionization techniques is both the unification of the raw data obtained and the interpretation of the final result of their processing. So, we present here an approach using database for comparing the high and low resolution spectra obtained by various ionization methods on different mass spectrometers and intended for identification of different tumor types.

Methods:
Tumor tissues were dissected during neurosurgery. The spectra were measured by: a) needle electrospray ionization [1] on ICR-based HR mass spectrometer LTQ FT Ultra; b) direct spray of lipids extracted from tissue by Folch method on Thermo LTQ-Orbitrap XL and Bruker Esquire 3000 plus. The high and the resolution was 56,000 and 1,000 at 800 Th, respectively. Information about ionization method, instrument type, tumor type, etc. was stored in NoSQL database.

Results:
A database of over 300 samples of different tumor tissues and surrounding tissues from over 250 patients was collected in the study. All of the samples were provided by the Burdenko Neurosurgical Institute. Collected spectra were filtered and stored in column-oriented database MonetDB. User interface allows for visualisation, comparison, transformation (PCA, ICA etc.) of the spectra. The integration with R/Bioconductor makes whole spectra of statistical analysis and machine learning techniques readily available from API as well as from user interface. To demonstrate the infrastructure performance 9 classifiers were trained on 20 astrocytoma and 9 unmodified brain samples with different settings. Comparative analysis of the results obtained from spray-from-tissue ionization experiments and extracts analysis shows that both methods demonstrate similar series of peaks. All major peaks are found in both types of spectra. Peaks in low resolution usually are larger when normalized to TIC.

Conclusions
Vast majority of selected features distinguishing the tumor tissue from the adjacent tissue was found in both high- and low-resolution spectra. We have created mapping schema, which allows using classifier trained to separate tumor samples from non-tumor brain samples on high-resolution spectra with data from low-resolution spectra with a minor decrease in accuracy.

Acknowledgements: Supported by the RSF (16-15-10431).

Novel Aspect:
Our cross-platform approach is a huge step to the unification of the data obtained at various types of mass spectrometry platforms, in particular, for LC-MS.

References
Combining GC-MS with Machine Learning for Non-Invasive Cancer and Disease Diagnostics
Dr Michael Cauchi, University of Limerick, Ireland.

Keywords: Metabolomics; GC-MS; Machine learning; Biomarker discovery; Medical diagnostics

Introduction:
The use of machine learning in biomedical applications has grown. Gold standard techniques are invasive, costly and time-consuming. A non-invasive approach measures the volatile analytes present in patients’ urine or faeces via gas chromatography mass spectrometry (GC-MS) in conjunction with optimised machine learning models that can distinguish between ill and healthy patients. Bladder cancer [1] and Crohn’s disease [2] are presented.

Methods:
Patients diagnosed with bladder cancer or Crohn’s disease were recruited and donated urine or faecal samples respectively. Samples were processed and passed through a GC-MS instrument. Each sample generated a data matrix from which the total ion count (TIC) chromatogram was attained. Normalisation was followed by peak alignment via correlation optimized warping (COW). The data were subjected to machine learning algorithms for diagnosis and biomarker discovery.

Results:
The three machine learning algorithms employed were partial least squares discriminant analysis (PLS-DA), support vector machines (SVMs) and random forests (RFs). The models were optimized via a bootstrapping process in order to ensure confidence in the results. The bladder cancer data (from urine) consisted of three control groups (no disease, infection and urological disease) and three cancer grade groups. Of great interest was to see if urological diseases could be distinguished from the cancer grades: 77%, 79% and 74% overall accuracies were attained for PLS-DA, SVMs and RFs respectively. The other control group comparisons attained higher accuracies (i.e. >85%). The Crohn’s disease data (from faeces) also produced a very good overall accuracy of 85%. Interrogation of the optimal PLS-DA models for the bladder cancer and Crohn’s disease suggested significant retention time peaks which led to the identification of possible markers.

Conclusions:
The application of GC-MS in conjunction with machine learning techniques offers a highly feasible, non-invasive procedure for cancer and disease diagnosis. In the case of bladder cancer there was an improvement on the gold standard technique of urine cytology. In the case of Crohn’s disease, results compared favourably with the gold standard technique of colonoscopy.

Novel Aspect:
Combining GC-MS with machine learning offers a non-invasive approach to cancer and disease diagnostics. Applications to other diseases/instruments are highly feasible.

References:

For information please contact: scientific@imsc2018.it
1296 - TISANE DENOISING ALGORITHM FOR LARGE DENSE SPECTRA

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Keywords:
Denoising, large datasets, Petroleomics

Introduction:
In Fourier Spectroscopies noise can critically hamper data analysis. In particular, for FTICR datasets characterized by a large number of points and in some cases, by a large density of lines, removing the noise is a non trivial task. Some denoising methods were devised (Chiron et al. (2014)) (Fabrice Bray and Rolando (2017)) able to cope with the size problem but for a large number of lines.

Methods
We present here a fast, parallisable algorithm TISANE which is able to tackle the denoising problem for both a large datasets and a large density of lines. This is done by performing the denoising by part, dividing the spectrum in small chunks on which the Sane algorithm is applied. The resulting denoised chunks are then reassembled to produce the whole denoised spectrum.

Results
Recently, the invention of the ultra high resolution - harmonized FT-ICR cells (Nikolaev et al. (2011)) led to FTICR datasets containing millions of points. Here we show the processing of a petroleomic dataset obtained with such a cell, with R >= 500k at m/z=400, 4194304 points, with an estimated total number of peaks of about 15000. For Sane, with a computer having a RAM memory of 30Gb, the rank for this dataset cannot exceed 500. It is about 30 times under the value needed for the theoretical correct denoising with this method. Here TISANE got around the memory limitation by running with the following parameters: 4000 intervals, an adaptive rank and 15 processing cores. The processing took 40 seconds. Finally we observe that TISANE’s robust cleanup gives access to the small isotopic patterns.

Conclusions
The processing with TISANE is quite fast considering the size of the dataset. It benefits from a double acceleration in comparison with the Sane algorithm by using both a divide and conquer approach and parallelization. Apart large and dense line data the algorithm can be applied to small datasets with the advantage of its speed.

Novel Aspect
TISANE can be useful for Proteomics where denoising is crucial for peptides identification with automatic search engine like MASCOT.

References
Keywords: Big-Data, Data Analysis, Isotopic Deconvolution, Pattern Matching, Compressed Sensing

Introduction
Complex analysis of large volumes of data is not unusual in MS analysis. However, signal processing approaches of the raw data in order to extract the best of the acquisition data, has been only partly explored. In the recent years, in particular thanks to advances in mathematics, new approaches related to Compressed Sensing or to Machine Learning, allow to the development of improved sensibility and results, or even to explore new acquisition procedures.

Methods
As an example of such optimisation, the natural sparsity of MS data can be exploited through the minimization of the $\ell_1$ or $\ell_2$ norms. Proximal algorithms can be then developed which rapidly converge without the need to compute a (costly) gradient. Discreet pattern dictionaries and pattern matching algorithms will be presented for the analysis of isotopic patterns, and fast rank reduction operation as a method to reduce the noise, or even complement missing values.

Results
The basis of the approach will be presented on resolution enhancement of Orbitrap experiments, obtained by a fast optimization of a sparsity score function, using proximal operators. These approaches being fast enough to allow a near-real time analysis.

The same approach is then applied to FT-ICR data-sets, in particular to the 2D FT-ICR method[1]. Here, sparsity is combined to random projections to remove unwanted noise[2], enhance resolution, and missing point reconstruction[3].

Finally, we present recent work where isotopic patterns in complex are recognized in overlapped spectra, without the need of separated peaks. The use of the averagine model[4] allows to build a fast approximation of the patterns, and a search in the Fourier space allows to considerably speed-up the analysis and reduce the memory burden[5]. Promising results are already obtained with this method on peptide mixtures with several charge states intermixed, or with large proteins for which precise determination of the monoisotopic value is obtained.

Conclusions
The methods presented here rely on the analysis of the raw data, as obtained before any further processing. They have to be inserted before the usual data analysis pipeline. This displaces the chemist habits but is certainly worth the burden. This research is from a joint action with the CASC4DE spin-off company. This company works on data analysis in the biosciences, and is chosen to develop the data analysis pipeline of the EU_FT-ICR_MS European infrastructure.

Novel Aspect
New mathematics in data processing can bring you unexpected results.

References
26 - ONE SMALL STEP FOR INFLUENZA –NEW MASS-BASED BIOINFORMATICS AND PHYLOGENETICS APPROACH REVEALS INSIGHTS INTO THE EVOLUTION OF THE VIRUS

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One Small Step for Influenza –New Mass-Based Bioinformatics and Phylogenetics Approach Reveals Insights into the Evolution of the Virus

Keywords: evolution, phylogenetics, influenza, epistasis, mass spectrometry

Introduction: (Limit of 400 characters)

The word “influenza” is Italian for influence since in ancient Rome it was widely believed that epidemics were caused by an unfavorable conjunction of the stars as viewed through Galileo’s telescope. It is now known that epidemics are associated with the generation of escape mutants. We have advanced a novel mass (or number) based phylogenetic approach to study protein mutations and epistasis associated with the evolution of influenza to help improve responses.

Methods: (Limit of 400 characters)

Full length (HA0) non-redundant H3 hemagglutinin sequences from all human H3N2 type A strains across the period from 1968-2008 were downloaded from the GISAID EpiFlu database and digested in silico with trypsin using the FluGest algorithm. Mass trees were constructed from the mass maps using a modified MassTree algorithm [1] that displays mutations along branches of the tree, when visualised with the Figtree algorithm, and calculates their frequency and score.

Results: (Limit 900 characters)

The mass trees were analysed to study mutation trends and identify consecutive or near-consecutive mutations typically associated with positive epistasis. Non-conservative mutations were the focus of the study since these cause the greatest structural and functional change associated with the evolution of the virus. Consecutive or near consecutive mutation pairs were identified along interconnected branches within an average evolutionary distance cut-off. Leading or parent mutations were found to predominate within reported antigenic sites of the protein while subsequent mutations resided exclusively in different antigenic regions, or elsewhere in the protein, providing the virus with a possible immune escape mechanism. The results also enable a “small steps” evolutionary model to be proposed where the most frequent non-conservative mutations exhibited less structural, and thus functional, change [2]. This favours virus survival over mutations that involve more substantive change which could cause or risk its extinction.

Conclusions (Limit of 400 characters)

An innovative mass-based phylogenetic approach provides a reliable and robust method to study the evolution of organisms at the molecular protein level. It adopts a global view to study the interconnectedness of mutations associated with epistasis irrespective of their position in a protein. The ability to display mutations on mass trees and better predict future evolutionary trends should assist in the production of efficacious vaccines ahead of influenza outbreaks.

Novel Aspect: (Limit of 150 characters)

Mass map data, vital for protein identification in proteomics, is herein used in a phylogenetics strategy to better understand and predict evolutionary trends of any organism.

References

Interoperable and scalable metabolomics data analysis with microservices

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Life scientists working with big data such as genomics, proteomics and metabolomics are beginning to experience numerous difficulties of handling, processing and moving information that has not been experienced before. These intensive computational tasks cannot be tackled by regular laptops and workstations. The high demand for more resources and increased flexibility has even turned the well-known shared high-performance computing unresponsive.

Cloud computing together with microservices architecture offer a compelling alternative to these old models, with the possibility to instantiate and configure on-demand resources such as virtual computers, networks, and storage, together with operating systems and isolated software tools.

We have developed an architecture which uses components for data analysis encapsulated as microservices connected into operating computational workflows. This solution provides users with complete, ready-to-run, reproducible and scalable data analysis environment that can be easily deployed on desktop computers as well as public and private clouds, without requiring special IT skills from the user.

We showcase the capability of this architecture using four demonstrators: in the first demonstrator we reproduce a large metabolomics data analysis and show how it can be effortlessly scaled up on cloud computing resources with little efficiency loss. In this case, 1092 computationally intensive tasks were performed in less than four hours which otherwise could have taken days to finish on a workstation. In the second demonstrator we provide new mass-spectrometry data from multiple sclerosis patients, and present for the first time, to the best of our knowledge, a complete start-to-end analysis of untargeted mass-spectrometry and identified novel biomarkers. The last two demonstrators describe NMR workflow and fluxomics workflows, emphasizing that microservice architecture is domain-agnostic.

The four demonstrators show the versatility, applicability and scalability of our method in metabolomics; however, the methodology can be applied to all scientific disciplines, paving the way towards for large-scale integrative science.
1267 - VALIDATION DATA PROCESSING BY ADVERSE 2.0 FREE SOFTWARE PACKAGE: ASSESSMENT OF THE ANALYTICAL REQUIREMENTS OF A GC-MS METHOD FOR THE DETERMINATION OF 16 PAHS IN SMOKED FISH

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Keywords: method validation, data processing, PAHs, smoked fish

Introduction
Polycyclic aromatic hydrocarbons (PAHs) are a wide group of carcinogenic environmental contaminants. Official methods are not set for food monitoring plans, therefore in-house developed procedures need to be validated to assess their compliance with the strict performance characteristics required by Regulation 836/2011 (1).

Methods
A new developed GC-MS procedure for the determination of 16-EU-PAHs in smoked fish was validated (2). The study was performed at three spiking levels (0.9, 3 and 7.0 µg/kg) in four different days for a total of 41 experiments. The data were processed with the in-house-developed package ADVeRSE 2.0 (Analytical methoDs Validation Results Speedy Elaboration) for the R statistical environment (3).

Results
In the European Union maximum levels are fixed in food for both, the sum of four marker PAHs, (benzo(a)pyrene, benzo(a)anthracene, benzo(b)fluoranthene and chrysene) and for benzo(a)pyrene alone (4). Linearity, precision, recovery, LOD and LOQ of a new method for the determination of PAHs in smoked fish were estimated using ADVeRSE, a free software package developed by analytical chemists and statisticians. Measurement uncertainty was also calculated applying a top-down approach. The results were comparable with those obtained with the traditional spreadsheets, confirming that the method was fit for purpose (1,2). However, using ADVeRSE, the Validation Report is produced in few seconds, overcoming tedious calculations and possible mistakes, especially when a large number of analytes is included in the method scope.

Conclusions
Thanks to the use of automated statistical tools to handle validation data, analytical chemists engaged in the official control can go back to their original job focusing the efforts to improve analytical protocols instead of studying possible approaches to uncertainty estimation or statistics methodologies to demonstrate the performances of their methods:

Novel Aspect
ADVeRSE package allows a true feedback cycle going back and forward from method optimization to validation.

References
610 - VALIDATION OF ANALYTICAL METHODS FOR THE DETERMINATION OF RESIDUES IN FOOD: THE STUMBLING BLOCK OF DATA PROCESSING

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Keywords: method validation, data processing, residues, food control

Introduction
Starting from the late 90s, the ISO accreditation standard along with the EU rules required a great effort to validate methods and to process the relevant data in order to demonstrate the fitness for purpose of internal developed procedures. Today, this aspect is even more critical because, thanks to MS detectors, multiclass methods including dozens of analytes are currently applied.

Methods
The free software package called ADVeRSE (Analytical methoDs Validation Results Speedy Elaboration) has been developed in R environment [1]. ADVeRSE produce the Validation Report processing the validation data put into a predefined Excel file. In the same file the legal requirements fixed for each coupling analyte/matrix (maximum permitted levels) are inserted, too.

Results
The first part of Validation Report (.doc) generated by the ADVeRSE package includes the descriptive statistics of results, i.e. mean recovery and precision at each validation level for each analyte. Linearity (linear regression) is assessed applying Mendel and lack-of-fit tests. In the second part, the precision is studied as a function of concentration; limits of detection and quantification are estimated using the results obtained at the lowest concentration of the validation study. With regards to the assessment of compliance with legal limits, the measurement uncertainty is estimated applying the top-down approach [2]. Finally, according to the specific requirements stated by Commission Decision 2002/657/EC (veterinary drug residues in food), the limit of decision (CCα) and detection capability (CCβ) are calculated both for banned and permitted substances [3].

Conclusions
The created Report allowed the overcoming of the long dataprocessing saving time and minimizing calculation mistakes. The few seconds required for its generation favor an immediate evaluation of method performance characteristics helping the analyst to eventually improve her/his analytical procedure or the experimental design adopted for the validation study.

Novel Aspect
This is the first package able to produce a Validation Report usable in the various legislative contexts of the official control of residues in food.

References
The Comprehensive R Archive Network. https://cran.r-project.org
Keywords: FTICR, GC, complex mixtures, data analysis, algorithm

Introduction:
The coupling of gas chromatography (GC) to a Fourier transform ion cyclotron mass spectrometer (FTICRMS) affords additional information about complex mixtures. Co-eluted components can be resolved due to the ultrahigh resolving power, also allowing extracted ion chromatograms (EICs) to be used for the observation of isomers. There is a current need for data analysis tools for processing these datasets, aiding data analysis, and extraction of key information.

Methods:
A petroleum-related sample was analyzed using GC coupled with FTICR MS. An algorithm was used for pre-processing of the data. Peaks were filtered with respect to m/z and retention time (RT) and an averaged list was generated prior to compositional assignments using the petroleomics software, Composer. The algorithm was subsequently used to incorporate the assignments into the processed data, generating a series of interactive plots.

Results:
The typical workflow for this type of data is based upon manually merging data over a range of retention times, extraction of a peak list, performing the compositional assignments, visualizing the results, and repeating for each retention time range. An environmental sample from the oil sands region of Alberta, Canada, was used as a testbed for a new algorithm, which was developed using the open source R language, coupled with the array of tools offered by the Tidyverse and Shiny packages. The algorithm was used to process the dataset, monitor peaks with respect to m/z and retention time, filter peaks of inconsistent m/z, and generate an averaged peak list of all the EICs detected. The data was then visualized according to heteroatom class, carbon number, double bond equivalents (DBE), and retention time. The algorithm also affords the abilities to screen for isomeric contributions and to follow homologous series and compound classes, instead of individual components, as a function of time.

Conclusions
Using a new algorithm, a time-consuming workflow has been replaced with one taking only a few minutes. It has become possible to follow both individual components and compound classes as a function of retention time, without the necessity for averaging multiple time windows and the accompanying loss of information. The algorithm is not only suitable for application to GC data, but also data produced using other separation techniques.

Novel Aspect:
New algorithm developed in R to process and visualize complex mixture data, acquired using hyphenated ultrahigh resolution mass spectrometry.
Introduction:
The desire to extract mass spectrometry data from commercial systems is becoming increasingly popular. Customers of such systems wish to use data in bespoke applications and incorporate data into existing repositories. An Application Programming Interface (API) is proposed that allows access to raw and processed mass spectrometry data contained within the instrument platform. An external application using the API to create a library of compounds is discussed.

Methods:
A representational state transfer (REST) API uses HTTP requests to move, change, create and delete data. Data acquired on an ion-mobility enabled instrument for a selection of 500 natural products were processed in the UNIFI mass spectrometry data system. An application, written using the UNIFI API, was employed to automatically interrogate the processed data, then to aggregate the reduced outcomes, to produce a library of compounds.

Results:
Manual analysis and collation of mass spectrometry data to create a library of compound information is a laborious task that is prone to error. An application, external to the UNIFI mass spectrometry data system, has been written that accesses data through the UNIFI API and automatically extracts processed data to produce compound library content. The library content produced by the application is rich in content and can optionally consist of retention times, multiple adducts, together with their individual collisional cross section measurements, and fragment ion m/z values which have been generated using an insilico structure fragmentation tool and matched to measured high energy ions. All quantities derived can be averaged over multiple replicate injections. The application transforms a manual process, which often takes weeks or even months, and is open to transcription errors, into an automated process that can be performed error-free in a matter of hours.

Conclusions
The UNIFI Application Programming Interface allows direct access to raw and processed data within UNIFI. A compound library generation application has been presented which accesses data through the UNIFI API and then uses the data to derive accurate library content. A library of commercially natural product compounds has been created and contains information on multiple adducts, collisional cross sections and fragment ions.

Novel Aspect:
Direct access to mass spectrometry data for use with third party applications. Automatic production of mass spectral library content through the analysis of processed data.
1026 - METABOLOMIC ANALYSIS OF HIGH DENSITY MS DATA

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Keywords: LC-MS, metabolomic analysis

Introduction:
Metabolomic analysis allows to systematically identify and quantify metabolites from a biological sample. Non-targeted analysis which refers to the analysis of all metabolites is typically performed by liquid chromatography-mass spectrometry (LC-MS). The resulting 3D-chromatogram contains thousands of features that need to be properly analysed and processed. This can only be achieved by automated computational approaches.

Methods:
MATLAB, a multi-paradigm numerical computing environment (MathWorks, Inc.).

Results:
A software for automated data evaluation was developed in our laboratory by using Matlab programming language. Besides basic chromatogram alignment, various postprocesses were designed and developed. One of them is grouping of so called “friendly ions” that consist of isotopic, adduct and fragment ions (originating from a single metabolite). Another postprocessing approach is the mass correction which is based on known background ions or identified metabolites. This allows lockmass-like correction with no measured lockmass data. The third postprocessing method is an algorithm for automated molecular peak identification. This approach is based on identification of typical fragments and adducts under defined experimental conditions. As it relies on correct determination of friendly ions, its effectivity is also affected by retention time stability and separation effectivity.

Conclusions:
The developed algorithms can help to process high density metabolomic data with improved accuracy and effectivity.

Novel Aspect:
The described algorithms take advantage of the access to raw MS data which greatly improves their effectivity when compared to current Methods:

Acknowledgement:
This work was supported by the Czech Science Foundation (project GA17-06613S).
A STRATEGY TO ANALYZE THE SPECTRAL DATASETS OF UNKNOWN COMPOUNDS USING MASS SPECTRAL SIMILARITY NETWORK

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Keywords: Informatics, Metabolomics, Natural Product, Fragmentation, Structure Analysis

Introduction:
In mass spectrometry-based small compound analysis, fragment spectra have been widely used to deduce the structures of analyte compounds. In recent years, approaches such as reference spectrum library searching and structure database-assisted spectrum matching have been used in many applications. However, systematic and high-throughput structure annotation for large scale spectral datasets is still quite difficult to accomplish.

Methods:
Mass spectral similarity network, also called as MS/MS spectral similarity network or molecular networking, is a method to assess and visualize the similarities of fragment spectra of structurally related compounds. Since similarities of fragment spectra can be correlated to the structural similarities of the corresponding compounds, this network is a useful tool to detect structural similarity of analyte compounds.

Results:
We have developed a data analysis framework employing mass spectral similarity network to organize, classify, (sub)structurally annotate and visualize the complex fragment spectral dataset of unknown compounds. Reference fragment spectra is acquired from public spectral library such as MassBank and used to create “reference” spectral similarity network. Further, in-silico fragment spectra are generated by applying fragmentation models to the subsets of chemical structure database to create “in-silico” mass spectral similarity networks. These spectral similarity networks are further divided into user-defined classes such as chemical classes, substructures, source types. The multi-layer representation of spectral similarity networks effectively visualizes and categorizes the spectra of unknown compounds in samples. Further, network informatics and cheminformatics methods are applied the network structure to extract and assign the structural information, providing the structural information of the unknown compounds.

Conclusions:
This versatile method can be applied to mass spectral datasets containing a wide range of small molecules. We demonstrate the application of this method using case studies.

Novel Aspect:
A novel mass spectral similarity-based method to detect, classify and annotate the structures of unknown compounds in large-scale datasets of fragment spectra.
A low pulse current measurement circuit for current monitor system at mass spectrometer

ZiXing He, Lihua Zhai, ChaoHui He, QingMin Zhang, Zhiming Li, Xianglong Yuan

Keywords: reverse charging; constant current source; blast pulse; Mass spectrometer

Introduction
A pulse beams at 105 particles per second have been generated by laser Resonant ionization at 10KHz. Beam current is normally measured with the aid of Faraday cup. However, the signal is difficult to be monitored by Faraday cup amplifier, which was developed to monitor the beam current at pA range. Simultaneously, a discriminator is inappropriate, due to the time of death[1] and response rates. Therefore, What has been designed is a low pulse current measurement circuit, which is used for current monitor system at mass spectrometer.

Methods
A low pulse current measurement circuit, applying synchronous charging module and reversed charging constant current source module, was designed for current monitor system at mass spectrometer. This circuit turns the strength of measuring signal into the amplitude of voltage[2]. As a modified charge integration circuit for periodic blast pulse, a reverse charging circuit is designed[3], aiming to discharge integral capacitor for the avoidance of saturation. At the same time, it requires to improve the efficiency of measurement. So, a controllable constant current source model will be designed.

Results
In circuit simulation once the integral capacitor’s voltage exceeds given threshold of 12V, the comparator will generate a pulse signal for discharging. Then the integral capacitor’s voltage will drop rapidly. At the end of discharging process, the integral capacitor’s voltage just drops to -10V. At the same time, the discharged signal is generated by the comparator as a counting signal sent to the counter. In experiments, A modified charge integration circuit for periodic blast pulse is verified by measuring the synchronous pulse signals that are generated by laser ionization. Verification results show that the experiment error is 2%.

Conclusions
By calculations, simulations and experiments, The results show that a charge measurement circuit for periodic blast pulse is very effective. It is also shown that the count rate of periodic blast pulse signals between 103 and 107periodic blast pulse can be measured by this circuit. At the same time, the circuit is effective in the continuous measurement of the periodic blast pulse signal. Measurement precision of this circuit is mainly affected by the slew rate of operational amplifier and the bypass capacitors of the input signal[4]. So, it is feasible to further improve the accuracy of measurement by perfecting the circuit.

Novel Aspect:
A modified charge integration circuit for periodic blast pulse, a reverse charging circuit is designed and dynamic range of the ion monitor is effectively expanded.

References


Introduction:
Local accumulation of amino acids and oligopeptides on a surface of mud sediments was visualized using DART imaging. A movable stage and He cone were specialized to the analyses of unstructured samples. The image of surface distribution of the building blocks of biomolecules would help to see the correlation between local geochemical conditions and abiotic amino acid evolutions, suggesting the possible requirements of emergence of life on the primitive earth.

Methods:
DART-OS (IonSense) was mounted on Q-TOF (Xevo QTOF, Waters) and Q-IMS-Q-TOF (Synapt G2, Waters). Activated helium supplied through a narrow hole (0.5 mm) ionized target molecules on a narrow surface, c.a. 2 mm², of the non-structured mud samples collected at Ege-Jigoku in Beppu, Ohita, Japan. The sample stage was moving with a constant speed in 0.2 mm/sec. DART Source temperature was set at 430°C and derivatization was also examined using DBD-Py-NCS or TFATBDMS.

Results:
Authentic oligoglycines, tetragnucine, glycylalanine, and alanilglycine dipeptides doped on the mud surface were detected with a spatial information. Heated Hegas dried and altered the wet mud surface locally, so that the position once ionized did not interfere the later ionizations at the next spot. The maximum sample size was limited to 3 cm x 3 cm due to the source configuration. The optimum distance between the sample surface and He cone was kept within 4 mm. The position of the target molecule was reconstructed by the calculation using the constant moving speed of the stage and detection times of ions corresponding to the targets. On-surface chemical derivatizations with DBD-Py-NCS or TFATBDMS worked not only for enhancing the signals but also selecting the specific structures to the amino acids or oligopeptides. Gas-phase ion chromatography in the ion mobility mass spectrometry and MSn also enhanced molecular selectivity to characterize isomeric peptides having different amino acid sequence.

Conclusions:
Unlike SIMS, MALDI, and DESI, DART ionization does not depend on the surface characters, such as electron conductivity or smoothness. DART ionization with the movable sample stage enabled us to visualize the accumulation of amino acids and oligopeptides on the unsmooth and heterogeneous surface of the unstructured mud sediment. On-surface chemical derivatization was assisted by the ionization heat and profitable to detect the signals specific to the targets.

Novel Aspect:
Amino acids and oligopeptide mapping of the heterogeneous surfaces of mud sediments was realized using DART imaging mass spectrometry.
955 - LC-MS1 METHOD FOR PROTEIN IDENTIFICATION AND QUANTITATION WITHOUT USING TANDEM MASS SPECTRA

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Keywords: proteomics, protein identification, LFQ quantitation

Introduction:
A major bottleneck of modern shotgun proteomics is limitation of fragment mass spectra acquisition speed. We present a novel workflow for protein identification based on LC-MS1 analysis with no using of tandem mass-spectra [1]. The proposed method has no drawbacks of accurate mass tags (AMT) or classic peptide-mass fingerprinting approaches.

Methods:

Results:
Here we present identification and quantitation efficiency of developed workflow. The MS1-based approach outperforms traditional MS/MS search in both number of identified proteins groups and sequence coverage for the HeLa cell line analyzed on Orbitrap Velos with 25 min LC gradient. We have shown that LC-MS1 based approach does not depend on the length of chromatographic gradient and can potentially replace traditional MS/MS approach in high throughput proteomics applications. Also, we have shown that MS1-based workflow successfully found changes in concentration of all 6 proteins from iPRG2015 data set without any false positives. For the blood plasma samples, the MS1-based approach reported concentration changes in 4 proteins which are known FDA-approved disease markers.

Conclusions:
The ability of using the developed method for short gradients with better efficiency compared with MS/MS can potentially be an interesting feature for the applications requiring high throughput analyses of clinical samples using relatively simple and inexpensive LS-MS setups.

Novel Aspect:

References
Keywords: Natural product, High resolution tandem mass spectrometry

Introduction:
Natural products are small molecules produced by organisms, including primary and secondary metabolites, which have complex chemical structures. Recently, advances in mass spectrometry (MS) with high sensitivity and specificity have enabled it to become a major tool in the analysis of natural products. However, the analysis of natural products is still challenging because of the insufficient reference data in tandem mass spectrometry. Therefore, providing a sufficient reference is necessary for the characterization of various natural products.

Methods:
We selected 1,400 natural products with the diversity of the structure. Then, we analyzed them using three different high resolution mass spectrometers including Orbitrap MS, Triple Time-of-flight (Triple TOF) MS and Quadrupole Time-of-flight (Q-TOF) MS. Tandem mass spectra were acquired at five different collision energies and they were used for the construction of spectral library.

Results:
Currently, the library contains more than 20,000 tandem mass spectra for 1,400 compounds. Comparative analysis of tandem spectral data showed different fragmentation patterns according to the structure of natural products, instrument types, and collision energies.

Conclusions:
High resolution tandem mass spectral library of natural products was constructed and used to identify unknown compounds or secondary metabolites in complex sample.

Novel Aspect:
A high resolution tandem mass spectral library for 1,400 compounds using authentic standards and purified compounds has been constructed.
404 - CHARACTERISING THE CATABOLISM OF PEPTIDES USING ION MOBILITY ENABLED HIGH RESOLUTION MASS SPECTROMETRY WITH MASS-METASITE INTEGRATION FOR DATA PROCESSING

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Keywords: Mass-MetaSite, WebMetabase, IMS QToF, Biotherapeutics, ADME

Introduction

A growing need exists to characterize and optimize the absorption, distribution, metabolism and excretion (ADME) properties of biotherapeutics. The challenge is that few software packages available to characterise the clearance and metabolic fate of biotherapeutics. Here, we mine ion mobility high resolution mass spectrometric data for the analysis of biotherapeutic drug metabolism using the Mass-MetaSite and WebMetabase software platform for processing.

Methods

The studied peptides are 14-amino acid analogues of the hormone somatostatin. They were incubated in human serum at eleven time points between 0 – 48 hr. Data were collected on an ACQUITY UPLC coupled to an IMS QToF using HDMSE. Data were processed in a prototype version of Mass-MetaSite 5.1.9 able to read data directly from UNIFI 1.9.2 using the built-in Application Programming Interface (API) and also uploaded onto the server based application, WebMetabase 3.2.9.

Results

A number of catabolites for the parent peptides were identified over the time course and could be attributed to hydrolysis of the peptide backbone, with the main catabolites identified attributed to hydrolysis of the non cyclic portion of the peptides. A measure of the ratio of structurally matched to mismatched product ions found by Mass-Metasite provides confidence in catabolite assignment through data acquired by data independent acquisition (DIA). Approximately 99 % of the parent was rapidly (<10 min) turned over generating three key catabolites with minor metabolites, possibly attributed to hydrolysis of cyclic portion of the peptide, formed over the incubation time course. The use of HDMSE showed an improvement in data quality through alignment of precursor and fragment ions in both RT and DT (drift time) thus leading to improved spectral quality and an increase in identified fragment ions over the number of false positives for several peptides.

Conclusions

Mass-MetaSite and WebMetabase were used to characterize metabolic fate of the parent peptides showing that data can be collected routinely, processed and reviewed efficiently across time courses and treatments. A tightly integrated, UPLC HRMS informatics platform with high chromatographic performance, high mass accuracy and fast scanning capabilities afforded by the IMS QToF enabled rapid and confident detection of peptides and any corresponding catabolites.

Novel Aspect:
Informatics and ion mobility HRMS workflow for detecting and reviewing peptide catabolites.
Introduction:
As the omics are rocketing we gain insight into the chemical transformations occurring in living beings. The lack of authentic standard substances hinders obtaining deeper quantitative information about these processes. In recent years, artificial intelligence is improving different fields of life. We propose an approach of applying machine learning to overcome the lack of authentic standard substances via predicting the ionization efficiencies (IE).

Methods:
Altogether, ionization efficiencies of 400 compounds were measured in electrospray ionization positive and negative mode in 29 mobile phases (altering the percentage of acetonitrile or methanol and covering a variety of the most used additives) in flow injection. For model development, descriptors from PaDEL and different machine learning approaches (multilinear regression, random forest regression and artificial neural networks) were used.

Results:
For both ionization modes, universal models over all mobile phases were developed. For positive mode regularized random forest regression gave the best model with a residual standard error of 0.54 logIE units and R² 0.82. For negative mode, multilinear regression gave the best model with a residual standard error of 0.58 logIE units and R² 0.77.

The coefficients in these models are expected to depend on the instrument. Therefore, we propose using a set of calibrants to transfer ionization efficiency prediction models between instruments. This means analyzing a smaller set of calibration compounds (6) and samples together using the corresponding instrument and mobile phase in one batch. The relative IE values measured in this way can be used to fit a model between the logIE values observed for calibrants and descriptors. As a result, the fitting coefficient for the model are obtained which describe directly the system used to analyze the samples.

Validating this approach with gradient elution resulted in a 3.7-fold mismatch of concentration.

Conclusions
Universal ionization efficiency prediction models help to reduce the need for authentic standards with less than 4 times mismatch in concentration compared to a conventional comparison of peak areas that can result in a mismatch of several orders of magnitude. The approach only requires 2D structures of the compounds and a small set of compounds for system calibration.

Novel Aspect:
Universal ionization efficiency prediction models are developed for positive and negative ESI, allowing semi-quantitative analysis on LC/ESI/MS with high accuracy.
MSIML – AN OPEN-SOURCE PROGRAM FOR THE VISUALISATION AND PIXEL-BY-PIXEL CLASSIFICATION OF MASS SPECTROMETRY IMAGING DATASETS

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Keywords: Imaging Mass Spectrometry, machine learning, bioinformatics, DESI, software

Introduction:
Mass spectrometry imaging (MSI) is used to interrogate the contents of biologically relevant samples often directly without sample preparation. The most readily employed methods (i.e. DESI and MALDI) generate vast amounts of data [1], often making the analysis cumbersome and time-consuming. In addition, the resurgence of machine learning and deep-learning methodologies offer an opportunity to mine MSI datasets and provide a need for user-friendly and accessible software solutions.

Methods:
MSIML was developed to enable rapid access to machine learning, clustering and multivariate methods for the analysis of MSI applications. The software suite is written in python and incorporates scikit-learn for machine-learning and neupy for neural-network training. Sections of whole mouse body, kidney and brain were analyzed using DESI on a Xevo G2-XS Q-ToF (Waters, UK) to demonstrate MSIMLs capabilities in the analysis of complex MSI datasets.

Results:
Unsupervised multivariate analysis techniques is shown to greatly improve the interpretation of large MSI datasets, as well as aid differentiation of spectral and anatomical features. MSIML provides easy access to most commonly used methods including (i.e. PCA, NNMF) as well as clustering (i.e. K-means, Birch) and embedding (i.e. Spectral Embedding, t-SNE or HSNE) algorithms. However the main strength of MSIML lies in its ability to interactively label MSI pixels of multiple files, enabling building of complex supervised classification models that can be easily applied to unknown samples [2]. Here, classification models were applied to segregate healthy and tumorous regions in mouse samples and differentiate tissue components. In addition, on-the-fly classification is demonstrated in a high-throughput environment where each mass spectrum is pre-processed and immediately labelled by previously build classification models, providing the user with more information and directions for further analysis.

Conclusions:
Here, we present open-source platform (MSIML), an all-in-one program that can be used for the entire analysis workflow, including loading of single or multiple .imzML or Waters .raw files, pre-processing, spectral analysis, data mining, machine learning and sample classification. MSIML offers easy to operate GUI environment, enabling rapid interrogation of large datasets and providing valuable bioinformatics information.

Novel Aspect:
Combined machine learning classifications and multivariate analysis of known and unknown imaging mass spectrometric datasets can enhance differentiation of complex samples.

References:
Keywords: atom probe tomography, Bayesian approach, automatic peak identification, peak labeling.

Introduction
Atom probe tomography relies on the analysis of mass-spectrometry data [1, 2] and requires preliminary identification of the mass-spectrum peaks. Establishing correct correspondence between the peaks in the spectrum and the particular atomic species is crucial for accurate interpretation of the measured data [3, 4]. Manual peak labeling is a time-consuming procedure and vulnerable to errors [4]. We propose a Bayesian approach suitable for robust automatic peak identification.

Methods
We apply the Bayesian approach, previously developed for search and match analysis of X-ray powder diffraction data [5], to the problem of peak identification in atom probe mass spectra. The optimal peak association can be found as the maximal likelihood estimate of the specimen model for the given experimental mass spectrum. The designed technique is applied to the measured time-of-flight mass-spectrometry data.

Results
The Bayesian approach provides a general framework for derivation of physically grounded methods for peak identification. The initial information about the investigated specimen (e.g. presence or absence of some elements, possible correlations between presence of different ions, etc.) is encoded in prior probabilities for the sample models. The expected inaccuracies of the measurement and peak search are characterized by the likelihood function. Then, based on the measured data, posterior probabilities are calculated for the specimen models. The optimal peak labeling is provided by the most likely model (having the maximal posterior probability). By varying the probability models, one can construct different peak identification algorithms, suitable for different types of measured input data.

For testing, a peak identification algorithm based on a simple model of mutually independent inaccuracies of positions and intensities of the peaks was designed. Applicability and robustness of the algorithm was demonstrated for a number of experimental datasets.

Conclusions
The proposed Bayesian approach is universal and suitable for construction of different peak identification algorithms by taking into account different aspects of the data acquisition process. It is capable of correct analysis of overlapping peaks, of making model- and probabilities-based decisions when the mass-to-charge ratio is the same for different ions, and of taking into account possible global systematic errors (e.g. miscalibration).

Novel Aspect
Instead of “guessing” an automatic analysis algorithm, suitable for superseding manual peak labeling, we demonstrate derivation of such an algorithm from first principles.

References
Introduction: (395/400 characters)
Match Factor is a term ubiquitous in mass spectral library-searching. It is a numerical measure of similarity between spectra used to sort a hit-list of potential identifications to a query. But what happens when multiple unique hits have very similar match factors? How do you decide which is correct? Here we detail how match factors used in library searching are computed [1,2], and present a decomposition method to help interpret hit-lists when ambiguities exist.

Methods: (358/400 characters)
An EI spectrum can be represented as a vector where indices are the integral-mass of fragment-ions and values are the corresponding abundances. The masses of all peaks considered in a match factor computation is a set. This set can be decomposed into subsets: (1) matching peaks, (2) query only peaks, (3) library only peaks, (4) zero peaks. The ratio of sums of peaks in each subset can be used to interpret hit-list ambiguities.

Results: (898/900 characters)
Simple and Hybrid match factors are computed as a function of peak intensities from the full set of peaks. This function is referred to as the modified dot product and differs from a standard dot product of two vectors in that the input values are rooted and the function output is squared and then scaled by 999. The difference between simple and hybrid match factors is in defining the subset of matching peaks. In hybrid match factors, this subset includes peaks from both spectra that differ by the molecular mass difference between the two compounds in addition to peaks that share the same mass.

When library spectra give the same match factors to a query, the ratio of sums from each subset of peaks can be used to discriminate hits. For example, a match factor of 800 due to peak intensity variability reducing the sum of matching peaks but with no unmatched peaks is more likely to be the correct identification of the query than the same score generated from a pair with many unmatched peaks in either the query or library spectra. Several example scenarios will be discussed.

Conclusions (352/400 characters)
Mass spectral library search algorithms use computed match factors to sort potential identifications of a query spectrum. When two or more hits have similar match factors, simply choosing the highest match factor may be incorrect. This presentation details a method of further decomposing match factors to assist analysts in this situation. An R implementation of this method is available through correspondence.

New Aspect: (138/150 characters)
The decomposition of peaks into subsets is a novel method of analyzing match factors and greatly improves the interpretation of ambiguous library search results.

References:

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TOWARDS AUTOMATED REPORTING AND REVIEW OF METABOLIC PATHWAYS FOR LARGE MULTIMODAL MSI STUDIES

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Keywords:
MSI, automated data characterisation, metabolism, cancer

Introduction:
Enormous effort has been made to understand the metabolic hallmarks of cancer [1]. MSI techniques are promising tools to visualise the spatial distribution of the molecules that constitute the metabolic pathways altered in cancer. Identifying and characterising which molecules can be measured is crucial to study their interactions. An automated tool to search the HMDB [2], collect and organise varied information regarding these molecules was implemented.

Methods:
Either the list of detected masses or those identified as drivers of a multivariate analysis or machine learning classification result can be fed to this tool. The input is compared to a list of adducts generated from all molecules present in the database. For each potential assignment, information such as name, metabolic pathways in which it is known to be involved, and tissues in which it has been found, for example, are collected and organised for further exploration.

Results:
This tool outputs lists of potentially measured metabolites or potential drivers of segmentation or classification results, as well as estimates of the metabolic pathway coverage (i.e. percentage of pathway constituents that can be measured), for example. Its outputs can be used to: (1) compare data acquired from the same sample but with different MSI techniques or with the same MSI technique but at different sites; (2) adjust the acquisition protocol to either maximise the number of measured molecules or to focus on a particular set of molecules (e.g.: those expected to be crucial to understand the particularities of metabolic alterations happening in tumours and surrounding tissues); and (3) restrict data post-processing analyses to particular sets of molecules. This tool will be part of an automated data processing pipeline developed to cope with the large amount of data generated by a 5-year multisite, multimodal, and multiscale project focused on improving our understanding of metabolic alterations occurring in various types of cancer.

Conclusions:
This automated MSI data characterisation tool can be useful not only for optimisation of MSI acquisition protocols, but also to understand the usefulness of MSI techniques to investigate biologically relevant questions, in particular, those leading to a better understanding of cancer-specific metabolic alterations.

Novel Aspect:
Automated collection and organisation of metabolic information available in MSI data, highly relevant to study the metabolic fingerprints of cancer using MSI data.

References:
Introduction:
Computational approaches to PTM identification using tandem mass spectrometry are severely limited when dealing with many types of modifications [1]. MODplus is a practical, unrestrictive modification search tool that works well for complete proteome databases, hundreds of modifications from the Unimod [2], multiple modifications in a peptide, partially tryptic peptides and so forth.

Methods:
Sequence tag approach significantly filters out useless peptides from a database [3], and improved spectral alignment based on aligned multiple tags rapidly identifies top N candidate peptides. The spectral alignment defines regions (called gaps) within a peptide that were not aligned with any tags but possibly contain modifications, and the mass differences defined over the gaps can be interpreted by enumerating modifications in Unimod or user-defined list.

Results:
We assessed the performance of MODplus with Q-Exactive data from human proteome (HEK293 cell lysate, ProteomeXchange: PXD001468) in terms of its search time and the number of identifications with varying the number of modifications. In case of a standard database search tool (MS-GF+ [4]), the search time scaled linearly with the number of input modifications and the number of identifications started to diminish with a dozen modifications. In contrast, MODplus search with ~700 variable modifications required only ~3-fold time than that with three modifications where MODplus search time was similar to that of the standard database search tool. Even as the search space got bigger with the number of modifications, MODplus lost almost no existing identifications (or identifications from a smaller search space) and yielded more identifications. We also demonstrated the utility of MODplus with TMT-labeled, phosphorylation enriched data (Human Skeletal Muscle, ProteomeXchange: PXD001543), which is of great interest in many laboratories.

Conclusions:
A tag-based approach could resolve many computational issues in an unrestrictive modification search. MODplus successfully addressed both scalability and reliability challenges in searching for hundreds of modifications from human proteome datasets such as HEK293 cells and TMT-labeled phosphorylation enrichment. MODplus can be easily integrated with any search strategy or workflow to play its role as a part of a data analysis pipeline.

Novel Aspect:
MODplus dramatically increases the depth of post-translational modification discovery by mass spectrometry-based proteomics.
References:

Introduction: Use of MALDI-TOF as high-throughput hydrogen-deuterium exchange MS platform

Hydrogen deuterium exchange followed by Mass Spectrometry (HDX-MS) is a biophysical tool capable of probing protein/ligand interactions, conformational changes, and protein dynamics. Despite an increased number of applications, the expansion of the technology has been slowed by its intrinsic technical and analytical complexity (e.g., sequential online pepsin digestion and HPLC separation at pH 2.5, 0°C). Although most HDX-MS studies have been conducted with electrospray ionization (ESI), matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometry turns out to be a convenient tool for this purpose. MALDI-TOF combines the advantages of high speed of analysis and excellent sensitivity and accuracy of mass measurements with the capability to analyse peptide maps in a single spectral acquisition. With recent advancements in automated sample preparation entire HDX-MS experiments can be executed within one hour [1].

Methods: Development of an R-package for the semi-automated analysis of large MALDI-HDX datasets

We have developed an R-package to help analyse large MALDI-HDX datasets. This software facilitates the assessment of the quality of spectral segments for accurate centroid mass determination of identified peptides. It allows the user to semi-automatically validate, visualize and compare the relative deuterium incorporation of peptide features. The resulting HDX dataset can also be exported in a format suitable for subsequent statistical analysis or 3D protein structure mapping using the MEM-HDX package [2].

Results: Development of a MALDI-HDX workflow for the analysis of peptide binding interactions

Coupling MALDI with HDX has been challenging because of undesired back-exchange reactions during sample preparation, spotting and analysis. In this report, we systematically evaluated different methods utilizing MALDI coupled with a commercially available high-throughput nanolitre dispenser capable of highly accurate and precise spotting of 96 samples in less than five minutes. We establish a sample preparation protocol using sub-zero temperatures and non-aqueous matrix solution that minimize back exchange. We applied our newly developed MALDI-HDX data analysis package to demonstrate specific binding of M2 anti-Flag antibody to FLAG or 3xFLAG peptides in a mix of 9 peptide calibration standards.

Conclusions: MALDI-HDX provides accurate linear epitope mapping data

The biophysical characterization of a monoclonal antibody recognizing different linear epitopes establishes the ability of MALDI-HXMS to provide accurate peptide binding data. This approach may be extended to glycopeptide or macro-molecular interactions, provided that peptide mapping is conducted carefully in order to minimize back exchange and confirm sequence coverage.

Novel Aspect: New software package facilitates the systematic analysis of MALDI-HDX data

We present a new R-package to help analyse large MALDI-HDX datasets. We used this software to systematically analyse peptide back exchange rates and other technical challenges associated with the coupling of MALDI and HDX. This has allowed us to establish a protocol utilizing a nanolitre dispensing system for rapid, automated MALDI sample preparation. This step is an essential pre-requisite for future use of MALDI-HDX as a high-throughput platform to study biomolecular interactions.

References


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790 - HTTP://MS.CHEMINFO.ORG : THE ULTIMATE TOOL FOR MASS SPECTRA PROCESSING ON-LINE

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Keywords: MS tool, high resolution, protein fragmentation, internal fragments, contaminants,m/z deconvolution, teaching

Introduction (Limit of 400 characters without spaces, currently 220):

Cloud based services are becoming of main importance for many applications like collaborative working, large databases analysis and IoT (Internet of Things). Nevertheless, few scientific implementations are currently available online. For the last 10 years, we are developing core functionalities to allow mass spectra processing directly in the browser [1]. Recently, we compiled various tools to solve usual tasks encountered in MS research laboratories, available on the website http://ms.cheminfo.org. Those tools are open-source (MIT licence), don't require any installation and are always up-to-date (just need to reload the web page). In addition to classical tools such as calculation of isotopic distributions and simulation of fragmentation ions, the tool assists the user in finding possible molecular formulas from experimental high resolution monoisotopic mass, associated with the list of PubChem candidates. Among others, the tool allows to check for the presence of known contaminants or compounds listed in a Google Spreadsheet that can be dynamically created by the user. This tool implements a matching algorithm that compares experimental and theoretical spectra in order to generate interactive reports that can be easily shared between colleagues. These algorithms were successfully used to analyze intricate systems involving for example protein metal complexes [2] or complex ionic liquid cluster deconvolution. http://ms.cheminfo.org also contains interactive teaching oriented tools that can be used in MS related lectures.

Methods (Limit of 400 characters without spaces, currently 77):

The libraries are implemented in JavaScript and available on GitHub. The main library can be downloaded on https://github.com/cheminfo-js/molecular-formula. The code is thoughtfully tested using jest and each commit is automatically processed in order to check for any broken code. Currently, there are over 150 tests that cover more than 80% of the code. Code syntax is enforced by over 200 eslint rules, that yields to a code easy to read, maintain and release under a MIT license.

Results (Limit of 900 characters without spaces, currently 411):

Several tools available on http://ms.cheminfo.org were unified and recently updated. These applications are daily used in our mass spectrometry facility of ISIC (EPFL), for a wide range of applications and projects. Among others:
Core function to generate isotopic pattern distributions. It can be calculated from a molecular formula or a chemical structure (Drawn or imported), with the possibility to select isotope abundances and charges. Based on the customizable FWHM, the system generates the theoretical isotopic distribution that can be compared with experimental data and exported as high quality figures (SVG).
Application to determine possible molecular formulas for a specific monoisotopic mass acquired by high resolution mass spectrometry (HRMS). It is possible to specify the molecular formula range for a single monoisotopic value but also to specify groups of atoms that, when combined, generate multiple combinations that will be matched and compared with experimental data. The system allows as well to specify the ion types (polarity, number of charges, adducts, etc.).
Easycont to track classical contaminants inherent to the sample preparation such as detergents (Tween, SDS, Triton...), polymers (PEG, PPG...), contaminants from containers (phthalates, plasticisers) and solvent interferences. The experimental mass spectrum can be rapidly screened against the updated ms.cheminfo.org database of contaminants. Easycont browses the results ordered by similarity (%) and gives information about each identified contaminant.
Apm2s calculates theoretical fragment ions (a, b, c, x, y, z and internal fragments) from a given protein/peptide sequence with any user defined modification such as post translational modifications, chemical modifications, non-natural amino acids, ligands or metal ions bound to the protein, and matches each individual theoretical isotopic pattern to the experimental mass spectra to provide a list of matches with similarity score. Very recently [2], this exhaustive Apm2s matching tool provided an abundance of data which gave critical insights into cisplatin-ubiquitin binding.

MS/MS fragmentation is a virtual and interactive fragmentation tool. Bonds can be selected from any chemical structure with a mouse click and all fragments and combinations of them are generated and compared to the experimental data.

The new deconvolution tool consider an experimental mass spectrum as a linear combination of spectra simulated from expected molecular formulas and this is achieved using SVD (Single Value Decomposition). This algorithm can deal with molecular formulas that yield to overlapping spectra, e.g. M+ and M2++. For example it has been used to shed light on the different clusters and their relative intensity in a complicated mixture of ionic liquids.

Conclusions(Limit of 400 characters without spaces, currently 98):
Contrary to beliefs, JavaScript proves to be suitable for creating fast and efficient scientific tools from any Web browser and indeed are competitive with most vendor’s applications. Many different analytical tools were gathered on the ms.cheminfo.org website such as isotopic pattern distributions generations, molecular formula finder from monoisotopic mass and several advanced tools to compare the experimental data to the automatically predicted isotopic distributions of thousands species. The applications associated with this automated workflow are endless and can certainly be of extremely valuable help for any user with internet access having complex high-resolution mass spectra data to analyze.

Novel Aspect(Limit of 150 characters without spaces, currently 72):
Complex problems involving MS measurements can not necessarily always be solved using commercial solutions. In top of that, commercial softwares are often expensive, inflexible, and their installation and upgrade procedures are generally time-consuming. In order to use the versatile ms.cheminfo.org toolbox, the only mandatory application is a web browser already installed on a computer. The free and open source applications of ms.cheminfo.org are in constant development, quickly evolving with the researchers requirements.

References:
COMPARISON OF HIGH- AND LOW-RESOLUTION MS DATA FOR DIRECT TISSUE PROFILING: MOVING FROM A LABORATORY INTO THE CLINICAL PRACTICE

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Keywords: clinical application, neurosurgical support, profiling.

Introduction:
High-resolution mass spectrometers are widely used in research facilities around the globe, but applying mass spectrometry to clinical use requires reproducing the results on the low-resolution instruments. But in practice high- and low-resolution spectra obtained from one sample are not so similar as one could expect. In our work we analyze feature selection approach to determine features that are common for the different resolution spectra.

Methods:
All experiments were performed on Thermo LTQ Orbitrap XL instrument using the direct method of extraction of lipids from brain tumor tissues followed by ESI. Multiple switching between Orbitrap and LTQ analyzers allows obtaining high-resolution spectra as well low-resolution ones almost simultaneously from the one sample.

Results:
Both types of spectra were processed through the identical data analysis pipeline for denoising, aggregation, normalization, peak picking and peak alignment. Two datasets were then aligned to each other and analyzed. The low-resolution spectra contain about one-third of the peaks, detectable in high-resolution spectra; however, all major peaks are found in both types of spectra. Analysis of peaks selected as features to distinguish the tumor tissue from boundary brain tissue demonstrate that vast majority of selected features were preserved in both types of spectra. Moreover, we have created a mapping scheme, which allows us to use a classifier trained to separate tumor samples from non-tumor brain samples on high-resolution spectra with data from low-resolution spectra with a minor decrease in accuracy.

Conclusions:
It was demonstrated that tissue profiling and identification techniques developed on the high-resolution mass-spectrometers could be transferred to low-resolution instruments that are commonly used in routine application.

Novel Aspect:
It is shown that low-resolution spectra preserve distinctive features of brain tumor samples and could be used for sample classification in clinical settings.
Introduction:
Electron ionization (EI), which accompanied by extensive fragmentation, is the most popular method used in gas chromatography-time of flight mass spectrometry (GC-TOFMS). The compounds are identified by mass spectral database search. On the other hand, the field ionization (FI) is also available in GC-TOFMS, which can obtain the molecular information from accurate mass. We have developed the software to integrate the GC/EI and GC/FI data obtained from GC-TOFMS.

Methods:
The commercially available vinyl acetate resin was used as a sample for GC/EI and GC/FI analysis. The GC-TOFMS, (AccuTOFTM GCx-plus, JEOL) combined with a pyrolyzer (PY-2020iD, Frontier lab) was used for data acquisition. The thermal decomposition products were injected with sprit mode (100:1) and separated using GC capillary column DB-5msUI. The EI/FI combination ion source was used for GC/EI and GC/FI measurement.

Results:
The data integration of GC/EI and GC/FI was performed with following steps. (i) The GC peaks were detected from GC/EI and GC/FI data separately. (ii) The mass spectra were extracted for each GC peak. (iii) Database search was performed for extracted GC/EI mass spectra using NIST mass spectral library. (iv) The mass spectra from GE/EI and GC/FI data were linked using retention time information. (v) The accurate mass analyses were performed by using mass of molecular ion shown in GC/FI spectra and the masses of fragmentation ions in GC/EI mass spectrum. The one useful point of the software is the confirmation of database search results with GC/EI mass spectra by molecular information of GC/FI spectra. The other is to support analysis of unmatched compounds by database search. Both elemental compositions of molecular ion and fragment ions, which represent substructure, can be estimated by accurate mass from GC/FI and GC/EI mass spectra, respectively.

Conclusions
We have developed the software to integrate high mass resolution GC/EI and GC/FI data. The functions are tested using GC/EI and GC/FI data obtained by Py GC-TOFMS analyses for vinyl acetate resin. The software is useful to overview the sample chemical information in complicated mixture.

Novel Aspect:
Development of integration software for GC/EI and GC/FI ionization data acquired by high mass resolution time-of-flight mass spectrometer.
Introduction:
The analysis of complex mixtures from energy related mixtures has been pushed forward during the last years by the development of powerful mass spectrometric machinery that is not only capable of producing ultra-high resolving and accurate mass data, but that is also of high sensitivity. Still problematic, is simple and coherent data analysis that allows to gather the relevant bits of data from the enormous variety of numbers acquired during a typical measurement.[1]

Methods:
Vendor software that enables the complete analysis and non-targeted assignment of complex FTMS spectra from crude oil or biofuels, especially in a time-resolved manner, is to date not available. Therefore a mixture of third party software and self-written tools needs to be applied. An essential part during data analysis is adequate grouping and visualization of the results to convey the necessary information.

Results:
The first step in data analysis typically involves the assignment of elemental compositions to each peak within a given spectrum. This step can be aided by the use of Kendrick mass scale[2] and the fact that within crude oil related samples one typically finds broad homologous series of compounds where the repeating units are either CH2 and/or H2. A first step in data accessibility involves grouping into so called heteroatom classes which define the amount of heteroatoms per molecule. For visualization and reevaluation of the data, as well as for the comparison of different datasets, several tools have been developed that first transfer the data into heavily structured MS Excel workbooks. From here graphs with varying degrees of detail can be easily created. An additional tool enables a straightforward overview of heteroatom class distributions by plotting the findings into a hexagonal grid.[3]
Most challenging is the handling of time resolved data for which a lab-built database approach was devised.

Conclusions:
Mass spectra of crude oil related samples reach an average peak density of 100-200 signals per mass unit over a range of 100 ≤m/z≤ 1200. For an aim-leading interpretation of such spectra not only mass resolution and accuracy are of utmost importance, also data interpretation and representation play a vital role. A broad and flexible set of visualization tools helps understanding the composition and the chemistry of such complex samples as crude oils.

Novel Aspect:
New software tools were developed to ease up the data analysis and visualization pathway of complex crude oil samples.

References
Organophosphates (OPs) are widely used as insecticides. Some of the most toxic organophosphates have been used in warfare causing thousands of fatalities. Acute and chronic exposure to organophosphate insecticides can cause adverse health effects. The mechanism of delayed toxicity is thought to involve off-target inhibition of serine proteases [1], although the precise molecular details remain unclear due to the lack of an analytical method for global detection of proteins that have been covalently modified by organophosphates.

Methods:
A novel mass spectrometry method was developed to identify OP-adducted proteins in a non-targeted fashion. Human plasma was incubated with a mixture of isotope labelled and unlabeled OP pesticides. Proteins were extracted, digested and analyzed by LC-MS/MS to detect “twin ions” of peptides adducted by OPs. The LC-MS/MS data was processed by a blended data analytics software, which can identify the amino acid residue of proteins modified by the labelled OPs [2-4].

Results:
We found that proteins can be modified covalently by OPs by both trans-methylation and phosphorylation. Transmethylation at Glu, Asp, Cys, His and Lys residues are identified as major reactive pathways which have not been reported in the literature to date. Seven protein targets of OPs from human plasma were identified. These amino acid residue target sites were also confirmed by NMR by reacting four commonly used OP insecticides with single amino acids.

Conclusions:
We discovered a new non-selective reaction pathway between OPs and human proteins. This transmethylation reaction may be responsible for the mechanism of delayed toxicity owing to organophosphate exposure. It is anticipated that this information will be useful for revealing the mechanism(s) of organophosphate toxicity.

Novel Aspect:
We report the first non-targeted identification of the proteins that react relatively non-specifically with OPs. This method is more reliable and efficient compared to the traditional pull-down method [5]. Transmethylation is identified as a new reaction pathway in addition to the well-known phosphorylation mechanism for OP-protein interactions.

References
721 - AUTOMATIC IDENTIFICATION OF METAL-BOUND BIOMOLECULES USING SNAP-LC, IMAGING, AND 2D-MS

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Keywords: FT-ICR MS, isotope analysis, LCMS, cancer therapeutics, data analysis

Introduction:
The majority of chemotherapy utilizes metal-based compounds to preferentially attack cancer cells. Proteomic investigations of compounds and effects on cellular components is widely sought after to understand the mechanisms of action for potential drugs, but difficult to interpret.1,2 To accurately identify and assign these species we present a new method to reveal metallo-drug-modified species within complex data sets such as LCMS runs, MS imaging, and 2DMS.3

Methods:
The SNAP Algorithm (V2.0), coded inside Bruker Data Analysis software (V4.0, 4.1, 4.2, and 4.3) was used to peak pick acquired data sets. SNAP-LC and MSi was coded using the internal visual basic environment inside DA to automate peak picking and processing.

All spectra were acquired using a 12T SolariX FT-ICR MS. LC-MS and MS/MS analysis was acquired using an EASY nLC II system coupled to a micro-ESI source, and used a water-CAN separation gradient and 15cm C18 nLC column.

Results:
SNAP-LC analyses each and every scan from large LCMS acquisitions and produces a “metal chromatogram” of the specified metal searched.3 This method was successfully applied to tryptic digest samples containing Pt,2 Os,4 and Ir metallo-drugs. SNAP-LC was then applied to a proteomic investigation of an osmium metallo-drug administered to a human ovarian cancer cell line, successfully identifying four modified drug target species within ~10,000 biomolecules observed, automatically and accurately.

SNAP-MSi reveals not only the location of all metal containing species (in a similar fashion to LA-ICP MS) but also the speciation information of the analytes detected, such as free drug identity or metal modified biomolecule, providing detail on drug targets and transformations. SNAP analysis of 2D data sets was of particular interest due to their complex nature and diagonal dependence of isotopes with respect to precursor and data point location.5

Conclusions
Accurate analysis of complex data sets is the key to understanding large biological systems, but is hindered by sample complexity and the ability to differentiate species of interest. SNAP-LC, MSi, and 2D analysis allows us to exploit the unusual isotopic patterns of metal chemotherapy agents and accurately identify drug targets in complex samples regardless of prior identity. These techniques are also applicable to such as halogenated species, pesticides, etc.

Novel Aspect:
Exploitation of metal isotope patterns using SNAP analysis reveals cancer drug biomolecule targets and drug transformations in biological samples.
References


Introduction:
Rapid and reliable bacterial typing is an urgent requirement for public safety [1]. Matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) is now widely used to analyze bacterial samples [2]. Identification of bacteria at species level can be realized by matching the mass spectra of samples against a library of mass spectra of known bacteria. Nevertheless, identification accuracy should be further improved [3].

Methods:
We propose a new framework for more accurate MALDI-MS based bacterial typing. This framework includes two steps. First, spectral pattern matching is applied to figure out the candidates of identification results, where we introduced two new measures for spectral similarity. Second, the identification results are further assessed by a novel bootstrapping model [4] to provide more reliable characterization.

Results:
The performance of the framework was evaluated with a general dataset containing the mass spectra of 1741 strains of bacteria and another challenging dataset containing 250 strains, including 40 strains in Bacillus cereus group that were previously claimed impossible to be resolved by MALDI-MS [5]. Compared to the commonly used cosine correlation [6], our new similarity measures showed better performance. With the help of the bootstrapping assessment, the sensitivity of bacterial identification at the species level increased from 41% to 88% at the same error rate (5%) on the data set containing 1741 strains. Our approach also enabled to resolve the Bacillus cereus group.

Conclusions
This framework could provide much more reliable bacterial identification at both the genus and species level with the bootstrapping assessment. The method could promote MALDI-MS based bacterial typing and may further be applied to many other spectroscopy based analytics, e.g., the MS/MS spectral pattern matching in proteomics and metabolomics.

Novel Aspect:
To the best of the authors’ knowledge, our method is the first to provide a statistical assessment to MALDI-MS based bacterial typing to improve accuracy and reliability.

References

For information please contact: scientific@imsc2018.it
Introduction: (Limit of 400 characters) (Title calibri 12)
Spectral library searching (SLS) is an attractive alternative to sequence database searching (SDS) for peptide identification due to its speed, sensitivity, and ability to include any selected mass spectra. While decoy methods for SLS have been developed for low mass accuracy spectral library, it is not clear that they are optimal to high mass accuracy spectra. Therefore, we report the development and validation of methods for high mass accuracy decoy libraries.

Methods: (Limit of 400 characters) (Title calibri 12)
Reverse and Random decoy libraries are found to be suitable for high mass accuracy SLS. The first constructs spectra by reversing and the second by randomly replacing all peptide sequences in the library while maintaining the library’s amino acid distribution. In both cases, the m/z’s of fragment ions are shifted according to the decoy sequences generated. No alteration to fragment ion intensities, peptide length, or precursor charge state are made.

Results: (Limit of 900 characters) (Title calibri 12)
The utility of Reverse and Random libraries for target-decoy SLS in estimating false positives and FDRs was demonstrated using spectra derived from a recently published synthetic human proteome project.1 For data sets from two large-scale label-free and iTRAQ experiments, these decoy building methods yielded highly similar score thresholds and spectral identifications at 1% FDR. The results were also found to be equivalent to those of using the decoy-free PeptideProphet algorithm. The Reverse method yields slightly higher thresholds presumably due to its keeping homology matches. However, differences in these methods are rather small. Using these methods for an example FDR estimation, MSPepSearch, which is a NIST-developed search software, led to 18% more identifications at 1% FDR and 23% more at 0.1% FDR when compared with other widely-used SDS engines coupled to post-processing approaches such as Percolator. An application of these methods for FDR estimation for the recently reported ‘hybrid’ library search2 method is also made.

Conclusions: (Limit of 400 characters) (Title calibri 12)
The methods reported here for determining FDR values for high mass accuracy spectra enable results of library searches to be directly integrated with those of more widely used sequence searching Methods: The availability of a reliable means for estimating these FDR values should encourage the use of SLS, which offers advantages in speed and sensitivity. It is shown that SLS can yield more peptide identifications than SDS, especially at the highest levels of confidence.

Novel Aspect: (Limit of 150 characters) (Title calibri 12)
Reverse and Random decoy methods for high mass accuracy SLS permit the merging of SLS and SDS results, increasing assignment of peptides, leading to deeper proteome coverage.

References:
Introduction: Olive mill wastewater (OMW), a by-product obtained during olive oil extraction, represents an important environmental problem for the high toxicity, the resistance to biodegradation and the high volumes processed. Instead of the pollution, OMW is a rich source of bioactive compounds, polyphenols, which could be found up to 98% of the total content of olive fruits.[1] Hydroxytyrosol is the main detected phenolic alcohol [2, 3], widely known due to the natural antioxidant and anti-inflammatory properties [4].

The aim of the present study is to explore and compare the qualitative and quantitative phenolic profile of two OMW treated by two different techniques: freeze-drying and spray-drying.

Methods: Two samples of OMW, Ava and Bava, from two different Sicilian oil mills and generated by three phase continuous systems, were centrifuged, the supernatant was dismissed while the solution was subjected to freeze-drying (FD) and spray drying (SD). The experimental conditions of these techniques were optimized.

The phenolic compounds were extracted by ethyl acetate and, finally, were analyzed by ultra-high-performance liquid chromatography-heated electrospray-high resolution mass spectrometry (UHPLC-HESI-HRMS). Results: Qualitative and quantitative phenols composition were evaluated by UHPLC-HESI-HRMS. For freeze-drying and spray drying samples were identified and quantified about eleven polyphenols. This new HRMS analyzer allows to characterize in complex mixtures small molecules with confident and rapid monitoring.

The qualitative and quantitative analysis showed an important complexity of this wastes, in which, thanks to the new HRMS analyzer, it was able to determine the high concentration of polyphenols belonging to phenyl acids, phenyl alcohols and secoiridoids derivatives. Hydroxytyrosol is the most important phenolic compounds, extracted in FD and SD samples, and the concentration, up to 18.11 mg/g (in FD OMW). This amount was determine for the first time with this high concentration. Elenolic acid and hydroxy-O-decarboxymethyl-oleuropeina aglycone were the second phenolic compounds most represented: 6.57 mg/g in Bava FD and 1.87 mg/g in Bava SD and the second one was 4.48 mg/g in Bava FD and 1.82 mg/g in Bava SD. Freeze-dry was the best methods to enrich in phenolic composition the OMW samples especially in hydroxytyrosol.

Conclusions: the possibility of using and recycling bioactive compounds from agroindustrial wastes would reduce pollution caused by the latter, and would also allow to realize pharmaceutical, cosmetic, food and feed products enriched with functional molecules. In this view, new technologies, proposed to treating of OMW, have been used to valorise these wastes as a low-cost starting material as predict by new bio economy.

The recovered polyphenols obtained by freeze-drying could lead towards better usage of by-products from olive oil production.

Novel aspect: these techniques allow to recycle the OMW, only, through physic treatment without any chemicals extraction or processes. For SD samples was added maltodextrin, wildly known as food additive, which necessary to maintain the stability over the time. Therefore, the agricultural industries could implement the FD or SD systems in order to obtain valuable health-products while containing costs, especially for SD.

References

USE OF ICP-MS-BASED TECHNIQUES FOR THE CHARACTERIZATION OF NANOMATERIALS USED IN FOOD-RELATED APPLICATIONS

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Keywords: nanoparticles, food additives, nutrient sources, single particle inductively coupled plasma mass spectrometry, asymmetric flow field flow fractionation-UV-MALS-ICP-MS

Introduction
Applications of nanotechnologies to agricultural production, food processing, and food contact materials are rapidly developing. Such applications may bring benefits but potential risks have to be assessed and excluded since, if nanoparticles persist as such after gastrointestinal digestion, they may be absorbed in the gut. In the NANO-PERSIST project the transformations and fate of food-relevant nanoparticles during human digestion and in lysosomal conditions are being studied.

Methods
In the project, carried out in collaboration with the EC-JRC, acellular in vitro tests are applied to assess the dissolution/biopersistence of nanomaterials used in the food and nutrition sector and form the basis of the risk assessment strategy for these materials. A multi-method approach is used for the physicochemical characterization of the samples, where the results of MS-based techniques (spICP-MS, AF4-UV-MALS-ICP-MS/MS) are compared with those of centrifugal liquid sedimentation (CLS) and transmission electron microscopy (TEM).

Results
Ten different samples, belonging to four material types (namely synthetic amorphous silica, titanium dioxide, iron oxides/hydroxides, and zinc oxide) were studied. The pristine materials were characterized after dispersion with a standardized protocol. Whereas TEM was essential to study particle morphology, MS-based techniques provided excellent performances for the size characterization of the materials after proper method development. In particular for spICP-MS, the use of μs dwell times combined with the use of ion–molecule chemistry for resolution of spectral interferences was essential to obtain quantitative data - with fast acquisition times - and with improved (lower) size LoDs.

Conclusions
ICP-MS based techniques are the most promising tools for routine characterization and detection of inorganic nanomaterials, also in complex matrices after proper sample preparation. Development of multi-technique approaches and combination of separation and sizing techniques appears to be the way forward for the physicochemical characterization of particulate materials used as food additives and nutrient sources.

Novel Aspect
For the first time a range of materials belonging to four different material types have been characterized with state-of-the-art MS-based techniques and the results compared with TEM and CLS.
Introduction:
The use of nanoparticles (NPs) in consumer products is not new. In our kitchen, inorganic NPs can be found as food additives with specific purposes: color or flavor enhancers, preservative agents and texture modifiers. Detection and characterization of NPs in these complex media is necessary to assess risk linked with their use; however, standardized protocols and methods are still lacking for NPs characterization in complex samples.

Methods:
Single-Particle Inductively Coupled Plasma Mass Spectrometry (sp-ICPMS) is used to measure elemental mass of individual NPs and particle number concentrations in complex matrices. To investigate matrix effects on NP measurements, we combined ICPTOFMS (icpTOF, TOFWERK AG, Switzerland), with a dual sample introduction system, in which microdroplets doped with analytes of interest are used for calibration [1].

Results:
Gold NPs were measured in diverse matrices with varying complexity, ranging from ultra-pure water to food samples (fruit juices & milk) and a buffer solution (PBS) used in biological research. In fruit juice, we observed few matrix effects—sizing of Au NPs was identical to that obtained in water. However, with Au NPs dispersed in milk or PBS, we observe an attenuation of NP and microdroplet signal intensities. Because the sensitivities of calibrant droplet and analyte NP are affected in the same way, Au NPs could be sized accurately despite this matrix effect. In contrast, without online microdroplet calibration, Au NP diameters would have been underestimated by 27% or 34% for milk and PBS matrices, respectively.

Conclusions:
Microdroplets (calibrant used to determine the instrument sensitivity) are introduced concurrently with the NP-containing sample and experience the same plasma conditions. This online calibration approach can be used to compensate for plasma-related matrix effects. This setup allows for the analysis of large volume samples, rapid switching of NP-containing samples, and calibration of a wide range of NPs.

Novel Aspect:
We demonstrate a successful strategy to detect and accurately size metallic NPs in various real samples with automatic compensation for plasma related matrix effects.

References:
Optimization of Espresso Coffee extraction with different particle size distribution and analysis through GC-MS and HPLC-VWD

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Optimization of Espresso Coffee extraction with different particle size distribution and analysis through GC-MS and HPLC-VWD

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Keywords: Espresso Coffee (EC), Particle Size Distribution (PSD).

Introduction
EC extraction depends deeply on ground and roasted coffee particles produced by the grinding and brewing process. To extract tempting aroma and taste in EC, the grinding process is a crucial step. The taste and flavor change in important manner owing to the particle size of ground coffee [1]. This research aims at optimizing the impact of different particle size on aroma and bioactive compounds.

Methods
Quantitative and qualitative analyses were carried out on Arabica and Robusta cultivars. Different particle size average (200 and 400 microns) were used for extraction. Volatiles and bioactive compounds analyses were carried out through GC-MS and HPLC-VWD, respectively. The usual espresso machine settings (9 bars and 92 °C) were kept [2].

Results
EC extraction times, kinetics of extraction and its dependence on water pressure and temperature were controlled during the analysis. Volatile compounds, determined in different particles, were divided into family groups and their characteristics were identified [3]. According to literature [4], particle size influences the amount of extracted bioactive compounds in EC. To study the effect of the particle size, EC samples were processed analyzing caffeine, nicotinic acid and trigonelline. EC extraction were in triplicate for each condition for each particle size. Results confirmed a good extraction efficiency of caffeine, which accounted for 170 mg and 90 mg per cup, on 200 and 400 microns, respectively. Hence, amount of bioactive compounds were increased extracting smaller particle sizes.

Conclusion
EC extraction of different particle sizes were analyzed for their content in bioactive components and volatiles normally found in EC. Extraction optimization will be developed by modifying of extraction variables and further studies will be conducted.

Novel Aspects
The feasibility study of particle size effect on extraction of EC has not been well established yet. Understanding particle size impact on EC will help in setting optimal extraction conditions.

References
1310 - STUDYING OF PHENOTYPIC AND PHYSIOLOGICAL INFLUENCES OF ABIOTIC STRESSORS (TiO₂ AND EXCESS IRRADIATION) ON TOMATO PLANTS.

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Keywords: environmental pollutant, Titanium dioxide (TiO₂), phytotoxicity, climate change, nanoparticle behavior

Introduction
Titanium dioxide (TiO₂) nanoparticles are widely used for skin care, food additives, solar cells, and photocatalysis. However, high demand of TiO₂ raise chance of the releasing into the environment as a pollutant. [1] Since nanoparticles including TiO₂ can arise toxicological effect based on their physicochemical activity, [2-3] studying and expecting the possible influence to the human health is important issue in recently.

Methods
Herein, we studied about influences of TiO₂ when it exposed to tomato plants as a pollutant under excess light condition, for simulating behaviors of TiO₂ under climate change. Plant cultivation was performed in hydroponic system, and phenotypic and physiological analysis were performed to monitore and to suspect any possible influences on the human health.

Results
Accumulation of TiO₂ in leaves were analyzed quantitative and quantitative measurement with STEM, XRD, and ICP-MS. Through the obtained result, we have confirmed clear evidence of TiO₂ presence based on the plant parts, i.e., high accumulation of TiO₂ was detected in leaves and root, while no storage was analyzed in stem.

Other physiological effects on the model plant were exhibited, for example, increased level of oxidative enzyme activity and antioxidant level, color and thickness change of leaves, enhancement on photosynthetic rate, and reproductivity, with respect to the abiotic stressors of TiO₂ and excess irradiation. Presence of TiO₂, especially, lead to increase of the photosynthetic efficiency by improving the activity that related with photosynthesis, such as quantum yield of PSII, electron transport rate, stomatal conductance and so far.

Conclusions
Abiotic stressors of TiO₂ and excess light induced more oxidative stress in comparison with control, and the increased stress expected arisen by direct physical damage as well as enhancement of photosynthetic efficiency. However, we confirmed raised antioxidant level, and higher growth efficiency and fruits production, of which can improve quality of fruit and fruit yield in agricultural field from the tomato plants grown under the stressful environment.

Novel Aspect
This study focused on not only phenotypic influence but also physiological effect of tomato plants to expect possible effect on human health.

References
1164 - MASS SPECTROMETRIC DETECTION AND CHARACTERISATION OF NANOPARTICLES

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Key words: nanoparticles, HDC, AF4, ICP-MS, spICP-MS

Introduction:
Nanotechnology is on its way to become a key enabling technology and is expected to stimulate industrial growth, innovation and development in the most diverse fields. Although potential beneficial effects of nanotechnologies are generally well described, still existing uncertainties concerning environment, health and safety aspects need to be addressed to explore the full potential of this new technology. One challenge consists in the development of methods that reliably identify, characterize and quantify nanomaterials, both as substance and in various products and matrices. Mass spectrometry plays an important role here.

Methods:
Recent applications of mass spectrometry for the identification and characterization of nanoparticles have been reviewed.

Results
Mass spectrometry, in the form of inductively coupled plasma mass spectrometry (ICP-MS) has been used in combination with hydrodynamic chromatography (HDC) to determine the presence and concentration of metal and metal oxide nanoparticles. This combination has been used to determine not only the presence of NP in environmental samples and food, but also to study the fate of silica nanoparticles following digestion. MALDI-TOFMS has been used to identify organic nanoparticles but can also be used to identify organic coatings on nanoparticles. In many occasions asymmetric field flow field fractionation (AF4) has replaced HDC as the separation method for nanoparticles. To detect and quantify nanoparticles, AF4 is often combined with ICP-MS. AF4-ICP-MS has been used to identify and characterise nanoparticles in environmental samples and in food and personal care products. A relatively new technique is single particle ICP-MS (spICP-MS) which serves as a screening technique for nanoparticles. spICP-MS determines the particle size and the particle number- and mass-based concentration. The attractive feature is that spICP-MS determines a number-based size separation, very much in line with European recommendation for the definition of a nanoparticle. spICP-MS can also detect nanoparticles at very low concentrations which is an advantage in sample processing. Hyphenation of spICP-MS with separation techniques is also studied. The combination of HDC and AF4 with spICP-MS allows for the determination of aggregates and agglomerates since HDC and AF4 determine the hydrodynamic diameter of a particle while spICP-MS determines the spherical equivalent diameter. Very recently, time of flight mass spectrometry has been introduced in spICP-MS allowing true multi-element detection and opening new possibilities.

Conclusions
Mass spectrometry is well on its way to become an important technique in the detection and characterisation of nanoparticles in the environment and in biological and food samples. Especially spICP-MS has become an useful screening method for nanoparticles and combined with electron microscopy as a confirmation method it forms a strong combination.

Novel aspects
The identification and characterization of nanoparticles is a new area of research and one that shows many novel aspects.
Abstract
Edible oil production process may cause the formation of degradation products like polycyclic aromatic hydrocarbons (PAHs) or 3-MCPD esters and glycidol esters. Other contaminants include bisphenol A or phthalates from the packaging materials, mycotoxins, such as aflatoxins (AFs), ochratoxin A (OTA), deoxynivalenol (DON) and zearalenone (ZEA) and in some cases antioxidants which are frequently added to cooking oil for prolong shelf life. These undesired contaminants, if present in significant levels in oil, can pose serious health risk to consumers. Traditionally, these different classes of compounds are usually analyzed by different methodologies. Present study explores the possibility of analyzing all of the above compounds by a simple workflow of dSPE clean-up sample preparation followed by UHPLC-MS/MS analysis.

Methods: Limit 120 words
Oil sample obtained from local stores were extracted with methanol, followed by dispersive solid phase extraction (dSPE) clean-up. Analytes detection were via a highly sensitive PerkinElmer QSight 220 UHPLC-MS/MS system equipped with dual ESI/APCI sources.

Chromatographic separation was performed on a BrownleeTM SPP C18 (100x2.1mm, 2.7um) column reversed-phase column using a gradient elution. Under the optimized conditions, the method was validated in terms of its specificity, sensitivity, linearity, accuracy, and precision. Recoveries from sample matrix were evaluated by fortifications of analytes at three different concentrations.

Preliminary results: Limit 300 words
Calibration curves were built by preparing several concentration levels of analytes standards in oil sample matrix (matrix-matched calibration) to overcome any matrix effects. The calibration curves obtained for all analytes were linear over three orders of magnitude with a R2 value greater than 0.990. The LOQs for these analytes ranged from 0.1 to 500µg/L.

The mean recoveries of those analytes from six samples spiked at different concentration levels were between 70% and 120%. The intra-day and inter-day variations, expressed as RSD, were less than 8%, respectively. The results demonstrated that the accuracy and precision of the present method were acceptable for routine monitoring purposes.

Identification and confirmation of the analytes in investigated real sample was achieved by comparing their respective retention times and peak area ratios between the quantifier and qualifier MRM transitions with those from the standards. Overall, the method developed in this study is simple, rapid, sensitive and robust.
performance indicates that such unified method can be applied to determination of certain undesired substances in edible oil.

Future work would include further development of LC method for better separation which includes optimization of mobile phase conditions and MS parameters optimization for better sensitivity.

Novel Aspect: Limit 20 words
Simultaneous determination of different classes of undesired substances in edible oil by high pressure LC-MS/MS.
150 - DEVELOPMENT AND VALIDATION OF AN LC-MS/MS METHOD TO QUANTIFY FUMONISINS AND HYDROLYSED FUMONISINS IN PORCINE SERUM

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Keywords
mycotoxin, fumonisin, serum, LC-MS/MS, 13C-labelled standards

Introduction
Fumonisins are mycotoxins that negatively impact animal and human health. The predominant form is fumonisin B1 (FB1), followed by FB2 and FB3. A novel feed additive, containing fumonisin esterase (FUMzyme®), can enzymatically remove the tricarballylic acid groups of fumonisins [1], dramatically reducing their toxicity [2]. We aim to quantify toxins and reaction products in biological matrices.

Methods
We describe an analytical method for the quantification of FB1, FB2, FB3 and all their fully (HFB) and partially (pHFB) hydrolysed forms in pig serum. Due to their low bioavailability, the concentration of these twelve analytes in serum is in the low µg/L range. The sample extraction with acidified methanol-acetonitrile mix was optimised, prior to analysis by HPLC-ESI-MS/MS in MRM-mode.

Results
To compensate for losses during sample preparation and for matrix effects in ESI-MS/MS analysis, 13C-labelled internal standards (IS) proved necessary. They were generated by enzymatic hydrolysis of fully 13C-labelled FB1, FB2 and FB3.

Moreover, the chromatographic separation had to be fine-tuned, especially for pHFBA/b and pHFB3/a/b: FB2 and FB3 are positional isomers and fragment ions of sufficient intensity are not isoform-specific.

The optimised method was validated by spiking the twelve analytes into blank serum in ten different concentrations on three different days in triplicates.

The overall recoveries were between 85 and 110% for all analytes. In contrast, recoveries without IS-correction were only between 40 and 75% for FB2, FB3 and their pHFBS. The limit of quantification was between 0.14 and 0.67 ng/mL, except for FB1 and FB2 (2.9 and 1.5 ng/mL, respectively).

Conclusions
The quantitative determination of biomarkers in different biological matrices is a prerequisite for detailed analysis of animal health studies.

The validation data demonstrate the usability of the developed analytical procedure and measurement method for accurate identification and quantification of fumonisins and their hydrolysis products in pig serum.

Novel Aspect
A validated method to analyse FB1, FB2, FB3 and their hydrolysis products in serum, using enzymatically produced, 13C-labelled internal standards.

References
Identification of Synthetic Food Dyes by Nanoelectrospray Ionization

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Keywords: dyes, food additives, mass spectrometry, nanoelectrospray ionization

Introduction:
Food color is an important parameter making a product more attractive for customers. Food dyes, natural or synthetic, are commonly used for compensation of the loss of natural colors due to the processing or storage of food. Synthetic dyes are strictly regulated. In adulterated food amount of dyes can exceed permitted limits or dyes are not mentioned in the list of ingredients [1,2]. Identification of dyes by nanoESI applying simple sample preparation is presented.

Methods:
Experiments were carried out using a Xevo TQD triple quadrupole mass spectrometer (Waters, Manchester, UK). The commercial inlet of instrument was replaced by a custom-made inlet with a sample cone and a heated capillary allowing nanoelectrospray ionization (nanoESI). Nanoelectrospray tips (2 ± 1 µm I.D., PicoTips emitter, New Objective, Woburn, USA) were filled with sample solutions. Standards of fifteen synthetic food dyes were obtained from Sigma Aldrich (Prague, Czech Republic).

Results:
Food dyes were successfully ionized by nanoelectrospray ionization (nanoESI). NanoESI was applied for its tolerance to components of matrix and lesser contamination of a mass spectrometer due to significantly smaller flow rate of sample solution in comparison to electrospray. Mainly sodium adducts [M+Na]+ or [M-Na+2H]+ were observed in spectra of dyes in positive mode. Better ionization was observed in negative mode, typical ions [M-Na]-, [M-2Na+H]- (generally [M-nNa+(n-1)H]-) and [M-3Na+H]2-, [M-3Na]3- were formed. Simple dissolution of dyes from real food samples was applied. For example, the presence of declared dye brilliant blue FCF was confirmed by nanoESI and MS/MS experiment in blue candy M&M’s after its dissolution in methanol:water (1:1, v/v).

Conclusions:
NanoESI allowed analysis of synthetic food dyes with simple and fast sample pretreatment. Typically better ionization efficiency was observed in negative mode. Applicability was confirmed by identification of dyes in real food samples.

Novel Aspect:
NanoESI and MS/MS experiments represent a useful tool for simple identification of synthetic food dyes in food samples.

Acknowledgment:
The authors gratefully acknowledge the financial support from the Ministry of Education, Youth and Sports of the Czech Republic (LO1305) and Palacký University in Olomouc (IGA_PrF_2018_027).

References:
DEVELOPMENT OF A RAPID METHOD FOR ANALYSIS OF HETEROCYCLIC AROMATIC AMINES, BY QUECHERS EXTRACTION AND UHPLC-APCI-MS/MS ASSAY.

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Keywords:Heterocyclic Aromatic Amines, QuEChERS, UHPLC-APCI-MS/MS, meat, cooking

Introduction:
Heterocyclic Aromatic Amines (HAs) are neo-formed compounds during the cooking of meats and are known or suspected to be mutagenic and carcinogenic for rodents and man. More than 20 HAs have been classified according to their structures and physico-chemical properties. They have been characterized at concentrations in the ng/g (ppb) range in most meat cooked under normal time and temperature conditions, but the analysis procedure is still long and tedious.

Methods:
We present the development of a methodology for the quantification of 16 HAs by UHPLC-APCI-MS/MS after QuEChERS extraction and SPE purification of beef meat samples. Protocols, using an internal standard, were optimized in order to reach quick analysis, a good repeatability and a maximum recovery yield for each HAs. Quantification was achieved by UHPLC-APCI-MS/MS (MRM mode) on a triple quadrupole instrument by monitoring two specific transitions for each HAs.

Results:
The objective of this work was to develop a simple and fast method to overcome the traditional time-consuming extraction method of HAs using diatomaceous earth and large volumes of solvent. For that, the performance of the QuEChERS) method combined with UHPLC-APCI-MS/MS was investigated. Due to, the particular physicochemical properties of the HAs, as well as the fatty nature of the beef matrix, the classical QuEChERS method was modified, by carrying out a solid phase extraction(SPE) in mixed mode combining a polymer adsorbent and a cation exchange, for the purification of the extract in order to minimise matrix effects and signal extinction.

This method takes advantage of the rapidity, specificity and sensibility of both QuEChERS and UHPLC-APCI-MS/MS. Its performances will be presented in terms of selectivity, sensitivity, linearity, carry-over, recovery, matrix effect, repeatability, accuracy and intermediate precision in doped beef meat extracts (15% fat), according to the European Medicines Agency (EMEA) validation guidelines.

Conclusions:
An original methodology has been developed to quickly and easily assess 16 HAs in complex beef meat matrices by UHPLC-APCI-MS/MS. After validation, this method will be applied to implement a strategy allowing a reasoned choice of curative ingredients (marinades …) to mitigate the formation of HAs during the cooking of meat products and provide possible recommendations to minimize the formation of HAs during meat cooking and to reduce consumer exposure towards HAs.

Novel Aspect:
Easy rapid and repeatable extraction and purification method suitable for complex and fatty meat matrix combined with a sensitive and specific UHPLC-APCI-MS/MS assay method.

References:
A STUDY ON MIGRATION OF HEAVY METALS FROM CERAMICS AND GLASSWARE USING INDUCTIVELY COUPLED PLASMA MASS SPECTROMETRY (ICP-MS)

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Title: A Study on Migration of Heavy Metals from Ceramics and Glassware using Inductively coupled plasma mass spectrometry (ICP-MS)

Keywords: ICP-MS, heavy metal, glassware, ceramics

Introduction:
When ceramics and glassware come in contact with food, a migration of heavy metals such as lead and cadmium may occur [1]. The presence of heavy metals in foodstuff represents a crucial problem around the world. The risk associated with the exposure to heavy metals in food products had brought widespread concern in human health. This study investigated the migration level of heavy metals (lead, cadmium, arsenic and etc.) from food contact articles into a food simulant.

Methods:
All the food contact articles (80 ceramics and 80 glassware) were purchased in domestic markets around South Korea. The articles were eluted with a food simulant (4% acetic acid) for 24hr at 25°C in laboratory oven [2,3]. Heavy metals including lead and cadmium that migrated from articles into a food stimulant were analyzed by Inductively Coupled Plasma Mass Spectrometry (ICP-MS).

Results:
The migration levels of heavy metals in all the samples were compared with the migration limits of Ministry of Food and Drug Safety (MFDS) in South Korea.
(in progress)

Conclusions
Analytical methods were successfully validated by evaluating linearity, LOD, LOQ, precision (intra/inter-day) and recovery.

Novel Aspect:
The result of this study can be used as valuable data for the safety control of the food contact materials.

References

For information please contact: scientific@imsc2018.it
Keywords: acrylamide, LC-MS/MS, carbohydrate-rich food

Introduction:
Acrylamide (2-propenamide) is a water-soluble molecule, which is formed by Maillard reaction during manufacturing or home-cooking of carbohydrate-rich food at high temperature [1], especially during browning process at temperature higher than 120°C, in food with high level of sugar and asparagine, like potato chips, French fries, crackers, bread, coffee and biscuit.

In 2002 the International Agency for Research on Cancer (IARC) classified Acrylamide in Group 2A as a probable human carcinogen [2], for this reason European Union established mitigation measures and reference levels for reducing the presence of acrylamide in food with the Regulation2158/2017 [3].

The aim of this work was to develop and validate a method in LC-MS/MS coupled with QuEChERS approach to carry out a monitoring of processed foods in North-West Italy from 2017 to 2018.

Methods:
An aliquot (2 g) of ground rehydrated sample was homogenized with 10 ml of acetonitrile. A prepared mix of QuEChERS was added and shaken for 10 min. The organic phase was separated by centrifugation. An aliquot of upper layer was dried in water bath at 45°C and 120 mbar. The sample was dissolved in a volume of Milli-Q water and filtered through a 0,22 μm PES membrane into vial, ready for analysis in LC-MS/MS.

Analysis was performed by an HPLC Accela 1250 pump (Thermo Scientific). Chromatographic separation was obtained by a reversed-phase Kinetex Biphenyl (100 x 2.1 mm ID, 2.6μm) (Phenomenex). The eluents were 0,1% formic acid aqueous solution (eluents A) and 0,1% formic acid in methanol (eluents B), the elution was carried out in isocratic mode (total run 7 min), at flow-rate of 200 μl/min at 20°C. The injection volume was 5 μl. Mass spectral analysis was performed on a TSQ Vantage triple quadrupole mass spectrometer (Thermo Scientific), equipped with a heated ion spray interface (HESI). Two mass transitions (72.1/55.1 and 72.1/27.4) were monitored for acrylamide, providing unequivocal identification of it and only one mass transition (75.3/58.6) was used for the internal standard (acrylamide-d3).

Method evaluation included: linearity, matrix effect, recovery, precision (as intra- and inter-laboratory RSD%) and LOQ, using certified reference materials (CRM) and spiked samples.

Results:
Method validation was performed at three concentration levels: 25 μg/Kg, 120μg/Kg and 625 μg/Kg. For the first level, we used a cereal based baby food spiked with a standard solution of acrylamide, we decided to fix the LOQ at this level; for the second level a biscuit based CRM and for the last level a potato based CRM, to cover all matrices of routine samples.

Linearity was evaluated over the concentration range from 25μg/Kg to 1000 μg/Kg by constructing a calibration curve in solvent. Matrix effect was verified by comparing the calibration curve in solvent with calibration curve in matrix-post for the matrices: potato chips, biscuits, flakes and mashed potatoes.

Fourteen samples of different cereals or potato based matrices were extracted and analyzed. The occurrence of possible interferences was tested by plotting the acrylamide selected-ion chromatograms at the relative retention time interval (±2,5%) expected for its elution. Satisfactory selectivity is achieved when area peaks was under 10% of medium area peaks of sample at LOQ in the corresponding retention time windows for the quantification SRM transition.
Intra- and inter-day precision were determined by assaying six replicates of sample at three concentration levels in two different analytical batches. Repeatability was calculated by applying the Horwitz equation and the acceptable criteria was based on this equation: intra-repeatability RSD = 0,66*inter-repeatability RSD[3]. The recovery was evaluated on the same samples and ranged from 90% to 101%.

Fifteen samples of crackers, chips, baked potatoes, rice cream for children, cereals, biscuits, bread, rusks were purchased on the North-West Italian market. Baby food, bread and rusks showed values lower than LOQ; the highest concentration of acrylamide was found in potato chips (average concentration of 600 μg/Kg), the baked potatoes showed an average content of 269 μg/Kg; breakfast cereals, biscuits and crackers had concentrations from 27 μg/Kg to 54 μg/Kg.

Conclusions
The study describes a quick and easy method for acrylamide determination by QuEChERS approach coupled to LC-MS/MS.
Validation of this method fulfilling recoveries (90%-101%), and showing a good linearity (from 25 μg/Kg to 1000 μg/Kg). Precision varies from 1,43% to 6,38% of intra-repeatability and from 3,73% to 10,36% of inter-repeatability. The method was applied to food matrices such as crackers, chips, biscuits, bread, rusks and baby food. Results confirm that acrylamide was found in ordinary consumer products, this underlines the importance of these monitoring to ensure consumer health.

Novel Aspect:
There are relatively few methods in the scientific literature addressed to the analysis of acrylamide in processed food, baby food included. Our protocol distinctively combines simplicity of sample treatment but effective, applicability to very different matrices and compliance of the sensitivity requirements expected by European and Italian legislations.

References
DETERMINATION OF PESTICIDES IN FRUIT, VEGETABLES AND FEED BY MULTIRESIDUE METHOS: ONE YEAR OF MONITORING IN NORTH-WEST ITALY.

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Keywords: pesticides, cereals, fruit, vegetables, monitoring

Introduction:

Pesticides are used in various combinations at different stage of cultivation or postharvest storage, to protect crops against a wide range of pests and fungi or to provide quality preservation. Numerous monitoring studies have demonstrated the presence of pesticide residue and their degradation products (metabolites) on crops, in environments and in the diet. These residue levels are usually low, below of effective action and interaction is not expected to occur. At the same time, literature data indicate that presence of pesticide mixtures in food and feed could be a potential risk to human and animal health.

The aim of this study was to monitor pesticides in fruit, vegetables and cereals through using of screening-quantitative multiresidue methods (GC MS/MS and UHPLC MS/MS) developed and validated for selected analytes. The survey was conducted in North West Italy (from 2016 to 2017) and these regions constitute one of the main cultivation areas of Italy. In these areas the main crops were cereal grains, rice, corn, fruit, vegetables and vineyards.

Methods:

Screening quantitative multiresidue method (GC-MS/MS and UHPLC-MS/MS) were developed and validated for selected pesticides and their metabolites in fruit vegetable and cereals. The homogeneous sample was extracted with the help of acetonitrile. Samples with low content (<80%) require the addition of water before the initial extraction to get a total of approximately 10 g of water. After addition of magnesium sulfate, sodium chloride and buffering citrate salts, the mixture was shaken intensively and centrifuged for phase separation. An aliquot of the organic phase was cleaned-up by dispersive solid phase extraction (D-SPE) employing bulk sorbents as well as magnesium sulfate for the removal of residual water. Following clean-up with amino-sorbents (e.g. primary secondary amine sorbent, PSA) extracts were acidified by adding a small amount of formic acid, to improve the storage stability of certain base-sensitive pesticides. The final extract was carried out by GC- and LC-based determinative analysis.

Two mass transitions were monitored for each pesticide, providing unequivocal identification of contaminants. Detected pesticides were then quantified using triphenyl phosphate as internal standard.

Method validation was carried out according to European guideline document SANTE 11945/2015 [1]. All the parameters studied (specificity, linearity, matrix effect, LOQ, recovery, precision and ruggedness) met the criteria set in the guideline at the three different spiking levels investigated (0,010 – 0,050 and 0,10 mg/kg). Also expanded uncertainty of the result was calculated using a top-down approach as indicated in the Codex Guidelines on estimation of uncertainty of results (CAC/GL 59-2006 amendment 2011).
Results:
National monitoring results of the 2016 year have shown that nearly 48% of the foods sampled had no pesticide residues detected. Pesticide residues were detected in 52% of samples analyzed and the results showed a different distribution of active substances in the various commodity: 76% in fruit/vegetable, 33% in cereals and 80% in wine grapes/wine. The most frequently detected pesticide was fungicide and insecticide, followed by acaricide and herbicide. The study showed that pesticide residues exhibited a seasonal variation in terms of detection frequency and concentration, with spring and summer as critical seasons.

In particular N.R.L. for pesticides in cereals and feed analyzed 93 samples of feed and cereals, in 56 samples, from one to six pesticides were detected in quantifiable quantities (>LOQ 0.010 mg/Kg) but below the MRL (Maximum Residue Level).

The most common detected substances are: pirimiphos-methyl, most times associated with piperonyl-butoxide (used as preservative and not yet normated) and cis-deltamethrin. While cypermethrin, chlorpyrifos-methyl, chlorpyrifos, endrin, malaoxon, desmediphamresmethrin, permethrin, azoxystrobin, fenazaquin and difeconazole were determined with less frequency. The most contaminated matrices are feed, rice and wheat.

Conclusions:
Pesticide residues were detected in different matrices examined at values below to the legal limit. Active substances were identified in high percent in fruit/vegetable and wine/wine grapes with fungicide and insecticide mainly detected. However, for national application in monitoring programs it is necessary to develop new methods in multi and mono residue screening which cover a broad spectrum of pesticides and metabolites.

References
1. Document N° SANTE/11945/2015 implemented by 01/01/2016
Introduction:
Pesticides are widely used in food production and to control and manage various unwanted species. The detection, quantification, and confirmation of pesticide residue in food at trace levels require sensitive, selective and robust analytical instrumentation.
With ever increasing pressure to analyze contaminants at very low levels in a greater number of samples and with shorter turnaround times, laboratories seek continuous improvements in analytical instrumentation in order to maximize productivity and reduce downtime.

Methods:
This study describes an optimized sample extraction and the analysis of multi-residue pesticides in baby food using femtogram-level sensitivity on a Thermo Scientific GC-MS/MS system. Sensitive detection and quantification of pesticides in baby foods is of particular importance as infants are more vulnerable to adverse health effects from these chemicals than adults. In this work, commercially available fruit and vegetable based baby-food samples were subjected to an optimized QuEChERS extraction method to isolate the pesticide residues using acetonitrile as the final extraction solvent. Direct analysis of extracts in acetonitrile is desired to avoid the need for solvent exchange to a more GC amenable solvent. The QuEChERS extracts were then analysed for pesticide residue content using a fast, targeted, timed-SRM method for >200 pesticides (inclusive of priority pesticides). The performance of the method focused on assessing various quantitative parameters for analysis of over 200 pesticides.

Results:
The results show outstanding LODs (as low as 0.005 µg/Kg), robustness (over 900 matrix injections with no maintenance) and linear responses over up to 5 orders of magnitude (0.025 – 250 µg/kg) for the compounds investigated.

Conclusions The data presented in this study demonstrates unprecedented method performance from sample preparation to sensitive and robust GC-MS/MS analysis in addition to automated data processing and reporting capabilities.
Phthalates are emerging food contaminants linked to endocrine disruption through ingestion of foods exposed to these common plasticizers. Due to their endocrine mimicking properties, phthalates are considered as priority pollutants and various countries around the world have already introduced maximum acceptable levels in food contact materials (FCMs) for compounds such as DMP, DEP, DAP, DIBP, DBP, DXHP, BBP, DCHP, DEHP, DINP, DNOP and DIDP.

Phthalates are lipophilic in nature and the propensity for these compounds to leach from FCMs into food products such as cooking oils is significant. Oils are complex mixtures of triacyl glycerides that are difficult to chromatograph and present a challenge in terms of selectivity, sensitivity and robustness for GC-MS analysis of phthalates.

Methods:
With the innovative design of the new advanced electron ionization source however less frequency of source cleaning is required as the axial ionization in this source leads to increased ionization efficiency. This means the source filament can be operated at a reduced emission current which in turn means less ionization of complex matrices in the source reducing the cleaning interval.

Results:
The enhanced sensitivity of the new source down to low ppt levels (0.01 ng/mL) also means that the sample matrix can be diluted more or the split ratio can be increased which further reduces the amount of contamination in the GC flow path.
Here the use of a novel AEI high performance electron ionization (EI) source in conjunction with a new single quadrupole mass spectrometer provides the sensitivity and selectivity to analyze phthalates in cooking oil at low part per trillion (ppt) levels with low liquid injection volumes.

Conclusions:
In this work the Thermo Scientific™ ISQ 7000™ GC with Advanced Electron Ionization Source mass spectrometer was evaluated for phthalate selectivity, sensitivity, recovery, repeatability, robustness and linearity of response.
255 - THE FRAGMENTATION BEHAVIOUR OF PYRROLIZIDINE ALKALOIDS AND ITS IMPLICATIONS ON FOOD ANALYSIS

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Keywords: pyrrolizidine alkaloids, fragmentation, isomers, food analysis

Introduction:
Pyrrolizidine alkaloids are natural toxins which are produced by numerous plant species and may contaminate food such as honey or herbal teas. This substance group exhibits a huge structural variety, which, however, can be clustered into several classes which share common structural elements. Several of these alkaloids are isomeric to one another, thus complicating their differentiation during food analysis.

Methods:
The analysis of pyrrolizidine alkaloids is commonly performed by LC-MS/MS. Therefore, the in-depth knowledge of their fragmentation behaviour is of great importance for a selective and sensitive analysis. The fragmentation patterns of more than 30 pyrrolizidine alkaloids were studied by low- and high-resolution mass spectrometry on a triple-quadrupole and a QTOF instrument. Food samples were analysed by LC-MS/MS utilising the previously obtained information.

Results:
The obtained product ion spectra and accurate masses of the fragments of the different pyrrolizidine alkaloid species allowed the deduction of the structures of the fragments and their assignment as characteristic product ions of certain pyrrolizidine alkaloid classes. These structural relationships will be presented and discussed in detail. It was observed that isomeric pyrrolizidine alkaloids – both stereoisomers as well as structural isomers – have all the same fragments; however, the relative abundancies of the different fragments were found to vary dramatically between the isomers. For a correct differentiation between these isomers in the course of the LC-MS/MS analysis a chromatographic baseline separation is essential, otherwise the obtained quantitative results will strongly depend on the ratio of the isomers and the analytes may fail common identification criteria based on MRM ratios. Results of real-life honey and herbal tea samples will be shown that highlight the need for a careful differentiation between the isomers.

Conclusions:
A detailed understanding of the fragmentation of pyrrolizidine alkaloids is of great value for a correct analysis of food products for these compounds. Stereoisomeric pyrrolizidine alkaloid species can exhibit significantly different mass spectrometric behaviours, thus calling for a sophisticated chromatographic separation to allow a careful differentiation between them in order to obtain a true picture of contamination levels in food products.

Novel Aspect:
The fragmentation behaviour of pyrrolizidine alkaloids was elucidated in detail and its impact on the correct determination of contamination levels in food demonstrated.
In vivo efficacy of a YCW-based product in reducing oral bioavailability of zearalenone in pigs

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Keywords: Yeast cell wall; efficacy; zearalenone; toxicokinetic; piglets; biomarker.

Introduction

The use of yeast cell wall (YCW) derivatives as mycotoxin binders (MB) can be considered a promising approach to protect livestock against the harmful effects of mycotoxin exposure. Several in vitro studies confirmed the potential of YCW in binding mycotoxins. As in vitro studies do not always predict in vivo results, the European Food Safety Authority (EFSA) guidelines state in vivo trials are needed. This work was aimed to determine the in vivo efficacy of a YCW, obtained from Lesaffre International, in reducing oral bioavailability of zearalenone (ZEN) in piglets.

Methods

In order to investigate the in vivo efficacy of a YCW in reducing ZEN bioavailability, a toxicokinetic study focusing on the plasma concentration-time profiles of ZEN was performed. The study was conducted on 32 healthy 5-week-old piglets, sexes equally divided. The pigs were randomly allocated in 2 groups of 16 animals, a control group (C) (treated with a single oral bolus of 0.3 mg/kg BW ZEN) and a treatment group (T) (treated with a single oral bolus of 0.3 mg/kg BW ZEN in combination with the detoxifier).

Results

Plasma concentrations of ZEN were quantified with a validated LC-MS/MS method. Limit of detection (LOD) and limit of quantification (LOQ) were 0.1 and 0.5 ng/mL, respectively. As plasma concentrations of ZEN after oral administration were below the LOQ, ZEN-glucuronide was quantified as plasma biomarker for ZEN. Non-compartmental toxicokinetic modeling was performed using Phoenix 64 (Certara, USA). The effect of the mycotoxin detoxifier on the oral absorption of the mycotoxin was evaluated by statistical comparison of toxicokinetic parameters between the C and T groups, with special emphasis on area under the curve from time zero to infinite (AUC\text{0→∞}), maximal plasma concentration (Cmax), time at maximal plasma concentration (Tmax) and relative oral bioavailability (F).

The mycotoxin detoxifier significantly reduced oral ZEN absorption in piglets (pvalue< 0.05).

Conclusions
A YCW based product, produced at lab and industrial level, was selected after showing high efficacy in adsorbing ZEN in vitro. In vivo efficacy testing of the YCW based mycotoxin detoxifier revealed a significant reduction of ZEN oral bioavailability in piglets.

Novel Aspect
In vivo efficacy testing of a safe YCW based product to be proposed as detoxifier for reducing ZEN exposure, and consequently estrogenic effects of ZEN, in farm animals.

Acknowledgments
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EXPLOITING THE POTENTIAL OF HR-MS FOR DECIPHERING MYCOTOXIN MODIFICATION IN MICROPROPAGATED WHEAT: THE CASE OF DEOXYNIVALENOL

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Keywords: deoxynivalenol, wheat, metabolism, HRMS, untargeted

Introduction
Deoxynivalenol (DON) is a mycotoxin produced by Fusarium spp. Plants mechanisms to counteract mycotoxins include phase I and II reaction. The resulting modified forms have different structures and chemical formulas, making difficult their detection by routine analytical approaches. In this study, liquid chromatography coupled to high resolution mass spectrometry (LC-HRMS) was employed to identify DON metabolites produced by in-vitromicropropagated wheat plantlets.

Methods
Two wheat varieties (Kofa and Svevo) were grown in a DON-spiked medium. After 14 days, media and plant samples underwent to LC-HRMS analysis. DON absorption and translocation into the aerial part were evaluated. Phase I and II metabolites were hypothesized, respectively, by Sites of Metabolism (SOM) prediction and based on the known plant detoxification pathways. Their identification was achieved using accurate mass, isotopic pattern and HRMS/MS spectra.

Results
At the end of the experiment, in vitro plantlets showed no visible damages. After 14 days, a reduction of 10% and 30% of DON was observed in the growing media of Kofa and Svevo, respectively. The more efficient DON absorption of Svevo is in accordance with previous results [1]. Quantification of DON was carried on different plant organs to evaluate the mycotoxin absorption and translocation. In the plant material, an extensive conversion of the DON into its conjugate forms, among them DON-3-Glucoside (DON-3-Glu) and other analogues, was observed. Untargeted profiling was carried out to identify the DON biotransformation products. Metabolites annotation was performed as already proposed by other authors [2]. Full identification was achieved when commercial standards were available. For the remaining DON metabolites, they were putatively identified considering accurate mass, isotopic pattern distribution and the comparison between experimental and in silico predicted fragmentation pattern (http://cfmid.wishartlab.com).

Conclusions
Although the conditions applied do not represent the complexity of an actual environment, in vitro wheat culture has proved to be suitable for the investigation of mycotoxin metabolism in plant. Svevo showed a larger DON absorption from the growing medium. However, no considerable differences were observed in the DON-3-Glu conversion ability, between the two varieties. A considerable number of the hypothesized metabolites was identified through the untargeted LC-HRMS analysis.

Novel Aspect
The integration of in-vitro plant cultures and HR-MS is an effective workflow for the elucidation of the metabolic fate of mycotoxins and other xenobiotics.

References
Bee Pollen and Pyrrolizidine Alkaloids: A Contamination Profile Analysis using LC-MS/MS

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Bee Pollen and Pyrrolizidine Alkaloids: A Contamination Profile Analysis using LC-MS/MS

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Keywords: Pyrrolizidine alkaloids, bee pollen, LC-MS/MS, food safety

Introduction:
Pyrrolizidine alkaloids (PAs) and their N-oxides (PANOs) contamination in foodstuffs and dietary supplements, such as honey and bee pollen, represents a potential health risk for consumers. This study aimed the development and validation of an LC-MS/MS method to quantify PAs/PANOs levels in bee pollen samples coming from local beekeepers (LB; Veneto/Italy), great retail and web (GR).

Methods:
The developed method was validated according to the Commission Decision 2002/657/EC. For this purpose, blank bee pollen samples were spiked with 17 PAs/PANOs standard solutions at three levels (2, 10 40 µg/Kg). PAs and PANOs were extracted from pollen using PLEXA cartridges (200 mg/6 mL, Agilent, USA) and the LC-MS/MS analysis was conducted on an LTQ-XL Ion trap operating in positive ion mode.

Results:
All results obtained for recovery, linearity, repeatability and reproducibility in the validation study were in agreement with the limits reported by Decision 2002/657/EC. The limit of quantification was 0.4 µg/kg for all analytes. The validated method was applied to 81 bee pollen samples, of which 50 were positive: 20 were from LB and 30 from GR. The average total concentration of PAs/PANOs in samples from LB and GR were 27.8 µg/kg and 470.9 µg/kg, respectively. The identified PAs/PANOs could be categorized into three classes and their average concentrations were as follows: Eupatorium-Type 42.90 µg/kg (LB) vs. 162.76 µg/kg (GR); Echium-Type 7.46 µg/kg (LB) vs. 465.54 µg/kg (GR); Senecio-Type 16.24 µg/kg (LB) vs. 176.62 µg/kg (GR). Most samples (17) from LB presented Senecio-Type PAs/PANOs, whereas most samples (24) from GR presented Eupatorium-Type PAs/PANOs.

Conclusions:
The validated LC-MS/MS method showed to be sensitive and suitable for the quantification of the 17 selected PAs/PANOs markers in bee pollen. Samples from LB and GR presented a very different PAs/PANOs contamination profile reflecting their different botanical and geographical origin. The average total concentration of PAs/PANOs was 17 times lower in samples from LB than in those from GR.

Novel Aspect:
This study should highlight any hazard due to the import/export of pollen and suggest management strategies to beekeepers to ensure a healthy product.

References:
Introduction
Ensuring food safety remains a major public health issue. Recent food safety crisis (i.e. eggs contaminated by fipronil in Europe) clearly illustrated the limits of analytical targeted approaches. The need for untargeted approaches has recently merged, especially for detecting non expected contaminants [1]. These new approaches raise many analytical challenges, due to contaminants diversity, low-levels concentrations (µg/kg) and food matrices complexity.

Methods
An untargeted foodomics approach was developed for blind food chemical contaminants detection. It relies on four pillars from metabolomics and chemometrics: (i) a broad-range UHPLC-HRMS method based on minimal sample treatment; (ii) feature detection based on XCMS and data cleaning tools; (iii) blind discrimination of sample groups by Independent Component Analysis; (iv) powerful data exploration strategies for annotation of suspect compounds.

Results
Tea has been chosen as a development food matrix since it is the most consumed hot beverage over the world, offering a high diversity and complexity as well. The proof-of-concept was initially developed on a rather simple case (known contaminants characterizing the potential diversity at different levels with a homogeneous matrix), and then successfully applied on more complex cases, such as the detection of an unknown contamination or the simultaneous analysis of samples from different brands of black and green tea. Efficient data filtration, normalization and scaling strategies are key issues since a discerning choice of tools enables a blind discrimination between control and spiked sample groups at levels as low as 10 µg/kg, as well as the detection of unexpected contaminants. Annotation step appears to be the main challenge, but in-house data mining tools are key factors to reduce data complexity and also provide the user a quick feedback about potential identity or property (presence of an isotope or potential adduct) of unexpected compounds.

Conclusions
In this work, a metabolomics-like strategy was set up to detect and characterized a contamination in complex food samples at level down to 10 µg/kg. On top of that, the approach, based on open and free tools, was shown to have the capability to detect unexpected contaminants, which is critical for future chemical food safety assessment. No doubts that our results will encourage the development of such strategies for chemical food safety assessment within the next years.

Novel Aspect
We propose an original methodology for untargeted food safety assessment (down to 10 µg/kg) validated on several contamination scenarios for a complex real food matrix (tea).

References
Introduction

Palytoxins (PlTXs) are produced by marine dinoflagellates, cyanobacteria and certain corals and is a neurotoxic compound that can end up in shellfish. PlTXs are within the classes of marine biotoxins the largest non-protein molecules with m/z 2700. To protect shellfish consumers EFSA established an acute reference dose of 0.2 µg/kg b.w. which can be translated to 30µg PlTX-eq/kg shellfish (400g shellfish by 60kg person). Sensitive methods for analysis are lacking.

Methods

Shellfish homogenate was extracted in triplicate with 50:50 v/v water/methanol. Subsequently extracts were purified with SPE clean-up. For the LC chromatographic separation a Phenomenex Kinetex (2.1 x 100 x 1.7) column was used. Mobile phases consisted out of 0.25mM LiI and 0.31mM formic acid in water and 0.25mM LiI and 0.31mM formic acid in 90% acetonitril. The LC effluent was directed into an Q-Orbitrap-MS or tandem quadrupole MS both equipped with ESI operating ESI+.

Results

When analysed by mass spectrometry complex spectra are obtained with multiple adducts, charges states and in source water losses. To gain sensitivity there was attempted to simplify the spectrum by the formation of lithium adducts. Without the addition of lithium in the mobile phase the most abundant ion present is less than 30% of the sum of all ions formed. However with the addition of lithium the main adducts formed were [M+H+2Li]3+ and [M+H+Li]2+ where the abundance of [M+H+2Li]3+ was around 60% of the total abundance of all ions formed. In MS/MS the most abundant fragments are mainly due to losses of H2O. Also more structural fragments were obtained, the most sensitive transition is [M+H+2Li]3+ m/z 897.8> 1215.7. When measured with MS/MS the method has an LOQ of 1 ng/mL for a standard solution. Subsequently the method was successfully validated for PlTX in shellfish at levels of 10, 30 and 60 µg PlTX/kg.

Conclusions

A sensitive method based on cationization with lithium for the detection of PlTXs in shellfish was developed. Subsequently the method was validated with good recoveries (70-120%) and repeatability RSDs below 20%. The method described is a novel method for the detection of intact palytoxins in shellfish which can measure these compounds at the relevant levels (µg/kg range).

Novel Aspect

Application of cationization in the field of marine biotoxin research for the development of an intact palytoxin LC-Q-Orbitrap and LC-MS/MS method.

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519 - THE SWEET DEAL OF ADDING ION MOBILITY TO FULL SCAN ACQUISITIONS TO PROFILE STEVIOL GLYCOSIDES KNOWNs AND KNOWN-UNKNOWNs IN FOOD MATRICES

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Keywords: Steviol glycosides, isomers, Collision Cross Section (CCS), ion mobility spectrometry (IMS), known-unknowns

Introduction:
In 2011, the EU authorised steviol glycosides as a sweetener food additive (E960), its natural origin is key to success in the food market. Like many botanical extracts, E960 is a complex mixture of compounds, with diverse physico-chemical/organoleptic properties and is a challenge to analyse. Hence its characterisation in complex food matrices requires a variety of analytical tools. Here, we present a simple screening method using ion mobility mass spectrometry.

Methods:
For the assays, RP-C18-Ion Mobility-HRMS analyses were performed on a Synapt G2-Si using standard solutions and real samples. The sample treatment comprises the extraction of the steviol glycosides, followed by C18-SPE clean-up prior to analysis. Mass spectra were collected from m/z 50 to 2000 (resolution 20000 FWHM). The mobility drift gas used was nitrogen and mobility cell calibration was carried out using poly-DL-alanine.

Results:
From the analysis of steviol +10 steviol glycosides standards, CCS values, Rt, and fragmentation patterns were derived for precursor, formate adducts and labile fragments ions. These results were entered into a screening library. Regardless of the molecular complexity of the food additive, co-eluting critical pairs were ion mobility resolved. Using the method, E 960 was profiled in food matrices. The results show the efficiency of CCS values in simplifying the screening workflow, while other isomeric steviol glycosides were detected. Characterization of these known-unknowns without standards was performed using IMS. Drift time aligned fragment spectra, confirmed the origin of the fragment ions and in combination with Zimmermann [1] fragmentation rules, sugar moiety positions were identified. These compounds were inserted into the library and used for retrospective analysis. The CCS specificity of known-unknowns enabled the distinction of multiple isomers within complex mixtures of food extracts, providing enhanced profiles.

Conclusions
Using IM-HRMS analysis methodology, multiple identification parameters for the same steviol glycoside have been determined. As a result, the screening workflow has been drastically simplified and successfully applied to the analysis of a variety of food matrices containing the E 960 food additive. The additional CCS metric has enhanced confidence in identification of steviol glycosides at trace levels, which is critical considering the sweetness potency of E 960.

Novel Aspect:
This work demonstrated the benefits of combining ion mobility to full scan acquisitions to build a comprehensive screening analysis, even when standard references are unavailable.

References

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1084 - CHARACTERISATION AND QUANTIFICATION OF KNOWN/UNKNOWN MIGRATING SUBSTANCES IN FOOD USING LC & GC HIGH RESOLUTION MASS SPECTROMETRY

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Keywords: Food Contact Material, Mass Spectrometry, semi-quantitation, food simulant

Introduction:
A method was developed that enables to 1) detect and categorise known and unknown migrating substances from packaging into food, 2) to semi-quantify these known and unknown substances using CAD and FID and 3) define an exposure threshold of unknown substances above which these will be identified. The methodology is applied to packaged food in comparison with measurements performed in so-called food simulants.

Methods:
The developed approach consist of a QuEChERS extraction applied to the food sample and a migration with food simulant of the corresponding empty packaging. Then the extracts are analysed using GC-FID/PCI-HRMS and LC-CAD-HRMS. A semi-quantitation based on FID and CAD detection is applied. LC-HRMS and GC-PCI are used for identification purpose.

Results: A process was developed that enables to classify detected substances into known and unknown substances that migrate from the packaging material into food. Using our quantification/exposure based method, it is possible to focus on substances that are relevant to be considered for risk assessment.

Conclusions
The strategy for sample preparation of unknown substances will be presented together with measurements in food and food simulant. Levels of migrating substances in food will be compared with those in simulant which helps to mitigate the concern of these migrating molecules in the risk assessment of packaging materials.

Novel Aspect:
The approach allows to analyse known/unknown migrating substances in food giving real levels of migrating molecules in contrast to approaches used today that rely on food simulants.
Introduction:
The link between food-toxic effect and disease occurrence requires, the most complete characterization of exposure. In food safety, consumption data crossed with food contamination data or biomonitoring data allow evaluating exposure only in a targeted way. Based on our previous results in exposomic [1], food or environmental toxicology should focus on mixture of compounds, mostly at low doses, and in an untargeted way to identify unknown compounds.

Methods:
We developed an untargeted method to profile electrophilic metabolites which are most of the time toxic, due to their chemical reactivity towards bio-molecules such as DNA. The main line of cellular defense against that is the conjugation to the tripeptide glutathione, which is metabolized in mercapturic acid conjugate to be excreted into urine. Therefore, mercapturate conjugates were monitored through their characteristic neutral loss observed in MS/MS.

Results:
This approach was applied to the study of different groups of rats fed diets containing various oils. According to our previous results on lipid peroxidation [2] these diets led to the production of different aldehydes conjugated to mercapturic acid. The most famous is DHN-MA which corresponds to the mercapturate conjugate of 4-hydroxynonenal (4-HNE), which is commonly used as a biomarker of lipid peroxidation [2]. Using our methodology, we were able to detect without a priori, dozens of mercapturate conjugates, including DHN-MA and other known conjugated aldehydes. Furthermore, our approach also allowed the detection of conjugates of unexpected aldehydes, and of other chemical classes, for which putative identifications have been proposed based on complementary structural analyses. Interestingly, multivariate statistical analyses of the HRMS signals carried out on the mercapturate conjugates yield a better characterization of the studied animal groups compared to results obtained from a classic untargeted metabolomic approach.

Conclusions
Using the “all ions MS/MS” mode (MSE) of a Synapt G2-Si mass spectrometer it is possible to detect all the metabolites displaying the characteristic loss of a mercapturate conjugate, which represents a reactive metabolites. Thus, among these numerous metabolites representative of our exposure, it seems possible by our approach to focus on toxic ones.

Novel Aspect:
We developed an untargeted profiling method of mercapturate conjugates, which are representative of reactive metabolites in our exposome.

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Pesticides are widely used in agriculture, growing of fruit and vegetables, and in lots of garden plots. The main aims are to protect the crops from pests and to control the growth of unwanted plants, both to minimize crop loss. The use of pesticides is strongly regulated to ensure that any hazards for humans, animals, and the environment are minimized. To monitor pesticide residues in food, LC-MS/MS in combination with QuEChERS sample preparation is today the method of choice for the majority of the relevant pesticides. With the SANTE/11813/2017 document, the European Commission published specific guidelines on quality control and validation procedures for analytical methods for pesticides in food and feed. In order to analyze more than 1000 pesticides, powerful data processing software, which ensures fast and easy data review, is strongly demanded by routine food testing labs.

The new SCIEX OS 1.4 software makes the data processing fast and easy. It includes powerful features like the automatic outlier removal feature, which will automatically fit the regression type and remove standard outliers from the calibration curve based on regression fit, accuracy, precision of replicates, and a user-defined outlier tolerance. This automatism tremendously reduces the time to check the calibration curves for all analytes.

Furthermore, the pesticide data have to be reviewed in accordance to the SANTE/11813/2017 document. All identification requirements of this guideline for both unit mass resolution and accurate mass measurement including retention time, mass accuracy of two ions and their specific ion ratio can be easily and fast reviewed in the software using a user-friendly traffic light system.

The SCIEX OS 1.4 software supports both QTOF data acquired with the SCIEX X500R QTOF system and the SCIEX TripleTOF® system and unit mass resolution data acquired with a SCIEX QTRAP® or SCIEX Triple Quad™ system.

SCIEX OS software 1.4 is perfectly fit for the analysis of pesticides in compliance with EU regulations.
Simultaneous determination of propoxycarbazone and its metabolite in food by UHPLC-MS/MS, applying a QuEChERS extraction based method

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Keywords: Propoxycarbazone, metabolite, 2-hydroxy-propoxycarbazone, QuEChERS, UHPLC-MS/MS

Introduction:
Nowadays, it is of great importance the monitoring of parent compounds as well as potential metabolites, otherwise the effects of the use of chemicals would not be fully studied. The analysis of pesticide residues including metabolites in food matrices is a challenge, especially due to the low concentration of analytes and the large quantities of interfering substances that can be coextracted.

Methods:
For the extraction of the compounds, several modifications were performed to the acetate-buffering QuEChERS method[1] dependent on the commodities tested (lettuce, beetroot, honey and soybean meal). For analyte determination, ultra high performance liquid chromatography coupled to a triple quadrupole mass spectrometry analyzer was used. In addition, the cleaning stage was optimized, evaluating the effect of several sorbents, as well as the effect of the matrix during the quantification stage.

Results:
The suitability of the method for the analysis of the target compounds in the different commodities was properly validated according to the SANTE document.[2] Linearity was evaluated using matrix matched standard calibration by analyzing spiked blank samples of each of the matrices under study at six concentration levels (from 5 to 200 µg/kg). R2 was 0.99 in all the tested matrices. Lettuce and beetroot did not show significant matrix effect; however, honey and soybean meal have shown a strong matrix-effect. Recovery and precision was studied at two concentration levels (10, 50 µg/kg), obtaining adequate recoveries (73-110%), intra-day (4 - 19%) and inter-day precision (5 - 20%). The limits of detection (3-6 µg/kg) and quantification (10-25 µg/kg) were lower than the maximum residue limits (MRLs)[3] in the matrices evaluated. The validated method was applied to the analysis of 20 samples, but residues of target compounds were not detected in any of the samples analyzed.

Conclusions
The modified QuEChERS approach followed by clean-up in combination with liquid chromatography coupled to triple quadrupole method provided suitable selectivity and sensitivity for the simultaneous determination of propoxycarbazone and its metabolite in lettuce, beetroot, soybean meal and honey. The method is appropriate for routine monitoring of residues in vegetables, cereals and food samples to ensure food safety.
Novel Aspect:
This is the first method that includes the simultaneous determination of propoxycarbazone and its metabolites in foodstuffs, as it is established by regulations.

References
SENSITIVE DETERMINATION OF TOXIC PYRROLIZIDINE ALKALOIDS IN SPICES AND CULINARY HERBS USING LIQUID CHROMATOGRAPHY TANDEM-MASS SPECTROMETRY

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Keywords: pyrrolizidine alkaloids, spices, culinary herbs, method development, tandem mass spectrometry

Introduction:
1,2-dehydropyrrolizidine alkaloids (PA) and their N-oxides (PANO) are phytotoxins with hepatotoxic and mutagenic potential. PA presumably occur in plant species worldwide and are responsible for various recorded food and feed poisonings. Main source of consumers’ exposition is food contaminated with seeds, pollen, nectar or parts of PA plants. Especially (herbal) tea infusions, food supplements, honey and, recently, spices can be contaminated with PA [1].

Methods:
In total, 210 samples of spices and culinary herbs from Europe, Asia, Africa and America were investigated for occurrence of overall 49 PA. Therefore, a sensitive LC-MS/MS method was developed and validation was done for each group of spice and culinary herb matrices. Samples were extracted under acidic conditions and extracts were cleaned using strong cation exchange cartridges. Quantification was performed via external matrix-matched calibration.

Results:
The method allowed the analysis of 49 PA within a total runtime of 16.0 min after a 6.5 min equilibration of the column. Limits of detection (LOD) and quantification (LOQ) ranged from 0.01 to 0.17 µg/kg and from 0.03 to 0.54 µg/kg, respectively. Precision of the method, expressed as the coefficient of variation of a six-fold determination of respective samples, was 0.9 % and 8.0 % for all analytes, except merepoxine and mernesskine and their N-oxides. Obtained recoveries lay between 45.1 % and 118.4 %, only single PA showed outlier data. After the investigation of 151 of the 210 samples, in 60.3 % at least one PA was found. The obtained sum levels of PA-contamination ranged from 0.13 µg/kg in a rosemary sample to 24.62 mg/kg in an oregano sample. Including all positive samples, mean PA-sum level was 967.62 µg/kg. The most abundant detected PA in the samples were europine/-N-oxide, heliotrine/-N-oxide and lasiocarpine/-N-oxide, which occur in plants of Heliotropium spp., a genus of the Boraginaceae family[2].

The final results will be reported.

Conclusions:
A LC-MS/MS method for the analysis of 49 PA in spices and culinary herbs was developed and validated. The applicability was successfully proven by investigating 210 samples of different origins. The study showed that spices could contribute to the overall exposition to PA/PANO. However, this contribution is generally quite low due to the small daily intake of herbs and spices in comparison to other food commodities.

Novel Aspect:
The developed method is the first target LC-MS/MS method being able to quantify 49 different PA and which was applied to a representative number of spice and culinary herb samples.

References
745 - ANALYTICAL METHOD DEVELOPMENT OF MULTI RESIDUE PESTICIDES IN LIVESTOCK PRODUCTS BY GAS CHROMATOGRAPHY-MASS SPECTROMETRY (GC-MS/MS)

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Analytical method development of multi residue pesticides in Livestock Products by Gas chromatography-mass spectrometry(GC-MS/MS)

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Introduction
The Gas chromatography-mass spectrometry(GC-MS/MS) multi-residue method for the analysis of 55 pesticides in livestock products has been developed. The monitoring of pesticide residues in food is nowadays a priority objective in order to get extensive evaluation of food quality and to avoid possible risks to human health. The pesticides included in this study belong to different chemical families of insecticides, herbicides, fungicides and so on. GC-MS is rapidly becoming an accepted technique in pesticide residue analysis for regulatory monitoring purposes. This survey was aimed to establish for determination of multi-residue method for 55 pesticides in commercial livestock products by GC-MS/MS.

Method
Briefly, the homogenized samples were mixed with 1% acetic acid in acetonitrile. The mixture was vigorously shaken, then added sodium chloride and MgSO4 repeated the shaking process, and cleanup. After a centrifugation step, the resulting extract with solid-phase extraction using Florisil cartridge. The extracts were transferred to vials for analysis by GC-MS/MS. Pesticides were confirmed by their retention time and multiple reaction monitoring of three fragment ions by GC-MS/MS.

Results & Conclusions
Recovery, precision, accuracy, linearity, limit of determination (LOD), and limit of quantification (LOQ) in the analytical method were validated in different matrices (Pork, Beef, Chicken, Egg, Milk). Calibration curves were linear for most of the compounds studied. The recoveries obtained at fortified levels of 0.001~0.05 mg/kg were 60~120%, with relative standard deviations (RSDs) of ≤30%, with a few exceptions. This method we have developed can quickly and accurately analyze 55 kinds of pesticides used in livestock products. The methods will be proposed in the Korean Food Standards Codex as the analysis of residual pesticides in livestock products.

Novel Aspect
A GC-MS/MS multi-residue method for the simultaneous analysis of 55 kinds pesticides in livestock products.

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600 - LIMITATIONS OF GAS CHROMATOGRAPHY–SINGLE QUADROPOLE MASS SPECTROMETRY IN THE IDENTIFICATION OF COMPOUNDS MIGRATING FROM FOOD CONTACT MATERIALS

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Keywords: food contact materials, migration, GC-EI-Q-MS, GC-EI-TOF-MS, non-intentionally added substances

Introduction:
Plastics are commonly used for food packaging. From these plastics potentially hazardous compounds can migrate into the food. It is difficult to establish the chemical structure of migrated compounds especially for the non-intentionally added ones. They are often detected with GC-EI-Q-MS and identified using NIST database without any further confirmation [1]. But to what extent can such results be confirmed?

Methods:
The ability of GC-EI-Q-MS to identify migrating compounds was tested using plastic food contact materials (FCM). Degradation and migration into a food simulant was assisted with microwave radiation. The extracts were analyzed by GC-EI-Q-MS. Preliminary identification of the migrants was based on the obtained mass spectra and a NIST library search. Confirmation was attempted using GC-EI-TOF-MS measurements and analytical reference materials, if available.

Results:
As a result of the microwave irradiation of the polypropylene samples plenty of compounds were formed and migrated to 90 V/V% isooctane and 10 V/V% ethanol (solution for simulating fatty food). With GC-EI-Q-MS at least four compounds could be detected for all 26 samples. Using the NIST mass spectral database 11 different compounds could be preliminary identified with good matching factors (>80%). Out of these, 8 could be confirmed with GC-EI-TOF-MS with less than 5 ppm mass difference for all occurrences. On the other hand the Irgafos 168 - a commonly used antioxidant - and a degradation of it was found in 24 cases with relatively low matching factors (40-55%). All these occurrences could however be confirmed with both GC-EI-TOF-MS and analytical reference materials. Furthermore another common antioxidant, the butylated hydroxyl toluene (BHT) was first identified in 12 samples, but always under 70%. All these samples also contained its degradation product that was consequently misidentified with the NIST library search.

Conclusions
Our results prove that the GC-EI-Q-MS is a powerful tool in the detection of compounds migrating from FCM. However, identification of these compounds cannot reliably be based solely on a NIST mass spectral database search even though it is a rather widely used approach. Reliability of the identification can notably be improved with GC-EI-TOF-MS and analytical reference materials.

Novel Aspect:
The identification of semi-volatiles migrating from FCM is often based on mass spectra of a single type of MS. Reliability of this approach is questionable.

References:
Introduction:
Food additives are widely employed by food industry to provide number of benefits. The use of food additives is regulated, for some of them maximum limits have been established. To control safe use of food additives and protect consumers' health, effective methods are needed. In line with current trends, this study was performed to replace number of ‘conventional methods’ based on LC-UV by multi-analyte method employing MS detector with single quadrupole analyzer.

Methods:
Presented method uses an ultra performance liquid chromatography (UPLC Acquity, Waters) coupled to a simple single quadrupole MS (QDa, Waters) using a gradient of 5 mM aqueous solution of ammonium acetate and methanol while separating analytes on reversed phase BEH C18 column (Waters).

Results:
The developed method enables simultaneous determination of number of synthetic food additives fairly differing in physicochemical properties. Synthetic dyes, sweeteners, preservatives and several other compounds, vanillin, ethyl vanillin, theobromine and caffeine, were involved on the analytes list. Method was validated for sweet drinks and fruit concentrates. Repeatability, expressed as relative standard deviation (RSD), is less than 10 % for all analytes with recovery ranging from 80 to 120 %. Limits of quantification range from 0.1 to 1 μg/mL which is still considerably lower than legislation limits for food additives which are often ten or hundred times higher. Considering all, QDa seems to be a suitable detector for this kind of analysis. Method was applied for the analysis of fruit concentrates and fruit wines revealing a wide use of food additives.

Conclusions:
QDa MS is an appropriate tool for rapid screening of synthetic food additives of which analyzed samples contained a significant amount despite not exceeding legislative limits.

Novel Aspect:
Presented method enables simultaneous determination of 19 synthetic food additives fairly using a relatively cheap and simple single quadrupole MS.
Aflatoxin M1 Residues Detection in Milk, Infant Food and Cheese by LC-MS/MS

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Keywords: Aflatoxin M1, milk, food safety, LC-MS/MS

Introduction
Aflatoxins are mycotoxins produced by some species of Aspergillus. In dairy cows, ingested Aflatoxin B1 is metabolized into Aflatoxin M1 (AFM1), which is eliminated through milk. Due to its high hepatocarcinogenic potential, the level of AFM1 permitted is strictly regulated: EU Commission has established a maximum limit of 0.050 g/kg for AFM1 in milk and a more restrictive limit of 0.025 g/kg in milk and food intended for consumption by nursing infants and children[1].

Methods
First, a liquid-liquid extraction (LLE) was performed on 20 g sample. As extraction solvent, ethyl acetate was chosen for milk and dichloromethane for infant food, dessert and cheese. A delipidation was needed for creamy infant food and cheese. LC-ESI-MS/MS analysis was carried out using a Zorbax SB Phenyl RRHT column (2.1 x 50 mm, 1.8 μm). The method was fully validated as screening assay; the validation as confirmatory quantitative analysis is in progress.

Results
Milk extraction was performed as reported by Biancardi et al [2]; regarding to infant food, dessert and cheese, LLE has to be performed by homogenization with 100 ml dichloromethane and 10 g diatomaceous earth; after filtration, 20 ml of raw extract was dried under a stream of nitrogen; the residue was dissolved in HCOOH 0.1%:CH3CN (90:10, v/v) and defatted with hexane. This clean-up step allowed a good recovery and a low matrix effect for all kind of food examined. Instrumental response was linear in 0.010-0.100 g/kg range.

Specificity was evaluated on 30 different milk sample (fresh, pasteurized, UHT, bovine, ovine, liquid or powdered), 20 different infant food (milk, milk flour, cheese, dessert, yogurt) and 20 different dairy products (cheese, dessert, yogurt, ice cream): no interference was found. Error was evaluated on the same samples spiked before the extraction with AFM1 at 0.015 g/kg for milk and infant formula and at 0.040 g/kg for dessert and cheese. AFM1 was identified in all samples, and error was less than 5% at the interesting level.

Conclusions
The Official Method [3], based on the use of immunoaffinity chromatography columns (IAC) clean up and HPLC-FLD detection, can’t to be used for infant food analysis, due to its higher detection limit. The developed method is able to detect AFM1 in infant food at concentration levels lower than 0.5 LM. Furthermore, in this case no IAC clean up is required, with a significant gain in time and in reagent costs, even for milk and cheese.

Novel Aspect
A new, faster and cheaper approach for official controls on food safety, based on a fast and very sensitive LC-MS method, AFM1 residues detection was developed and validated.

References
[1] Regulation 2006/1881 EC
Keywords: Veterinary drugs; Multi-residue; Fish tissues; Extraction optimization; Orbitrap

Introduction:
There is no doubt that veterinary drugs are very important for aquaculture. But inappropriate and commonly subtherapeutic use of veterinary drugs may result in the presence of drug residues in the fish tissues even food products. Most of the published multi-residues detection methods commonly employed the LC-triple-quadrupole tandem MS techniques, which requires extensive compound-dependent parameter optimization. And due to the great differences in the physicochemical properties of the various targets, we need an appropriate extraction procedure to ensure that all compounds can be extracted effectively. On the other hand, protein, fat and many other interferences are abundant in fish. There is an effective clean-up procedure still needed to eliminate background matrix interferences.

Methods:
A simple, rapid and sensitive multi-residue analytical method was developed and validated for 80 veterinary drugs in fish tissues using ultrahigh performance liquid chromatography-Orbitrap high resolution mass spectrometry (LC-HRMS). The analytes belong to 12 different families include benzimidazoles, β-lactams, lincosamides, macrolides, nitromidazoles, quinolones, sulfonamides and trimethoprim, tetracyclines, triphenylmethane dyes, amphenicols, nonsteroidal estrogens and steroid hormones. The sample preparation was optimized base on QuEChERS (quick, easy, cheap, effective, rugged and safe) procedure. A very simple and sufficient preparation procedure without salting-out and complex clean-up process was studied. It had been proved that water in the extract was helpful for extracting hydrophilic compounds and precipitating the lipids during the subsequent cleaning process. In addition, an appropriate percent of methanol was necessary to some analytes.

Results:
Finally, a mixture of acetonitrile, methanol and water (3:1:1, v/v/v) which include 1% acetic acid and 10 mMethylenediaminetetraacetic acid disodium salt 2-hydrate was selected as the extraction solvent, and the clean-up step consisted of a low temperature procedure and two times of high-speed centrifugation to deproteinize and remove lipids. The detection and quantification of all compounds were performed by ultrahigh performance liquid chromatography coupled with electrospray ionization quadrupoleOrbitrap high resolution mass spectrometry in positive and negative ion mode. This methodology was validated according to the Commission Decision 2002/657/EC and SANTE/11945/2015. The recoveries ranged from 60.74%-109.85% with relative standard deviations (RSDs) < 20%. The limits of quantification (LOQs) were 0.25 to 25 ug/kg, for the analytes which the MRL or MRPL had been established in fish tissue, the LOQs were all lower than their own legal tolerances. The values of decision limit (CCα) and detection capability (CCβ) were in the range of 1.91-1001.13 ug/kg and 3.52-1002.26 ug/kg, respectively. This validated method has been successfully applied on the determination of veterinary drugs in real commercial oplegnathuspunctatus samples.

Conclusions:
A very simple, rapid and efficient multi-residue and multi-class analytical method for the simultaneous determination of 80 veterinary drugs in fish tissue by ultrahigh performance liquid chromatography coupled with quadrupole Orbitrap high resolution mass spectrometry has been developed. To the best of our knowledge the proposed method is the simplest way reported in literature for multi-residue and multi-class determination in fish. The method was validated with linearity, recovery, precision, sensitivity and tested the CCα and CCβ for each analyte. The satisfactory performances were achieved mainly depended on the high efficiency of extraction solution
and the excellent selectivity and sensitivity of Orbitrap. This efficient and rapid method is a good way to screening for large batches of fish samples.
Keywords: plant toxins; pyrrolizidine alkaloids; UHPLC-MS/MS; food supplements; method

Introduction:
Pyrrolizidine alkaloids (PAs) and their N-oxides (PANOs), secondary metabolites of plants, are hepatotoxic to humans and animals and their ingestion in some cases might lead to death[1]. One of the sources of PA-intoxication is herbal remedies and food supplements that (un)intentionally contain material from PA producing plants. The objective of this study is to develop a simple and sensitive method for analysis of 35 PAs and PANOs in different food supplements.

Methods:
The analytical method is based on ultra-high performance liquid chromatography (UHPLC) coupled to mass spectrometry (MS), as this technique can detect both tertiary PAs and their N-oxides. LC separation of the analytes is performed on a 1.7 µm BEH C18 column using alkaline mobile phase. Detection is done by a triple quadrupole MS (Xevo TQ-S, Waters) operated in positive electrospray ionization mode. The data are acquired in MRM mode.

Results:
The method targets different PA types, namely senecionine-, lycopsamine, heliotrine- and monocrotaline-type compounds. The herbal dietary supplements are divided into several groups. In order to represent all the groups model matrices, namely seed powder of milk thistle, propolis, vegetable oil and herbal infusion, are selected for method development. These samples are free from PAs and PANOs or contain trace amounts. The optimization of sample preparation includes several techniques, such as a dilute-and-shoot approach, a QuEChERS-based protocol and different sorbent materials for solid-phase extraction (SPE). The QuEChERS-method and SPE with ion-exchange mode as well as SPE with reversed-phase mode show promising results with regards to recovery; however, matrix effect is unavoidable in all cases. The ultimate aim is to select a procedure that has a minimal number of involved manipulations, assures good recovery of the target analytes and is applicable for the analysis of a variety of food supplements. The developed method will be in-house validated and limits of quantification, linearity, recovery, repeatability and reproducibility will be determined.

Conclusions:
This analytical method allows for the simultaneous determination of 35 PAs and PANOs in food supplements at low levels, and can be used as a tool for collection of PA occurrence data, which is highly demanded by the European Food Safety Authority in order to perform more accurate exposure assessment.

Novel Aspect:
The outlined method is able to provide information about the quality of herbal food supplements.

References

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Comparison between HPLC-ESI-QTOF-MS and SPME-GC-MS technique for acrylamide determination in potatoes chips

Silvia Marzocchi, Sara Marziali, Maria Fiorenza Caboni

Keywords: HPLC-ESI-QTOF-MS, SPME-GC-MS, acrylamide, potatoes

Introduction:
In April 2002, researchers at Swedish National Food Administration and Stockholm University published data about acrylamide content in fried and baked starch-based food [1,2]. Acrylamide formation in food is attributed to the Maillard browning reaction; because of the interaction between amino group of asparagine free amino acid and carbonyl group of reducing sugar [3].

Methods:
Acrylamide in two different potatoes varieties, after frying, was determined by HPLC and GC coupled with MS. In the first case a HPLC-ESI-QTOF-MS was used, SPE extraction and chromatographic conditions were according to Calbiani et al [4]; using acrylamide-d3 as internal standard. The method used for SPME-GC-MS was according to Ghiasvand & Hajipour [5] using a calibration curve for quantification.

Results:
Fragmentation of acrylamide and acrylamide d3 involves in NH3 release, leading to a product ion at m/z 55 and 58 [4]. Regarding HPLC, positive electrospray proved to be the most sensitive mode for acrylamide determination [6]. Acrylamide identified in the samples was quantified using a calibration curve based on response ration of acrylamide/acrylamide d3. Considering GC, the quantification was made using a calibration curve, in the linear range 2-500 mg mL-1 with a correlation coefficient (R2) of 0.997. The two different analytical techniques allowed to determine different values for the same potato variety, due to the different sensitivity of the two instruments, but only with HPLC-MS were obtained results according to literature [4,7]. In both cases the two different potatoes varieties have shown the same content difference, about 30%, probably due to the different asparagine content.

Conclusions:
Both liquid and gas chromatography are able to determine acrylamide in food. This study confirmed HPLC-ESI-QTOF-MS as the most suitable chromatographic technique to determine acrylamide in potatoes sample, according to literature. At the same time, also SPME-GC-MS is able to determine acrylamide in food, but without the same sensitivity as the previous technique.

Novel Aspect:
SPME-GC-MS technique was tested for acrylamide determination and quantification to find an alternative to the well-established HPLC-ESI-QTOF-MS method.

References:

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1029 - A MULTI-RESIDUE CONFIRMATORY METHOD FOR THE DETERMINATION OF SEDATIVES BY HPLC-MS/MS

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Keywords: mass spectrometry, HPLC, sedatives, validation

Introduction:
Sedatives are injected right before transport of the animals to reduce the stress of animals generated during handling and transport to the slaughterhouse. Thus, animal tissues have become major targets for residue analysis of sedatives and adrenergic blockers. Therefore, simple and reliable analytical methods are required to monitor these drug residues in food of animal origin. LC-MS/MS was the technique of choice due to its high specificity and sensitivity.

Methods:
Samples were homogenized and fortified by addition of Internal Standard Mixture. Liquid-Liquid extraction was carried out by using acetonitrile. SPE was performed on silica gel cartridges. The HPLC-MS system consisted of HP 1290 Infinity (Agilent Technologies, USA) and 6500 QTRAP (Sciex, Canada) hybrid linear ion trap/triple quadrupole mass spectrometer. MS/MS parameters were optimized for each compound. The analysis was carried out with gradient elution.

Results:
Several types of liquid-liquid extraction and SPE cartridges were tested during the study. The method was validated for muscle, liver and milk matrices. The validation study consisted of 4 individual experiments with two factors: operator and storage time before LC-MS/MS analysis. The validation levels for muscle matrix were 1, 10, 20, 100, 200 ng/ml for azaperol, azaperone, acepromazine, haloperidol, detomidine, carazolol, xylazine, medetomidine, meperidine, triflurpromazine and fluphenazine. 5, 25, 50, 100, 250, 500 ng/ml for diazepam, metoprolol, promazine, propionylpromazine, romifidine, and chlorpromazine. Quantification was performed using individual matrix calibration curve for each series with the same concentration levels for each analyte. In order to prove the specificity and the lack of susceptibility to matrix interferences, several blank samples fortified with internal standard were additionally analysed in each series. The recovery was in the range of 45 – 74 % for all analytes in the validated concentration range.

Conclusions:
Method was developed and succesfully implemented for analysis of sedatives in samples of animal origin in the frames of the National Program of Monitoring of prohibited and regulated substances in food in Russian Federation.

Novel Aspect:
A rapid and sensitive method for the determination of sedatives in meat, plasma and liver by HPLC-MS/MS was developed.
1400 - COMPARISON OF DIFFERENT STRATEGIES FOR THE DETERMINATION OF VETERINARY DRUG RESIDUES IN FOOD BY UPLC-MS/MS USING ORBITRAP ELITE

Pavel Metalnikov (1) - Renat Selimov (1) - Alexey Efimov (1) - Ilya Batov (1) - Ayshat Dzhatdoeva (1) - Alexander Komarov (2)

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Keywords: Mass Spectrometry, Orbitrap, Veterinary Drugs

Introduction:
Analysis of veterinary drug residues in food matrices usually is performed for a specific group of drugs. Now, with the advent of high resolution mass spectrometry, it’s getting possible to use accurate masses, measured during analysis, for unambiguous identification of potential contaminants, and so-called “general unknowns” as well. The aim of the work was to compare different strategies for identification of analytes of different drug groups in large scale.

Methods:
Samples of food matrices (milk or meat) were originally homogenized, fortified with veterinary drug mixture solutions, and subjected to liquid-liquid extraction. Then, Solid Phase Extraction (SPE) was applied. Analysis was done on UPLC-Orbitrap system (Ultimate 3000 – Orbitrap Elite (Thermo Scientific, USA)) in gradient mode.

Results:
Orbitrap is a complex mass spectrometer, measuring masses of peaks with accuracy about 1 ppm and providing MS/MS data of high quality. Veterinary drugs were identified either by determination of their elemental composition based on accurate mass determination from spectra, detected in electrostatic ion trap (FT mode), or by fragmentation spectra, registered in linear ion trap (IT mode). Fragmentation spectra matched to in-house built Spectra Library or to other available data bases.

Conclusions:
Despite accurate mass determination is enough for compound identification, in some cases (e.g. overlapping peaks) confirmation by fragmentation spectra is important.

Novel aspect:
New strategies for veterinary drug residues determination in food matrices were developed.

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Introduction
Fenpyroximate is used to treat honey bee colonies in beekeeping farms. This not only deteriorates the quality of the honey, but also affects the health of humans who ingests it [1-2]. However, since there is no effective analytical method for the residual fenpyroximate in different types of honey, this study aims to establish an LC-MS/MS-based analytical technique after simple and rapid pretreatment with a minimum amount of sample.

Methods
5 mL of a saturated aqueous NaCl solution and 15 mL of purified water were added into 5 g of honey sample and then 10 mL of dichloromethane was added. After mixing and centrifuging, the dichloromethane layer was then transferred to a new tube. 2 mL of the dichloromethane extract was loaded onto a SPE cartridge, and then eluted with 6 mL of dichloromethane/n-hexane (65:35%, v/v). The eluate was redissolved with 0.2 mL of acetonitrile and injected into the LC-MS system.

Results
Experiments were carried out to determine the optimal values of parameters such as sample pH, type of solid-phase cartridge, type and volume of extraction solvent, loading sample volume, type and volume of eluent for effective sample extraction, purification, and concentration. Chromatographic separation was performed using a C18 (3.0 mm i.d. × 50 mm length, 2.7 μm particle size) column under isocratic conditions of water and acetonitrile (30:70). The optimized parameters for the extraction of fenpyroximate are as follows: sample pH, 7; type of sorbent solid phase, aminopropyl; volume of extraction solvent, 20 mL; volume of loading sample, 2 mL; eluent, dichloromethane/n-hexane (65/35%, v/v); eluent volume, 6 mL. The limits of detection and quantitation for spiked honey samples were 1.56 and 5.00 μg/kg, respectively. Linear correlation coefficient (r²) obtained from a concentration range 5.00–50.0 μg/kg was 0.9914–0.9977, while the accuracy and precision were 87.99–119.06% and 0.68–14.71 % (RSD), respectively.

Conclusions
The LOQ for the established analytical method was 5.0 μg/kg, which is 10 times lower than the EU-regulated concentration of 0.050 mg/kg. After monitoring 30 honey products, obtained from the market, using the established method, no residual fenpyroximate was detected in any sample. Compared with other studies, the novel aspects of this study are that method validation of four species of honey (acacia honey, wild honey, manuka, and chestnut honey) has been performed.

Novel Aspect
The LLE-SPE combined method does not require additional cleaning procedures, and good sensitivity can be obtained even with low-capacity cartridges.

References
Introduction:
Gum Arabic is a dried exudate obtained from several Acaciaspecies from the sub-Saharan region of Africa [1]. It is a complex mixture of macromolecules, mainly carbohydrates and proteins, widely used as an edible ingredient in various types of food as well as for non-food applications [2]. For oenology, OIV approves the use of both A. senegal and A. seyal gums. Europe indicates a maximum of 200 mg/L as the recommended technological dosage [3].

Methods:
The study was performed using a Thermo Ultimate R3000 UHPLC equipped with a byphenyl column (3 x 150 mm, 2.7 μm). Separation was obtained at a flow rate of 0.3 mL/min with a ternary mobile phase with 2% formic acid, acetonitrile and water. Mass spectra were acquired through a full MS experiment at 70,000 FWHM resolution using a Q-ExactiveTM HRMS equipped with a heated electrospray ionization (HESI-II) interface working in both positive and negative ion modes.

Results:
The full scan MS profile, both in positive and negative ionization, of 45 gum Arabic samples was evaluated in order to check if specific ions could selectively characterise the botanical (A. seyal and A. senegal) and/or the geographic origins of gums (Kordofan, Hashab and Verek). The PLS-DA statistical approach indicated 18 masses as possible predictive origin markers, allowing the correct reclassification of 98% of the samples to the corresponding cases. Moreover, we analysed 40 micro-winery genuine wines (8 varieties) and then reanalysed the same ones after randomly adding 200 mg/L of gum Arabic of the 2 origins (seyal, n=10; senegal, 10). Comparing their ion profiles, 5 of the previous 18 masses, not detected in genuine wines, permitted the identification of the addition of gum Arabic. PCA based on the 5 masses allowed us to distinguish between wines added with A. senegal and A. seyal. Finally, each isotopic patterns and ion fragmentation allowed us to define their molecular formulas and structures.

Conclusions
The HRMS untargeted approach allowed us to identify, through PLS-DA analysis, 18 masses (m/z) that could be proposed as possible markers for the differentiation of the commercial gum Arabic samples, both with respect to the botanical (A. seyal and A. senegal) and geographic (Kordofan, Hashab and Verek) origin. 5 of these 18 masses (m/z 152.1067, 166.1225, 181.0459, 585.1428, 643.1403) were also effective in detecting the presence of 200 mg/L gum Arabic in wine.

Novel Aspect:
A new rapid untargeted high resolution MS approach allows us to predict gum Arabic origin and detect its presence in wine.

References
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1187 - DIRECT FOOD ANALYSIS BY (TRANSPORTABLE) AMBIENT IONIZATION MASS SPECTROMETRY

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Keywords: food analysis, ambient ionization, mass spectrometry, sample preparation, on-site detection

Introduction:
Many health and food related apps have been developed in recent years, followed by the development of wearable sensors for blood pressure, heart beat, moisture and biochemical parameters such as blood glucose levels. Obviously, mankind gets used to measuring all kinds of parameters themselves and subsequently take control over their own life, based on analytical data. In the near future, also the pre-screening of residues and contaminants in food will be performed on-site at the farm, in industry, in retail and, eventually, even done by consumers at home. Typically, three different approaches to selectivity in portable food analytics (each at different development stages), can be distinguished: (1) generic spectral profiles from smartphone-linked optical spectroscopy sensors, such as near infrared (NIR) combined with chemometric models; (2) smartphone-based biorecognition assays; and (3) higher resolution spectral data, such as available from mass spectrometry (MS). With respect to the MS option, there is an urgent need for (trans)portable MS, elimination of tedious sample clean-up, simplified sample introduction, and data evaluation tools that communicate wireless to smartphones, tablets and servers of relevant stakeholders, including the consumer. Sample introduction can, at least in theory, partly be replaced and simplified by one of the >70 Ambient Ionization MS (AIMS) techniques.

Methods:
In this research, different AIMS techniques with potential for coupling to (trans)portable mass analyzers have been critically investigated for their applicability towards the analysis of pesticides, banned veterinary drugs such as β-agonists and steroids, regulated drugs such as analgesics and antibiotics, and natural toxins in various food matrices. Four different AIMS techniques were explored: Matrix Assisted Ionization (MAI), Handheld Desorption Atmospheric Pressure Chemical Ionization (DAPCI), ID-CUBE Direct Analysis in Real Time (DART) and Coated Blade Spray (CBS), either in combination with a benchtop Orbitrap or a transportable single quadrupole MS.

Results:
MAI is one of the most simple ionization options since no high voltage, lasers or gas supplies are needed, the sample or extract is mixed with a matrix and simply tapped against the opening of the cone or heated inlet of the MS. Secondly, a handheld battery powered DAPCI device, just using ambient air, can be used to ionize analytes directly from a sample surface. Third, a mini-DART setup (a modified version of the ID-CUBE) can be used but would require a helium gas supply. With this technique the sample is simply transferred onto a metal mesh sample card having the size of a credit card. Fourth, the recently developed CBS technique, only requires a high voltage. Both the metal mesh card and the CBS technique offer the benefit of trace enrichment following coating with a selective adsorbent. In this presentation an overview is given about the requirements, challenges and preliminary results of direct food analysis by these different (transportable) AIMS approaches.

Conclusions
All tested devices were able to successfully ionize some of the tested analytes, some of them even in various food commodities. A general drawback of these ambient ionization techniques is the poor reproducibility, but, where feasible, isotope dilution of the sample may compensate for that to a large extent. Detection limits will be typically (much) higher when compared to state-of-the-art high-end MS instruments so applications may be limited to regulated substances having relatively high maximum limits values, unless selective enrichment approaches, such as CBS, are being exploited. Nevertheless, the initial applications underline the great potential of ambient ionization.
with (trans)portable MS. Hence, it is realistic to envisage that portable MS, already successful in battlefield- and forensic applications for many years, will soon show up in food control as well.

Novel Aspect:
A paradigm shift in food control, from the laboratory to on-site pre-screening using, among others, (trans)portable MS is upcoming.
Introduction:
Comprehensive analytical techniques are required to meet the increasingly stricter regulations on food safety. Supercritical fluid chromatography with mass spectrometric detection (SFC-MS) was assessed for analysis of food dyes. Fourteen synthetic dyes representing azo, triphenylmethane, xanthone, indigoid, and quinoline classes and covering a broad range of polarities (logD from -8 to 3.5) were selected from the list of permitted additives [1].

Methods:
An Acquity UPC2SFC system coupled to a Xevo TQ-S triple quadrupole mass spectrometer with electrospray ionization (all from Waters) was used for analysis. A sharp gradient (5 to 100%) of methanolic ammonia modifier was employed on a 2-ethylpyridine column. Pseudo-SPE extraction on nylon syringe filters was utilized for real samples.

Results:
Owing to the presence of multiple sulfonic groups as Na+ salts, multiple charged ions were observed and development of SRM transitions required careful optimization. Addition of 10 mM ammonia and 5% water into a modifier allowed separation of a wide range of analytes in a single run without using ion-pairing agents. Thus, MS compatibility, good sensitivity and wide linear range were maintained: LLOQ 4-36 ng/ml, except Brilliant black BN 255 ng/ml; ULOQ 2000 ng/ml. Mobile phase properties changed continuously from supercritical to subcritical and liquid state and did not affect the method performance, which is confirmed by validation parameters: retention time repeatability (RSD<0.6%) and peak area precision (RSD<8.9%). Brilliant blue dye was determined in a sports drink (1.9 µg/ml) and in candies (10.5 µg/g), as well as indigocarmine in candies (5.9 µg/g), which fits well within the legislative limit. The total analysis time including equilibration was 16 minutes.

Conclusions:
A new SFC-MS method was developed for quantitative analysis of permitted synthetic food dyes and its essential validation parameters were evaluated. The results indicate that SFC-MS might serve as a reliable tool for analysis of both moderately polar and highly polar dyes in the context of food safety.

Novel Aspect:
SFC-MS was used for analysis of ionic sulfonated dyes. A sharp modifier gradient suggested operation far from supercritical region but results were reproducible and quantitative.

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References
SIMULTANEOUS SCREENING OF 6 DIFFERENT ANTIBIOTIC FAMILIES IN MEAT USING LDTD-MS/MS QUANTITATION AT 9 SECONDS PER SAMPLE

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Keywords: Antibiotic, Meat, screening, LDTD-MS/MS

Introduction:
Food quality is a fundamental aspect of public health and is determined by innocuity associated to it. The absence of contaminants can only be validated by adequate measurement. Antibiotics in meat represent a challenge in terms of sample complexity. Until now, only a statistical sample of the total animals is tested due to the throughput. We present a new approach to screen 6 different families of antibiotics simultaneously with LDTD-MS/MS at 9 seconds per sample.

Methods:
1g of pork meat is weighed and placed in a 24 wells plate. Sample preparation is accomplished at a low temperature and reduced light. Extraction solution of MeCN:Water (80:20) is added using a CERTUS FLEX to create a concentration of 1g/mL. The samples are blended in parallel for 3 minutes and centrifuged for 3 minutes. 30µL of supernatant is mixed with 150µL of Methanol:Water (75:25) and vortexed for 30 seconds. 4µL are deposited and dried into the analyzing Lazwell plate.

Results:
Strategies have been applied to find adequate markers for thermally unstable molecules. Beta-Lactam Amoxicillin and Ampicillin are detected at the methanolic adduct formation. Ceftazidime undergoes a thermal fragmentation which is reproducible and the fragment containing the beta-lactam ring allows a sensitive measurement for screening purposes. The other molecules tested are detected at their pseudo molecular mass M+H+. The extraction efficiency using a simple approach for the 6 types of antibiotics (Beta Lactam, Sulfonamide, Phenicol, Lincosamide, Tetracycline and Macrolides) ranges from 67 to 93%. QC samples at 50, 100 and 150% of cut-off values for each antibiotic do not overlap and exhibit sufficient signal levels for detection. Calibration curve linearity (R2) reaches values greater than 0.993. Accuracies between 86.5% and 112.3% are observed and reproducibility ranges from 7.2 to 13.7%. Automation is optimized to achieve 96 samples in less time (12 minutes) than LDTD-MS/MS analyses (14.4 minutes) at 9 seconds per samples.

Conclusions:
A complete workflow solution has been developed to extract simultaneously 6 types of antibiotic in meat. Mass spectrometer allows simultaneous specific quantitation of those compound at 9 seconds per sample using LDTD-MS/MS. Thermally unstable compounds are monitored on reproducible fragment. Sample preparation automation match the analyzing speed to enable real high throughput analysis.

Novel Aspect:
Simultaneous LDTD-MS/MS screening of 6 antibiotic families in meat

References
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Keywords: PBDEs, HBCDs, NDL-PCB, mussels, temporal trends

Introduction:
Mussels harvesting is a relevant commercial activity along the Adriatic Sea. Mussels are often used as sentinels for pollution monitoring, too. The aim of this study was to compare “old” contaminants like PCBs and contaminants not yet included in monitoring programs (PBDEs and HBCDs), studying temporal trends in M. galloprovincialis collected along the Marche Coast (2001, 2011 and 2017).

Methods:
Thirteen sampling stations (9 breeding and 4 wild) along Marche Coast (Italy) were included in this study. Breeding were distributed along the Coast from North to South, while the wild ones were around Ancona. Thirty-three mussel samples were analyzed for a series of environmental pollutants: 6 PCBs, 15 PBDEs (GC-MS/MS) [1,2] and 3 HBCDs (LC-MS/MS). All data were processed using STATA software.

Results:
The average concentrations of 6 PCBs were 3.75, 3.42 and 2.75 ng/g for mussels collected in 2001, 2011 and 2017, respectively and the predominant congeners were 153>138>101. No significant concentration differences were highlighted among the years. Mussels collected in the wild sites showed PCB levels significantly higher respect to the breeding ones (p<0.001) irrespective of the sampling period. The wild sites are closer to the coast (<1 km) and therefore subjected to higher anthropic impact. The PBDEs levels significantly decrease going from 2001, 2011 to 2017 (p<0.001) [3]. The 15PBDE medium bound decreases from 458 pg/g in 2001 to 432 and 306 pg/g, respectively, in 2011 and 2017. PBDEs contamination pattern was dominated by tetra- and penta-BDE: 47, 99 and 100 were measured in all samples and the PBDE-47 was the highest, accounting for 60% of the quantified congeners. The results were comparable with those reported in literature for European mussels [4,5]. No significant differences were observed for HBCDs among the three sampling periods. The HBCDs were 273, 448 and 227 pg/g in 2001, 2011 and 2017, respectively. -HBCD was measured in all the mussel samples, accounting for roughly 90% of the HBCD contamination. In 20 out of 33 mussel samples, also -HBCD was detected [4].

Conclusions:
NDL-PCB, BDE-47 and -HBCD were detected in 100% of the analyzed samples. PCBs did not reduce significantly in mussels with time, but the wild sites seems more contaminated than the breeding ones. The results obtained show a PBDE decrease in the years. The BDE-47 levels were generally lower than -HBCD’s.

Novel Aspect:
This study reports for the first time the concentrations of HBCDs in Adriatic mussels, confirming the predominance of the -HBCD isomer.

References:
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709 - AUTOMATED SOLID PHASE MICROEXTRACTION (SPME) WITH UHPLC-MS/MS TO DETERMINE VETERINARY DRUGS IN CHICKEN MEAT

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Keywords: Multiclass multi-residue method, automated solid phase microextraction (SPME), UHPLC-MS/MS, veterinary drugs, chicken tissues

Introduction:

Veterinary drugs are used in animal production to treat diseases and protect growth of animals. However, improper use of drugs can lead to residue violations in food products and possible health risks. Regulatory agencies around the world have established maximum residue levels or tolerance levels of veterinary drugs in foods. A common goal in drug analysis is to get acceptable results for many analytes by a cost-effective method in a single run. However, it is challenging to develop such a method for animal tissues due to the complexity of sample matrices and diversity of analytes from various classes of chemical properties. Sample preparation has always been the major bottleneck in any analytical procedure for the determination of chemical residues in food products. For veterinary drugs analysis, the most often used method has been the modified QuEChERS method with d-SPE clean-up [1-2]. However, some analytes could be lost during sample preparation due to adsorption to the sorbents. To overcome this problem, the method of direct injection of the sample extract without d-SPE cleanup has also been applied for veterinary drugs analysis recently [3]. Solid phase microextraction (SPME) is a solvent-less, effective and versatile sample preparation technique, which has been widely used in environmental and other applications including foods [4-5]. Recently, with the development of new extraction phases and devices, SPME has found increased applications in food analysis [6]. In this study, a multiclass, multi-residue method for analysis of 73 veterinary drugs (covering 13 different classes with various physical and chemical properties) in chicken muscle was developed by coupling automated SPME sample preparation with UHPLC-MS/MS. To get good analyte recovery, reduce sample matrix effects and carryover, the parameters affecting SPME performances were evaluated and optimized, such as different SPME extraction phases, extraction and desorption conditions. The method was validated according to regulatory guidelines and compared with the currently used sample preparation Methods:

Methods:

Chicken samples were homogenized and ground with dry ice to obtain uniformed powder and kept at -20 °C overnight to allow sublimation of residual dry ice and then stored at -20 °C until analysis. Certified organic samples were used as matrix for method validation. 2 g of sample was weighed into a 15mL centrifuge tube, spiked with internal standards and fortified with targeted analytes, vortex for 1 minute and leave the sample on an agitator for 1 hour. Finally equilibrate the samples overnight in the fridge before extraction by SPME and other Methods:

SPME sample preparation method: place sample tubes in the agitator for 1 hour, then add 6 mL of LCMS grade H2O for each sample and vortex for 1 min. Transfer 1.5 mL of sample to 96 well plates, perform static extraction for 60 min and 20 min desorption in 400 µL of desorption solvent (25:37.5:37.5 H2O: MeCN:MeOH + 0.1%FA). Other sample preparation methods: samples were extracted with 10-mL of extraction solution (MeCN/Water, 4/1 in v/v). After centrifugation, the extract was analyzed by LC-MS/MS directly or after d-SPE clean-up with different sorbents.

UHPLC-MS/MS method: analytes were separated using an UHPLC column on a PerkinElmer UHPLC system with a gradient program with mobile phase A (water containing 0.1% formic acid) and B (MeCN containing 10% MeOH and
0.1% formic acid). Mass detection was carried out on a PerkinElmer QSight™ 220 triple quadrupole mass detector with a dual ionization source. Optimization of MS/MS parameters (choice of ions, collision energies and so on) was done by infusion of standards and use of the software. Source conditions were optimized by flow injection (FIA) method. Since the mass detector is capable of fast polarity switching, both positive and negative ionization modes were used. All instrument control, data acquisition and data processing was performed using Simplicity 3Q™ software. Calibration curves were built by standards prepared in reagents only (RO) and in sample matrix. Matrix effects were evaluated by comparison of the slopes obtained from RO and sample matrix.

Results: (Limit 900 characters without spaces)

Mass detection method for 73 drugs was developed using time-managed-MRM algorithm of the QSight Simplicity 3Q™ software; the optimum dwell time of multiple MRM transitions was generated automatically for the targeted analytes, which saved time in method development and improved data quality and analytical performance. All calibration curves built from both the reagents only (RO) and chicken sample matrix showed good linearity (0.1X to 2X, X are the tolerance levels) with correlation coefficient (R²) larger than 0.99 for most the analytes studied. In this study, sample matrix effects were evaluated by comparing the slopes of calibration curves obtained from RO with slopes obtained from chicken sample matrix. Sample matrix effect (%) was calculated by the percentage difference between the slopes. When the percentage difference is positive, there is a signal enhancement effect, whereas a negative value indicates signal suppression effect. It was found that sample matrix effects are significantly reduced during SPME sample preparation (only 4 out of 73 analytes have matrix effect > 20%) compared to other sample preparation method (12 out of 73 analytes have matrix effects >20%). To overcome matrix effects and reduce variations in analytical results, matrix-matched calibrations were used in all the methods for quantification of all analytes. For SPME method, the method accuracy (or relative recovery) was evaluated by spiking the analytes to the samples at three concentration levels of 0.3X, 0.9X and 2.5X, respectively and the recoveries of analytes ranged from 70% to 120 % with RSD < 20% for almost all the analytes studied, demonstrating good accuracy and precision of the method. To compare with other sample preparation methods (direct injection and d-SPE clean up), the recoveries of analytes were also evaluated by spiking the analytes to the samples at two concentration levels of 0.5X and 1X, respectively and the recoveries of analytes ranged from 70% to 120 % with RSD < 20% for most of the analytes studied when the extract was analyzed directly without clean-up. However, the recoveries for several classes of analytes are lower after d-SPE clean-up, such as for fluoroquinolones, tetracyclines and some tranquilizers. Similar results were also obtained using different sorbents such as end-capped C18 and Z-Sep+ for d-SPE in this study, which are in good agreement with the published results[1-3]. The limits of quantification (LOQs) for the drugs studied are all below the tolerance limits or the maximum residue levels (MRLs), but SPME method provided lower LOQ compared with other Methods: The developed SPME-LC-MS/MS method can be applied for the fast screening and quantification of multi-class veterinary drug residues in chicken samples.

Conclusions (Limit of 400 characters without spaces)

A multiclass, multi-residue method for analysis of 73 veterinary drugs in chicken muscle was developed by coupling automated solid-phase microextraction (SPME) sample preparation with UHPLC-MS/MS. To get good analyte recovery, reduce sample matrix effects and carryover, the parameters affecting SPME performances were evaluated and optimized. The method was validated according to regulatory guidelines, taking into account Canadian maximum residue limits (MRLs) and US maximum tolerances for veterinary drugs in meat. A multi-day validation demonstrated that the developed method is suitable for fast and reliable quantitative analysis of 73 veterinary drugs in chicken muscle at half or below the maximum regulatory levels. Compared with other sample preparation methods such as direct injection and the frequently used modified QuEChERS method, SPME method demonstrated the following advantages: it requires less sample and solvents, easy to be automated, has less sample matrix effects and has the potential to be used for in-vivo animal sampling and fast screening by direct coupling to mass spectrometry. The selectivity and sensitivity of SPME can be tuned by using different extraction phases.

Novel Aspect: (Limit of 150 characters without spaces)
A new, fast, effective and automated SPME sample preparation method coupled with UHPLC-MS/MS for high throughput multi-class multi-residue analysis of veterinary drugs in chicken

References

Title: UHPLC-HRMS Analysis of Theobromine in Costa Rican Theobroma Cacao

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Keywords: High Resolution Mass Spectrometry, Costa Rican Cacao, UHPLC-HRMS, Theobromine

Abstract:
INTRODUCTION: Costa Rican Cacao has been a staple within Costa Rican culture for many centuries. Within the past 10 years, Costa Rican cacao has made a return for chocolate enthusiasts, helping to provide antioxidants to humans and satisfy sweet cravings. One of the major components of chocolate is Theobromine. Theobromine is a bitter alkaloid/flavonoid beneficial in the treatment of hypertension, arteriosclerosis, and angina pectoris.

METHODS: The samples were centrifuged and vortexed numerous times to give a purified product. A three-minute gradient method with a flow rate of 300 uL/min was developed on the UHPLC-HRMS using HPLC-grade water and acetonitrile. Quantitative analysis on Theobromine was completed on the Thermo Scientific UHPLC and LTQ Orbitrap Discovery equipped with an ESI ion source. 2-Hydroxyethyl theophylline was used as the internal standard.

RESULTS: In a previous study from our laboratory, Headspace GC-MS analysis was used to identify flavonoids from Cocoa Beans. Theobromine was the major component found. To give the precision of the theobromine extraction process, the recovery analysis was 86%. Raw, unroasted, roasted, and 100% cacao was analyzed in concentrations of ng/mL. Raw cocoa had a concentration of 586 ng/mL and unroasted cocoa had a concentration of 747 ng/mL. Roasted cocoa had a concentration of 521 ng/mL and 100% cacao had a concentration of 1018 ng/mL. Roasted cocoa had the lowest concentration of theobromine.

Unroasted cocoa had a higher concentration of theobromine prior to roasting cacao. Theobromine could have been lost based on the roasting process or choice of roasting. The 100% cacao chocolate bar had the highest amount of theobromine. Additional theobromine existed and contributed to a more bitter and rich cocoa flavor. Theobromine levels in popular commercially available chocolates such as Lindt, Hershey, Mars, etc will be discussed.

CONCLUSIONS: Theobromine levels in popular commercially available chocolates such as Lindt, Hershey, Mars, etc. were determined by UHPLC-HRMS and compared between raw, unroasted, and roasted chocolates.

NOVEL ASPECT: A sensitive and selective UHPLC-HRMS method to quantify Theobromine levels in chocolates.
Simultaneous Quantitation of Fat soluble Vitamins in infant milk formulae using 5500 QTRAP LC-MS/MS system

Ashley Sage (1), Jianru Stahl-Zeng (2), Alka Verma (3), Anoop Kumar (3), Manoj Pillai (3)

SCIEX, Market Development, Warrington (1) - SCIEX, Market Development, Darmstadt (2) - SCIEX, Market Development, Gurgaon (3)

Introduction:

Fat-soluble vitamins play an important role in the stimulation of synthesis and degradation of nutrients enhancing immune function and growth performance of living organisms. In recent years there has been great emphasis on simultaneous analysis of fat-soluble vitamins. The most commonly used methods of sample preparation for the analysis of fat-soluble vitamins in foods and pharmaceuticals include sample saponification and later extraction of the vitamins from the unsaponifiable matter. Saponification process exhibits disadvantage such as oxidation, low recovery, decomposition of vitamins labile under alkaline condition. Also there are few methods reported for simultaneous determination of fat-soluble vitamins in biological fluids and food matrices, the reported methods have only enabled determination of few vitamins, which usually have been vitamins A and E and hydroxy metabolites of D2 and D3 vitamins because other vitamins are present at extremely low concentrations. The present work aims at development of highly selective and sensitive LC-MS/MS method in order to determine complete profiles of fat-soluble bioactive compounds and to design a sample treatment method without hydrolysis for the simultaneous determination of fat soluble vitamins in infant milk formulae.

Methods:

Standard Stock Solution of each vitamin (A, A acetate, A palmitate, D2, D3, E, E acetate, E Succinate, K1, K2) was prepared by mass in ethanol containing 0.025% (m/v) of BHT to provide a concentration of 1 mg/mL for all the fat soluble vitamins. Chromatographic performance was optimized using different combination of mobile phase applied to variety of C18 column to achieve optimum chromatographic resolution. Best chromatographic performance was achieved by using Phenomenex Kinetex 2.6 µm C18 (100X4.6) mm column with mobile phase A: 2mM Ammonium tetra fluoro acetate and B: 100 % Methanol. Sciex QTRAP® 5500+LCMS/MS mass spectrometric system was used in multiple reaction monitoring (MRM) mode. Analysis was carried out in positive polarity with electron spray ionization mode. Multiquant™ 3.0.2 software was used for quantitation analysis.

Preliminary Data

Protein precipitation followed by liquid-liquid extraction was used as extraction technique. Ethanol containing 0.025% of Butylated hydroxytoluene was used as precipitating agent and Dichloromethane was used as extraction solvent. Calibration curve with the range of 5 to 500 ppb was plotted with linear regression coefficient (R2) value greater than 0.99 for each analyte. Percent recovery ranged from 50 to 85 % for all the vitamins. Matrix effect was also calculated in terms of matrix factor and percent CV was found to be less than 15 % for each analyte. Multiquant™ 3.0.2 analysis revealed 6 orders of linear dynamic range of quantitation. Limit of quantitation (LOQ) was achieved in nanogram level for all of the vitamins. Ion ratio of quantifier to qualifier was used for quantification.
Novel aspect:

Highly sensitive and selective method with good chromatographic resolution for simultaneous quantification of fat soluble vitamins in the concentration range of 5 ppb to 500 ppb in infant formulae without hydrolysis (saponification) has been developed.
Introduction
Multiclass approaches are more and more appreciated since they improve the cost effectiveness of monitoring programs to detect residues in food. Starting from our previous works [1; 2] in which we developed procedures for the determination of 62 antibiotics in meat and milk, the aim of this study was the widening of the method scope including 46 new veterinary drugs.

Methods
Sample preparation and instrumental conditions were those reported by Moretti et al. [1; 2] with some modifications. The analysis was performed using liquid chromatography/quadrupole-orbitrap mass spectrometry. The acquisition was carried out in positive ionization mode (ESI+), except for closantel and nitroxinil (ESI-).

Results
Some slight modifications of sample treatment and chromatographic conditions were required to obtain satisfactory quantitative performances (accuracy) for the new 46 analytes, most of which were antiparasitic drugs such as avermectins and benzimidazoles. Only an example: prior to LC injection the dried residue had to be dissolved in a mixture containing ammonium acetate/acetonitrile (50:50, v/v), instead of ammonium acetate as previously optimized [1; 2]. In fact, the introduction of lipophilic drugs such as ivermectin or closantel required a certain percentage of organic solvent to avoid insufficient solubilization of these analytes and, therefore, scarce accuracy. At the end of the development, a full validation study was carried out. The observed recoveries were generally higher than 70% and precision (inter-lab reproducibility) lower than 20% both for meat and for milk.

Conclusions
The widening of the number of analytes (from 62 to 108) included in our previously developed multiclass methods for antibiotics in food has been successfully achieved. The validation study demonstrated that the new procedure is fit for purpose [3] and therefore applicable in the official monitoring programs.

Novel Aspect
The flexibility of multiclass approaches must be experimentally verified when quantitative confirmatory methods are developed, as here demonstrated.

References

Acknowledgements
The authors gratefully acknowledge financial support from the Italian Health Ministry (“Determination of veterinary drugs in food: multiclass methods 2.0” IZSUM RC 022015).
INTRODUCTION:

Attack of the grapevines by fungi is one of the main challenges facing producers as it compromises the production volume and quality of grapes destined for wine production. Due to these diseases, fungicides are routinely used. Thus, the objective of this work was to evaluate the presence of fungicides in 190 wines produced in southern South America to promote discussion regarding the importance of monitoring pesticide levels in wines.

METHODS:

A total of 190 red wines were analysed; 41 of which were Brazilian, 75 were Chilean, 30 were Uruguayan, and 44 were Argentinian. The fungicides were analysed by LC-MS/MS and GC/MS using a method validated according to the SANTE recommendations [1]. This work proposed an advancement in the quantification of dithiocarbamate in which the levels are determined in mancozeb equivalent.

RESULTS:

Of the 190 wines analysed, 76 contained fungicide residues (40%), and of those, 31 (16.3% of the total analysed) presented values above those allowed for the grape, which would lead to them being classified as irregular. Among the 44 pesticides evaluated, dithiocarbamate was the most prevalent as it was found in 16.3% of the samples (n = 31); the next most common was azoxystrobin, which was found in 14% of the samples (n = 26), and of those, 11 were irregular. Carbendazim was found in 13% of the samples (n = 25), of which 12 were outside the limits set for grapes. Almost 20% of the samples (n = 32) contained residues of 2 or more pesticides. One of the wine samples (a blend from 2014) had five different pesticides, carbendazim, metalaxyl-M, azoxystrobin, dithiocarbamate and dimethomorph. In addition, 8 samples showed cyprodinil residues, a fungicide prohibited in grapes.

CONCLUSIONS:

The main fungicides found were dithiocarbamates, azoxystrobin, carbendazim, difenoconazole, dimethomorph and cyprodinil. Of the 190 wines analysed, 40% contained at least one pesticide, and 16.3% had levels above the MRL (irregular). The present work emphasizes the importance of the creation of laws regulating pesticides in wines, which will facilitate foreign trade.

NEW ASPECT:

To date, this is the largest study on pesticide residues in South American wines.

REFERENCES:

A Robust and Sensitive Method for the Direct Analysis of Polar Pesticides in Food and Environmental Samples

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1. Nofalab Laboratories, Schiedam, NL; 2. SCIEX, Darmstadt, DE

Introduction

The prevalence of multi-residue LC-MS/MS analyses for the quantification of pesticides in food and environmental samples has been steadily increasing for many years, and they are now considered to be a minimum requirement of most laboratories working in these fields. Modern tandem quadrupoles are capable of detecting such regulated compounds at very low levels with minimal sample preparation, such as QuEChERS, thereby enabling labs to process large numbers of samples for many analytes with a fast turnaround. However, some very polar analytes which are not amenable to the extraction procedure, chromatographic method or are poor ionisers require additional single-residue methods which involve time-consuming preparation and separation and often involve derivatisation to improve detection.

Recent increase in public concern regarding the presence of glyphosate has significantly increased the requirement to analyse it and its metabolites in food, feed and the environment, so has accelerated the need for a more efficient and robust analytical method. The extraction and chromatography of these compounds is well described in the EURL-QUPPE method, but the separation is not robust in practice, so system and method maintenance are intensive. Several different HPLC or HILIC based methods have failed to address the issues of reproducibility and sensitivity, so FMOC derivatisation prior to analysis is often still employed for glyphosate, AMPA and glufosinate. Although possible to automate, this procedure is still time consuming or expensive, and is not applicable to the other polar pesticides of interest.

Method

Nofalab is an independent sampling and testing laboratory based near Rotterdam, NL specialising in the fields of food, feed and environmental safety. The increasing pressure to provide fast, quantitative analysis has driven them to invest in additional LC-MS/MS instrumentation and develop a new method which covers as many of these polar pesticides in a single analysis as possible. Ion chromatography has been shown to be beneficial for separation, but the need for a suppressor is detrimental to MS analysis and the problem of changing inlet systems on a heavily used mass spectrometer makes it impractical in a busy lab performing primarily reverse-phase LC. So, the final method, presented here, makes use of an IC column in a method-switching RP system with MS amenable mobile phases at pH 9. Such conditions configure glyphosate ideally for MS detection with good retention and separation of the other analytes and matrix interferences. The method meets the Sante requirements of reproducibility (<20%) and recovery (80-110%), and the LOD of the method is below 0.01 mg/kg. Excellent long-term stability and robustness were achieved throughout the validation of this method for food samples extracted by the QUPPE procedure.

Results

Where environmental samples require testing, the regulatory limits are much lower and interference from matrix more problematic in traditional analyses with a short retention time, so derivatisation is often the only option. However, since glyphosate is well retained in this new method, the potential to further develop it for direct large-volume injection was investigated in collaboration with SCIEX. By modifying the gradient conditions and optimising the injection parameters, a second method specific to environmental water samples has been developed. Although the LVI is more susceptible to changes in pH (for example, due to evaporation of mobile phase) robustness has been shown to be similarly good, and allows detection of the same suite of analytes with a LOD of <0.02 ng/l.
Non-targeted fast screening of hundreds residual pesticides on fruits, eggs and soil using ambient mass spectrometry

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Keywords: non-targeted screening, pesticide, ambient mass spectrometry, miniature, high-throughput

Introduction:
Thermal desorption-electrospray ionization (TD-ESI) source[1] combined with a miniature triple quadrupole mass spectrometer (Ultivo, Agilent) was applied to rapidly screen hundreds of residual pesticides on fruits and in soils and eggs without or with minimal sample pretreatment.

Methods:
The TD-ESI/MS system consists of sampling probe, heated oven, electrospray device, and mass analyzer. For sampling analytes on the fruit, a stainless steel probe gently scrapes through the fruit surface for 2 cm. Since soil contains complicated components, ca. 2 uL of the organic solvent extracts was collected on the sampling probe after rapid extraction. The probe was then inserted into a heated oven to thermally desorb the analytes on it, the analytes were subsequently carried by a nitrogen gas stream to join in an ESI plume, the analyte ions were formed by ion-molecule reactions between the charged species (H3O+, CH3OH2+, (H2O)2+, etc) in the ESI plume and analytes. The analyte ions were detected by the miniature triple quadrupole mass spectrometer attached to the TD-ESI source.

Results:
Without redundant extraction and separation processes, the analysis of each sample is completed in 15 s. Pesticide database was well-established by using TD-ESI coupled with a miniature triple quadrupole mass spectrometer. In addition, approximate 200 pesticides can be simultaneously detected in one analysis. The detection limit of pesticides in fruits were in sub ppm level and below the legal limit of their respective concentrations.

Conclusions:
Thermal desorption-electrospray ionization/mass spectrometry is a sensitive, reproducible, qualitative, and rapidly analytical technique; it is also suitable for large-scale screening and high throughput analysis of fruits and soil to assure consumer’s health.

Novel Aspect:
Non-targeted screening of hundreds residual pesticides can be rapidly done by using thermal desorption-electrospray ionization/mass spectrometry.

References:
Introduction: (Limit of 400 characters)
Polybrominated diphenyl ethers (PBDEs) are flame retardants [1-2]. Numerous studies have proved that PBDEs showed the hepatotoxicity, embryotoxicity, thyroidogenic and estrogenic effects on animals [3-5]. So the residue levels of PBDEs in human are of great concern because of their potential toxicity. In this study, 7 PBDEs in breast milk from Beijing were determined, and the variation trend from 5 donors during the first six months lactation was dominated.

Methods: (Limit of 400 characters)
55 breast milks were collected from 30 donors living in Beijing over 5 years. Five of donors provided one sample per month and sampled for six months. The sample was treated with liquid-liquid extraction method (LLE). In general, 10 ml of sample was extracted by mixture of ethanol, diethyl ether and hexane, and then cleaned up by packing columns and detected by GC-NCl-MS. An external standard method was applied to qualitative and quantitative analysis.

Results: (Limit 900 characters)
The results showed that PBDE28, PBDE47 and PBDE153 were detected in samples, and the detected rates were 23.3 % for PBDE28, 26.7% for PBDE47, and 60.8 for PBDE153, respectively. Except for PBDE28, the result was similar to the research result reported by Li J.G. [2]. The concentration of PBDE47 was ranged from 0.21 to 1.21 ng/g fat, with 0.78 ng/g fat of mean value (n=30). Compared to PBDE47, PBDE28 and PBDE 153 were detected with higher average value, which were 5.40 ng/g fat for PBDE28, and were 2.73 ng/g fat for PBDE153, respectively (n=30).
The analytical results of PBDE28, PBDE47 and PBDE153 in breast milk, during the first six months lactation, indicated that the three compounds were not always detected, and the concentrations don’t decrease gradually, but shaved.

Conclusions (Limit of 400 characters)
The level of PBDEs was lower than the value reported by Li in 2010. This indicated that the residue level of PBDEs in local residents or exposure risks of local residents to PBDEs have decreased. It was well known that the diet exposure was the main exposure pathway. So we take the daily intake from diet into account for the waving change of PBDEs in breast milk during the first six month lactation.

Novel Aspect: (Limit of 150 characters)
The updated residue levels of PBDEs in breast milk from Beijing.
A time trend of PBDEs in breast milk was monitored during the first six months lactations.
The level of PBDE in breast milk may not, in some extend, showed its accumulated level in human body.

References
Screening and Quantitation for Food Matrices using scanning SWATH acquisition.

David M. Cox, Jeff Rivera, Holly Lee, Vanaja Raguvaran, Jianru Stahl-Zeng,

Introduction
MSMS data is important in confidently identifying a compound in a screening assay. Obtaining an MSMS for every compound in a run can be a challenge. SWATH acquisition can acquire MSMS that represent every precursor mass of interest at every time point. However, the deconvolution of MSMS requires that each fragment ion have a different chromatographic profile or elution time. Information dependent acquisition (IDA) will consistently trigger on the most abundant compounds, but can occasionally miss some candidates. Combining SWATH with IDA improves the identification of compounds in screening assays.

Methods
X500R, LC, pesticide kit. Acquire SWATH, IDA, and SWATH+IDA data. Process with SCIEX OS.

Preliminary Data
SWATH acquisition uses precursor isolation windows that typically range from 5-25 Da wide. Even when two or more compounds fall within a SWATH precursor isolation window, and have similar elution times, deconvolution of the MSMS is usually possible using a PCA/PCVG based technique. This MSMS is used for library searching and subsequent compound identification.

With IDA, the precursor isolation window is 1 Da wide, which greatly reduces, but does not eliminate, the chance of two compounds being in the same window. However, if IDA does not trigger on a candidate, there is no MSMS data to confirm a compound identification with.

The amount of time spent on each cycle of acquisition is limited by how many points across an LC peak are required. With SWATH acquisition, this limit determines how wide the precursor isolation window for each SWATH experiment must be. Wider windows increase the chance of deconvolution failing, narrow windows take more time. With IDA, this limit determines how many candidates can be selected each cycle. Fewer candidates increases the chance that a candidate ion is missed, but more candidates take more time. In both cases, this trade-off follows a typical pareto principle (80/20- rule) where the majority of performance is achieved early (wide SWATH windows or small number of IDA candidates). Combining these two scan modes into one acquisition method enables a few wide SWATH windows and a small number of IDA candidates to perform well.

Novel Aspect.
Data independent acquisition (SWATH) combined with IDA to improve screening assay erformance.
HBCDs are used as additives in plastic polymers to reduce flammability; they can be released in the environment and accumulate in the food chain. Technical mixtures contain mainly diastereomers $\alpha$, $\beta$ and $\gamma$ [1]. Both gas chromatography (GC) and liquid chromatography (LC) are used for HBCDs analysis, but GC does not resolve the diastereomers. The aim of this study was to set up the best analytical method for HBCDs in food comparing GC-MS/MS and LC-MS/MS.

Methods:
HBCD were analyzed in isotopic dilution after QuEChERS-like extraction, acidic Extrelut-NT3®/Si-SPE and GPC clean up. Instrumental analysis was performed in GC-MS/MS on a RTX-1614 (15 m x 250 $\mu$m x 0.10 $\mu$m) column and PTV injection (10 $\mu$L) [2]. A LC-MS/MS method was also developed, using a Kinetex XB-C18-100Å column (2.6 $\mu$m, 100 x 2.1 mm) and, as mobile phase, MeOH (phase A) and 2 mM CH3COONH4/MeOH (50:50) (phase B) [3]. The method was validated in muscle at 10, 20, 50 and 100 pg/g.

Results:
Literature reports that GC does not enable the resolution of $\alpha$, $\beta$ and $\gamma$ isomers: HBCDs can be quantified only as sum. Furthermore, the development of the GC method is extremely tricky because the three isomers inter-convert at $T>160^\circ$C and decompose at $T>240^\circ$C or in dirty GC systems [4]. The performances for GC-MS/MS method were not satisfactory, because of the impossibility to separate the three isomers yielding in a not completely accurate quantification. During method set up, we experienced LC-MS/MS to be the method of choice for HBCDs analysis, because of the absence of thermal degradation and the ability to resolve the $\alpha$, $\beta$ and $\gamma$ isomers. Therefore the analytical performances were systematically studied in LC-MS/MS. Accuracies, in intra-laboratory reproducibility conditions, were satisfactory for all the isomers: apparent recoveries were between 92 and 107% and precisions between 9 and 22%. Recoveries of the surrogate labelled standards were higher than 70%. No laboratory background contamination was measured.

Conclusions:
The results confirmed LC-MS/MS as the method of choice for HBCDs analysis enabling to reach good performances also at 10 pg/g, which was set as method LOQ. The procedure performs well in fish products and lean terrestrial muscles. The applicability to other food matrices should be further tested.

Novel Aspect:
Few data are available in Italy about HBCDs in food, therefore this method enables an extensive monitoring at very low contamination levels.

Acknowledgments
The authors gratefully acknowledge financial support from the Italian Health Ministry - Projects Codes: RC0052014 and RC0052014.

References:
EFSA Journal, 9(7):2296, 2011
Introduction:
PBDEs and HBCDs are brominated flame retardants (BFRs) used as additives in plastic polymers to reduce their flammability. They can be released in the environment and accumulate in food chain. Few data are available in Italy about their environmental levels and dietary exposure [1,2]. In this study, levels of PBDEs and HBCDs were measured in marine fish samples collected in local markets.

Methods:
This study involved the analysis of 15 PBDE congeners (28, 47, 49, 66, 77, 85, 99, 100, 138, 153, 154, 183, 197, 206, 209) and 3 HBCD (\(\text{B}, \text{S}, \text{Sa}-\text{HBCD}\)) in 18 marine fish samples collected from local markets in Central Italy. The samples were analyzed either by GC-MS/MS (PBDEs) or LC-MS/MS (HBCDs) in isotopic dilution, performing a QuEChERS-like extraction followed by a two-step clean-up [3].

Results:
PBDEs and HBCDs were measured above the MDL in most of the samples. Their concentrations were generally in the range of ppt, only BDE47 reached the ppb level in one sample. BDE47 is the dominant congener in all species and its concentration was >LOQ in 16 out of 18 samples; in 12 samples also BDEs 100 > 99 > 49 and 154 were measured. Total PBDE contamination was estimated as sum of the 15 congeners adding 0.5 LOQ in case of not quantified. The highest PBDE levels were measured in a spiny dogfish and mackerel (Sum: 3057 and 874 pg/g respectively), while cod and hound shark showed the lowest levels (Sum: 301 and 311 pg/g respectively). The highest HBCD isomer was always the \(\text{B}\), which was quantified in 13 of 18 samples [4]. Only in one mackerel also \(\text{S}\)-HBCD was identified. The total HBCDs content ranged from <LOQ (cod and hound shark) to 3160 pg/g (mackerel).

Conclusions:
The average concentration (N=18) was 616 pg/g for PBDEs and 104 pg/g for HBCDs. The highest levels for both classes were measured in spiny dogfish (lipid 7.7%), confirming that fatter species are generally the most contaminated [1] while lean ones (cod and hound shark-lipid <1%) had lower concentrations. No data are available in spiny dogfish in literature while comparable results are reported for all the other species [5,6].

Novel Aspect:
Among food, fish highly contribute to PBDEs and HBCDs human intake. Few data are available in Italy about BFRs in fish, therefore these results may help in assessing background levels.

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Aznar-Alemany O. et al, Food and Chemical Toxicology 104:35-47, 2017
Trabalon L. et al, Food and Chemical Toxicology, 104:48–56, 2017
702 - ORGANIC (PBDES,NDL-PCBS) AND INORGANIC (PB, CD, HG, AS, NI, MN) CONTAMINANTS ASSESSMENT IN WILD BOAR (SUS SCROFA) BY GC-MS/MS AND ICP-MS

TAMARA TAVOLONI (1) - ARIANNA STRAMENGA (1) - VALERIA CASTRO (2) - MARTINA CIRIACI (1) - MICHELA CONQUISTA (3) - FRANCESCO GRIFFONI (1) - PAOLO PALOMBO (1) - GIAMPIERO SCORTICHINI (2) - TOMMASO STECONI (1) - ARIANNA PIERSANTI (1)

ISTITUTO ZOOPROFILATTICO SPERIMENTALE DELL'UMBRIA E DELLE MARCHE "TOGO ROSATI", SEZIONE DI ANCONA, ANCONA (1) - ISTITUTO ZOOPROFILATTICO SPERIMENTALE DELL'UMBRIA E DELLE MARCHE "TOGO ROSATI", SEDE DI PERUGIA, PERUGIA (2) - ISTITUTO ZOOPROFILATTICO SPERIMENTALE DELL'UMBRIA E DELLE MARCHE "TOGO ROSATI", SEZIONE DI TOLENTINO, MACERATA (3)

Keywords: PBDEs, NDL-PCBs, Heavy metals, wild boar, hunting.

Introduction:
Wild boar is an omnivore species often used as environmental pollution sentinel. It is hunted and consumed as food, therefore may expose population to relevant levels of contaminants. This study investigate the presence of some organic (PCBs and PBDEs) and inorganic (Cd, Hg, Pb, As, Ni, Mn) pollutants in wild boars caught in Central Italy.

Methods:
Samples were caught in a rural area close to the Appenninimountain in the Macerata district (Marche). Muscle, fat and liver of 44 wild boars hunted in 2017/18 season were selected. The samples were grouped by organ type, sex and weight to yield 21 laboratory pools. NDL-PCBs and PBDEs were analyzed by GC-MS/MS [1,2], Pb, Cd, As, Ni and Mn by ICP-MS and Hg by AAS (FIMS).

Results:
PCBs, Pb, Cd and Mn were above LOQ in all the selected tissues, PBDEs only in muscle, As was not detected in almost all the samples while traces of Ni and Hg were measure only in livers. PCBs were all below MRLs [3] and the highest levels measured in muscle (mean 13 ng/g fat), in liver mean PCBs was 0.5 ng/g; congener 153 was dominant, followed by 138 and 180. The concentration increases with weight in males while decreases in females, where the levels are generally lower, probably because they can transfer contaminants to their progeny [4]. Also for PBDEs, muscle was more contaminated than liver where almost all the BDEs were below LOQ. In muscle dominant congeners were 47> 99> 100. 15PBDEs in muscle (mean 848 pg/g) was generally higher than the ones reported from other authors [5]. Cd in muscle (mean 0.028 mg/Kg) was lower than MRL (0.050 mg/Kg) except for two samples (0.057 and 0.053 mg/Kg). None of the livers exceeded MRL (mean 0.25 mg/Kg) [3]. The results are comparable with previous studies [6, 7]. Pb values fell between 0.025 and 6.99 mg/Kg in muscle while in liver the mean level was 0.04 mg/Kg. Six out of 7 muscles exceeded Pb MRL; this could be related to Pb dispersion in muscle due to bullet fragmentation. Mean levels of Mn were 0.482 (muscle) and 1.774 mg/Kg (liver).

Conclusions:
Muscle resulted to be the most contaminated tissue respect to liver and fat for all the considered contaminants. Pb residues in muscle were very high as a result of fragmentation of bullets used in hunting.

Novel Aspect:
Few data are available about contamination of wild fauna in Central Italy, therefore these results may help to start assessing background levels in Italy.

Acknowledgments:
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References:
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LC-MS/MS METHOD TO EVALUATE THE PRESENCE OF EXOGENOUS GLYCEROL IN WINES

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Keywords: LC/MS/MS, glycerol, wine, cyclic diglycerols, 3-methoxypropane-1,2-diol

Introduction
Glycerol is a natural constituent of wine being produced by yeast during fermentation and its concentration ranges between 5 and 11 g/L depending on yeast strain. It plays an important role on the sweet taste of wine [1]. The addition of synthetic glycerol to wines in order to increase this parameter is not allowed by the European oenological legislation [2]. Actually, the strategy to investigate the presence of exogenous glycerol in wine is based on the detection of impurities produced by the industrial processes on its production [3], which are absent in wines. Synthetic glycerol contains the following impurities: 3-methoxypropane-1,2-diol (3-MPD) and cyclic diglycerols (CycDs) formed during the purification of the rough industrial product conducted through a distillation process. Currently, the determination of these impurities is carried out by an GC/MS method after an extraction step in ethyl ether. As an alternative to this “old” method, a proposal has been made for a new approach based on liquid chromatography coupled to the mass spectrometry (LC-MS/MS).

Methods
The chemical analysis of 3-methoxypropane-1,2-diol (3-MPD) and cyclic diglycerols (CycDs) has been performed using an Acquity Liquid Chromatograph (UPLC Waters Corporation, Milford, MA, USA) coupled to an Xevo TQ Mass Spectrometer (Waters Corporation, Milford, MA; USA). Chromatographic separation has been performed with an C18 HSS T3 column (2.1 mm x 100 mm, 1.8µm); flow rate 0.45 ml/min; Eluent A, Water + NH4Ac (5mM); Eluent B, Methanol. Mass spectrometer was equipped with an electrospray ion source operating in positive ion mode; capillary voltage 1.5 kV; nitrogen gas flow, 1000 L/h; source temperature, 150 °C. Acquisition were carried out in MRM (multiple reaction monitoring).

Results
Gas chromatography mass spectrometry (GC-MS) is the analytical method adopted by the OIV to detect the fraudulent addition of glycerol by measure these two contaminants [4]. To the best of our knowledge, this is the only validated method to detect and quantify 3-MPD and CycDs in wine. However, the application of the GC-MS method shows serious difficulties: sample preparation and time analysis (runtime about 42 min); dirty injections with early consumption of the column (particularly in the case of sweet wines); mass spectrometer ion source must be cleaned very often to improve the required sensitivity. In order to overcome these limitations, a simple LC-MS/MS method has been developed.

Conclusions
The proposed method could be a useful tool to rapidly screening wine suspected of fraudulent glycerol addition. Keeping GC-MS analysis to further confirm positive results.

Novel Aspect
High throughput LC-MS/MS method respect the official method (using GC/MS) proposed by OIV.

References
Introduction:
Food packaging materials can contain a wide range of known and unknown substances [1]. Chemicals such as impurities of ingredient, by-products, and degradation products are also plausible. These chemicals are referred to as non-intentionally added substances (NIAS). In this study, NIAS from polyethylene terephthalate (PET) bottles were examined by liquid chromatography high-resolution mass spectrometry (LC/HRMS).

Methods:
To understand migration of NIAS to bottled beverages, non-volatile polar NIAS was assigned as a main objective in this study. An LC/HRMS system comprising ExionLC AD and X500R (SCIEX) was used for non-target analysis. X500R mass spectrometer, which had a mass accuracy of 2 ppm, was used to acquire the HR mass spectra with a mass resolution of 30,000. Material tests and long storage tests of PET bottles obtained in Japan, were carried out.

Results:
Several compound series with a constant interval of m/z 192.042 were present. This value corresponded to C10H8O4, a monomer unit of PET. The mass spectrum includes much information such as fragmentation, exact mass, neutral loss, adduct ion formation, and isotopic pattern. Each element sticks to fundamentals and overlapped one another, so that the mass spectrum looks complex. It is cumbersome but usually possible to interpret the mass spectrum to determine a structure. The congener with the highest abundance in the material tests was cyclic PET trimer. Monomers and oligomers whose one terminal was carboxy group were also identified by the interpretation of mass spectra and confirmed by reference standards. One-year long storage tests with simulated food solvent showed a gradual release of these substances to the contents. PET bottles obtained from 8 manufacturers showed roughly similar migration profiles.

Conclusions
Unlike other plastic materials and articles, PET bottles obtained in Japan included less additives. In the examination by non-target analysis using LC/HRMS, PET congeners were the main migrants from PET bottles. One-year long storage tests with simulated food solvent showed a gradual release of these substances to the contents. The highest concentration of 4-[(2-Hydroxyethoxy)carbonyl]benzoic acid was 4.6 ng/mL and detected in one-year long storage test sample.

Novel Aspect:
NIAS from PET bottles was examined by non-target analysis with LC/HRMS. Several PET congeners were identified from HRMS data and confirmed by reference standards.

References
VALIDATION OF AN ACCURATE MASS SCREENING METHOD FOR PESTICIDE RESIDUE ANALYSIS IN COCOA BEANS AND EVALUATION OF COLLISION CROSS SECTION VALUES USING ION MOBILITY QTOF MASS SPECTROMETRY

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Keywords: Cocoa beans; Pesticide residues; Ion mobility; Collision cross section; QuEChERS

Introduction:
Multiresidue screening method for the analysis of pesticide residues in dried cocoa beans was validated and applied to imported and domestic cocoa beans samples. The screening method covers 130 pesticides (insecticides, fungicides, herbicides) of different chemical classes.

Methods:
The method was based on modified QuEChERS (Quick Easy Cheap Efficient Rugged Safe) extraction method [1] and detection using ion mobility quadrupole time-of-flight mass spectrometry (IMS-QTOF). Acetonitrile was used as extraction solvent and the effect of d-SPE clean-up procedure was studied relative to detection rate and screening detection limit.

Results:
In the present work, pesticide multiresidue screening method was proposed based on the application of UPLC-IMS-QTOF-MS. The screening method was validated and applied to the screening of 130 pesticides in cocoa beans samples and was found to be more selective towards the studied pesticides. The optimized screening criteria consisted of mass accuracy (± 5 ppm), RT (± 0.2 min) and CCS (± 2%) could decreased the false positive rate while maintain its detection rate at 98% compared with analysis without CCS values. Under this screening conditions, 33% of the investigated pesticides gave SDL of 10 µg/kg, whereas 44% and 15% of them presented SDL of 50 and 150 µg/kg, respectively. Finally, the automated screening workflow was applied to real cocoa beans sample through a monitoring study and 20% out of 60 samples were tested positive, in which drift time filtering improved the detectability and identification of the pesticides.

Conclusions:
IMS allows for the measurement of the CCS values, which provide information on the charge, shape and size of the pesticides [2]. Additional information in the form of CCS value provide more confidence in the identification process and lessening the probability of false positive during the pesticide screening process. Finally, a database of pesticide residues that consist of accurate mass, retention time, fragment ions, and CCS values was developed for cocoa beans.

Novel Aspect:
Intensive monitoring has been reported in this study using accurate mass screening with the help of CCS database for cocoa beans across wide range of different chemical classes.

References
Keywords: food analysis, external sample contamination, ubiquitous environmental phenolic compounds, UPLC-ESI-MS/MS

Introduction:
Phenolic compounds belong to a group of substances that might be of synthetic or natural origin. Synthetic phenolic compounds are present in our environment in pharmaceuticals, preservatives, personal-care products (e.g. synthetic fragrances and surfactants), as pesticides and miscellaneous industrial chemicals and byproducts (e.g. from plastic industry) [1,2].

As such, they can be detected in food and beverages in which they might origin from additives, food processing and packaging materials or as contaminants from the environment including the analyst. Many of the synthetic phenolic compounds are suspected to have the ability to affect the human body acting as estrogen mimics and are collectively termed endocrine disrupting compounds (EDCs). EDCs are substances that “interfere with the synthesis, secretion, transport, binding, action or elimination of natural hormones in the body that are responsible for the development, behavior, fertility and maintenance of homeostasis (normal cell metabolism)”[3]. EDCs are still not regulated at the EU level, but the work is ongoing to develop scientific criteria and properties for regulation. This causes intense discussion over these substances’ toxicity and mutagenicity which is reflected in ever growing interest in the question of their presence in foods. Thus, analytical methods for reliable quantitative determination of ubiquitous environmental phenolic compounds in food are desirable, but the analysis represents a substantial challenge due to the overwhelming risk of unintentional sample contamination from the laboratory environment. Such contamination complicates the quantitative measurements of the initial amounts of phenolic compounds in foods, especially when they are present at low nanogram levels.

Method:
The method is an UPLC-ESI-MS/MS based multi-analyte method that applies multiple reaction monitoring (MRM) and isotope labeled internal standards for quantitative determination of phenolic compounds.

Results:
This poster focuses on analysis of a selected number of most relevant phenolic compounds that can be found in foods, as well as approaches to minimize and control the external contamination from the laboratory environment during sample handling and analysis. The method is not validated yet and the findings from pre-validation measurements of phenols in some food samples, after preventive measures to reduce the external contamination sources in our laboratory were implemented, are presented. The levels of the found compounds were clearly higher than in the reagent blank.

Conclusions:
According to the present experience, the analysis of phenolic EDCs at low concentration levels in mixed and complex food matrices using regular analytical methods and laboratory equipment seems to be practically unfeasible until all the environmental sources of these chemicals are identified and maximally reduced to minimize their recurrence and impact on the analysis in order to ensure the validity of the data.

Novel Aspect:
The method used to analyze phenolic compounds in food is fast and straightforward. It allows analyzing different groups of phenolic compounds in the same analysis. The method also comprises important measures that have to be considered to minimize external contamination during the sample pre-treatment and thereby ensure contamination free extraction and reliability of the measurements.

References


Introduction
The color is considered an important characteristic for consumers to evaluate olive oils quality. Pigments such as carotenoids and chlorophylls are the main responsible of this property but the color changes from green to yellow-brown due to the production processes or inadequate storage. In order to regreen the olive oils, instead of adding 20-30% of extra virgin olive oil, as it is established, different dyes or oil mixtures are used to adulterate [1,2].

Methods
A UHPLC-APCI-HRMS (Q-Orbitrap) method was developed to quantify chlorophylls, chlorophylls derivatives and carotenoid pigments and to detect adulterated olive oil samples due to the addition of dyes. A SPE procedure was performed using Silica SPE cartridges to clean-up the sample extracts. The UHPLC-APCI-HRMS method was achieved using an Accucore C18 column and working in positive ion mode and full scan acquisition mode (range 200-1000 m/z, Rs 70,000).

Results
The chromatographic separation of pigments was achieved in less than 15 min under gradient elution with a quaternary mobile phase. All pigments were ionized with APCI in positive ion mode. Chlorophyll and its derivatives generated the protonated molecule [M+H]+ as base peak of the mass spectra with no significant in-source CID fragment ions. Concerning carotenoids, most of them also generated [M+H]+ as base peak with significant in-source CID fragment although, in the case of lutein, the in-source CID fragment ion [M+H-H2O]+ dominated the mass spectrum. Moreover, tandem mass spectrometry has been used to obtain structural information for further confirmation of these analytes in olive oil samples.

The UHPLC-APCI-HRMS/MS method was applied achieving a pigment profile (relative concentrations between the different pigments) useful for the detection of adulterated olive oils. Additionally, the pigment profile of different potential fraudulent colorants was also studied in order to identify further adulteration evidences.

Conclusions
The proposed UHPLC-APCI-HRMS/MS method showed good quality parameters and allowed the accurate pigment profile of olive oil samples. Moreover, this method provided enough evidences for the detection of adulterated olive oil by the addition of dyes.

Novel Aspect
This is the first time that UHPLC-APCI-HRMS is applied for the olive oil pigment profiling to detect a fraud due to addition of dyes.

References
621 - ISOTOPIC MAPPING OF MILK AND GROUNDWATER IN THE PRODUCTION AREA OF PARMIGIANO REGGIANO FOR FOOD TRACEABILITY

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Keywords: isotopes, milk, origin, Parmigiano Reggiano, feeding regime

Introduction
Determination of the geographical origin of foodstuffs is becoming of increasing interest to consumers and producers, since it may be used as a criterion for certifying quality and authenticity. The Protected Designation of Origin (PDO) trademark has been assigned to numerous local products based on their area of origin. In order to obtain this designation, the raw materials must have been produced and processed in the specific region from which the product gets its name. [1][2]

Methods
To make a check at the base of the production of "Parmigiano Reggiano" we began to collect monthly samples of groundwater, milk, hay and fodder from ten cattle-sheds in the production area. Sampling will last for at least a year. The stable isotope composition of the elements, such as O, H, C, N, have been evaluated with the aim of characterizing the milk of the Consortium areas and defining the isotopic fractionation of water ingested/milk produced and milk produced/food supplied.

Results e Conclusions
From the first results obtained it was possible to notice a differentiation of the isotopic values of O and H between plain and mountain milk. Even, the hay produced locally by breeders is different compared to the one purchased by import. Even, these greater characterizations could increase the added value of one producer’s cheese.

Novel Aspect
The results of the present study could be helpful for consumers, the food industry and government regulatory agencies as it can prevent fraudulent labelling of organic food. [3]

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Keywords: HS-SPME, GC-MS, wine, volatile organic elements, aromatic molecules

Introduction
Gas chromatography-mass spectrometry is widely employed to obtain important information on the aromatic profiles of wines, which are widely employed for differentiation and classification of wines based on geographical origin and grape cultivar. Wine is recognized to be very important in the human diet: the worldwide consumption of red wines reached over 240 mhl in 2017 [1].

Methods
On the basis of the general interest for local and sustainable products, we decided to focus our attention on some not yet studied Piedmont hilltop wines. The volatile molecules were extracted by means of headspace microextraction (HS-SPME) with a triphasic fiber (CAR/PDMS/DVB 30/50µm), and were quantified by means of gas chromatography-mass spectrometry. This extraction technique has gained universal approval and is employed in many applications such as aromas and fragrances recognition in food area, toxicology, environmental and biological matrices [2].

Results and Conclusions:
Multivariate chemometric approach allowed the identification of the representative profile of the selected typical Piedmontese wines, which will be useful for traceability and safeguard from food frauds.

References:
WINE TRACEABILITY: "VIGNETO ITALIA" PROJECT

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Keywords: wine, traceability, ICP-MS, Chianti

Introduction:
The project aims to classify wines on the basis of different geographical origin of the grapes. 246 and 393 grapes from known provenance are collected during 2013 and 2014 harvests. Then they are turned into wine by a vinification procedure that simulates the industrial process. Elemental composition of these samples is analyzed with ICP-MS and the resulting data are used to train a classification model that discriminates Chianti from other wines.

Methods:
Two quantitative procedures are used depending on the concentration level of the analytes. Most elements (10 ppb - 1000 ppm) are analyzed directly after 1:20 dilution and standard nebulization into the ionization chamber. For ultratrace elements (conc. < 100 ppt) the sample is diluted 1:5 after mineralization of the matrix; in this case the sample introduction system (Apex-Q with Spiro TMD) is designed to increase analytical sensitivity and to reduce interferences.

Results:
After statistical pre-processing of the data, three classification models are obtained through chemometric elaboration of ICP-MS analysis results: Linear Model and Similarity Model are based on supervised pattern recognition methods; Non Linear Model is based on Artificial Neural Networks (ANNs) which is one the most promising techniques to cope with the problem of automatic and intelligent classification. An additional model (Consensus) calculates the average between the results of the previous three models. The predictive models are validated by testing 134 wine samples with known geographical origin: the Consensus Model shows a non-error rate (NER%) of 89,9% with 18,7% of non-classified samples. Wine adulteration is also evaluated by analysing Chianti and non-Chianti samples blended at different rates: the results demonstrate that Consensus Model is able to detect down to (at least) 25% addition of non-Chianti wine to a Chianti sample, since it classifies such mixture as non-Chianti.

Conclusions:
The predictive model Consensus shows a satisfying percentage of correct classification. Some regions (such as Sicily) are difficult to differentiate from the Chianti area. But many regions with high wine production (Sardinia, Piedmont, Puglia, Marche, Campania, Basilicata, Umbria) are correctly identified. Besides the total overlapping of samples from vintages 2013 and 2014 suggests that correlation is not affected by vintage.
PROTEOMICS APPROACH FOR THE DIFFERENTIATION OF TUNA SPECIES

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Keywords: Authenticity, Food, Proteomics, HPLC-MS/MS, Tuna

Introduction:
Worldwide studies prove that food fraud of fishes is a relevant issue[1][2]. Tuna species (Thunnus spp.) are often affected caused by limited availability and high price differences [3]. As methods for the differentiation of tuna species like polymerase chain reaction (PCR) and isoelectric focusing (IEF) are not sufficient, the aim of this project is to develop a method for tuna differentiation based on species-specific marker peptides.

Methods:
Tryptical digested peptides of each species were measured with HPLC-MS/MS by information dependent acquisition (IDA). The identification of de novo peptides was done using the software PEAKS, comparison was done manually. The identified potential marker peptides were verified by targeted experiments using HPLC-MS/MS. Finally, a targeted method for the differentiation of all relevant tuna species was developed.

Results:
In this project, we included all eight tuna species (Thunnus spp.) and two close related tuna species, skipjack tuna (Katsuwonus pelamis) and mackerel tuna (Euthynnus affinis). After manually comparison of all de novo peptides, species-specific marker peptides which are present in all analyzed fishes of these species were found for all relevant species. After verifying these potential marker peptides, it was possible to develop a targeted method using HPLC-MS/MS.

In addition, marker peptides for the group of all three Bluefin tunas (Thunnusthynnus, Thunnusmaccocyii and Thunnusorientalis) and also for the genus Thunnus were found.

Conclusions:
Although tuna species are close related it had been shown that tuna species differentiation is possible using marker peptides analyzed with a targeted approach. In contrast to molecular biological methods, species mixtures can be also detected. After validation, this method could be implemented for routine analysis.

Novel Aspect:
This developed method is the first method for the differentiation of all tuna species based on mass spectrometry using a Proteomics approach.

References:
Introduction
Polyphenols are a large family of aromatic secondary metabolites well-known for their health beneficial effects and for contributing to sensorial attributes [1]. These compounds are mainly distributed in food of plant origin such as paprika, which is a dried and ground spice obtained from red pepper. In Spain, only La Vera and Murcia varieties are distinguished with protected designation of origin (PDO) and their polyphenolic content may allow to classify them.

Methods
A UHPLC-ESI-MS/MS (QqQ) method was developed to determine 37 polyphenols in paprika samples. A simple sample extraction procedure using H2O:ACN (20:80, v/v) was employed. The chromatographic separation was optimized using an Ascentis Express C18 reversed-phase (10 cm × 2.1 mm, 2.7 μm) fused-core column (analysis time of 30 min). Paprika polyphenolic contents were subjected to principal component analysis (PCA) using PLS_Toolbox 7.8.2 (Eigenvector Research).

Results
The developed UHPLC-ESI-MS/MS method showed satisfactory limits of detection (down to 0.012 µg/L), linearity (r² ≥ 0.995), run-to-run and day-to-day precisions (RSD ≤ 20%), and trueness (relative errors below 15%). 111 paprika samples from La Vera and Murcia (Spain), and from Czech Republic were analyzed, and the polyphenolic content quantified by external calibration. Chlorogenic, ferulic and p-coumaric acids, hesperidin, homoplantaginin, rutin, and vanillin were found in all the samples. Polyphenolic composition was considered as a source of potential descriptors to be exploited for the classification of paprika according to the PDO and region of production. The plot of scores showed a successful separation in accordance with both PDO and production region. Moreover, a discrimination between Murcia and Czech Republic paprika varieties (sweet and spicy) was also possible. The PCA loading plots revealed that homoplantaginin, betulinic acid and kaempferol were among the most relevant polyphenols to achieve this discrimination.

Conclusions
A simple and reliable UHPLC-ESI-MS/MS method for the quantitation of 37 polyphenols in paprika samples was developed. The characterization and classification of paprika samples according to the PDO and production region was achieved by employing polyphenolic content as chemical descriptors for PCA. This method will be useful to guarantee PDO attributes and for the prevention of frauds in paprika samples.

Novel Aspect
Fast UHPLC-ESI-MS/MS method for the determination of polyphenols and the classification of paprika samples in the authentication of PDOs.

References
64 - A NOVEL MULTI-PLATFORM HIGH RESOLUTION MASS SPECTROMETRY NON-TARGETED APPROACH FACING EXTRA VIRGIN OLIVE OIL ADULTERATION

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A NOVEL MULTI-PLATFORM HIGH RESOLUTION MASS SPECTROMETRY NON-TARGETED APPROACH FACING EXTRA VIRGIN OLIVE OIL ADULTERATION

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Keywords: Non-targeted mass spectrometry; Extra Virgin Olive Oil; Food frauds; LC-HRMS

Introduction
High resolution mass spectrometry was used in the past for the detection of frauds on extra virgin olive oil products [1] [2] [3].
However, at the moment, scientific studies are not largely focused on the detection of soft refinements of Olive Oils, even if the dilution of EVOO with these samples is probably one of the most common frauds applied on the market. This inter-laboratory work would like to identify some chemical markers responsible of this process.

Methods
Refined oils (deodorized and deacidified) were created on a laboratory scale starting from low quality olive oils and were analyzed together with a set of pure EVOO samples.
Sample preparation was executed according to previous works [4] [5].
UHPLC- HRMS analyses were performed with a SCIEX® triple TOF 6600 analyzer and with a Thermo® Q-Exactive Orbitrap analyzer.
Instrumental conditions were the same for all the instruments involved in the project.

Results
Raw data were processed with PeakView® (for triple TOF data) and Compound Discoverer® (for Orbitrap data); chemometric data analyses were performed with Markerview® (Sciex) and SIMCA® software (Umetrics).
Different PCA models were created and the scores plots clearly highlighted a separation between the pure Extra Virgin Olive Oil samples and the soft refined samples.
The features responsible of these clusterizations were selected and an attempt of compound identification was performed.
A group of molecules related to the adulteration processes were identified: they were at least upregulated in the refined oils or even only detected in these fraudulent samples and in their mixtures.
The markers selected applying the same experimental design with different instruments in different laboratories were compared.

Conclusions
This inter-laboratory research represents a robust untargeted metabolomic approach able to identify specific compounds that can be considered markers of soft refinement processes in Extra Virgin Olive Oils. The detection of these molecules (performed with easier “target” methods) will be a proof of fraudulent issues in commercial samples.

Novel Aspect
Chemical markers for this important fraud in EVOO have not been largely studied yet. The multi-platform non-targeted approach could also be used for retrospective analyses.

References
Introduction:
Regular consumption of chocolate is associated with beneficial effects on health [1]. Cocoa processing processes degrade the polyphenols it contains; the fermentation alone reduces by 90% the content of the polyphenols. It is therefore important to know its impact on the content and / or composition of polyphenols. Polyphenols were extracted from the post-harvest cocoa beans and the chemical compounds present in the cocoa extracts were identified and quantified by LCMS.

Methods:
Identification of polyphenol were done by using UPLC separation coupled with high-resolution MSMS (QToFanalysyer). Identification of compounds were based on their exact mass ans MSMS profile based on literature. Some of the flavanols identified were quantified by UPLC-MRM (triple quadrupole) with external calibration [2].

Results:
The post-harvest cocoa beans were divided into two batches (fermented and unfermented). In order to study difference between these two batches we achieved to build a robust chromatographic method [3] in order to get a good separation of compounds contained in the extracts.
In unfermented extract we observed twenty different compounds (eg.Catechin, procyanidin B...) while in fermented extract we only observe seven compounds.
From these observed compounds we choose three of them (catechin, epicatechin, procyanidin B) well identified to achieve quantification in both extract. Quantification results show decrease in the concentration of this compound in fermented extract. For example, epicatechin is present at 5.9µg/g of cocoa in unfermented extract and at 0.21µg/g of cocoa in fermente extract.

Conclusions:
Cocoa is a natural substance rich in polyphenols [4,5,6]. Fermentation is one of the essential processes in the processing of cocoa beans. This study demonstrated that this process degrades the polyphenols responsible for the health effects of cocoa. However, because of conflicting data reported in the literature, in-depth studies to fully identify different methods of cocoa fermentation are required [7]. In addition, to gain benefit, the research should take into account all the possible parameters such as the temperature, the fermentation time and the room in which the beans that deposit the initial content of the polyphenols during the fermentation processes are deposited. This would make it possible to avoid uncontrolled phenomena of natural, artificial or accidental origin that interfere during the fermentation of cocoa.

Novel Aspect: Relationship between post-harvest treatment of cocoa and its composition in polyphenol

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1155 - SHRIMP FRAUD: EVALUATING CHARACTERISTIC METABOLOMIC FEATURES FOR AUTHENTICATION BY HIGH AND UNIT MASS RESOLUTION SPECTROMETRY

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Keywords: Food fraud, Food authenticity, LC-HRMS, LC-MS/MS, chemometric

Introduction: 
Unfortunately fishery products occupy the second highest ranking position among commodities that are at most risk of food fraud. Among these, marine shrimps and prawns accounts for more than 17% of the global seafood consumption. As per Article 35 of the EC regulation 1379/2013, commercial designation of the species, production method and geographical origin have to be provided to consumers.

Methods:
Authentic samples were collected either directly from aquaculture farms or through local supermarket chains. Six species of shrimps namely Tiger Prawn, King Prawn, Indian Prawn, Pink Speckled Shrimp, Argentinian Red Shrimp and Red Prawn were included in the study. Among Tiger Prawns, wild caught prawns from India and Madagascar; and farmed prawns from Vietnam were sources as were farmed King Prawns were from India, Thailand, Vietnam and Honduras.

Results:
Methanolic extracts of freeze-dried samples were analysed in full scan positive and negative ionisation mode using a Waters Acquity I-Class UPLC coupled to a Xevo G2SQTof. Chromatographic alignment, peak picking and feature identification of the acquired data were carried out on Progenesis QI software followed by multivariate analysis in SIMCA-P software. OPLS-DA chemometric models could discriminate between species, geographical origin and production method. The R2 and Q2 values for all the models were >0.9. Further, characteristic metabolomic features for each species were determined by group wise comparison of the data. These features were evaluated in a targeted, triple quadrupole LC-MS/MS method and at least one exclusive biomarker for each species was selected. Subsequently, a confirmatory LC-MS/MS method for authentication of species was developed. An OPLS-DA model built using the characteristic features of Tiger Prawns and King Prawns was shown to discriminate between the geographical origins of these species.

Conclusions
The data generated gives a strong indication that the routine testing for species authenticity as well as the geographical origin of the prawns can be achieved using triple quadrupole LC-MS/MS.

Novel Aspect:
This innovative methodology based on LC-HRMS and LC-MS/MS can present an alternative to DNS testing or isotope based assays

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OPTIMIZATION, VALIDATION AND APPLICATION OF GC-FID AND GC-IRMS METHODS FOR THE DETERMINATION OF FATTY ACIDS WITHOUT DERIVATISATION

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Keywords: Fatty acids, FFAP, stable isotope ratio

Introduction:
The fatty acid (FA) composition of food is a recurring point of attention in government policy concerning nutrition and health, since they are source of energy, components of biological membranes, precursor for many different molecules and as transporters for vitamins such as A, D, E and K [1]. Further the stable isotope composition of carbon and hydrogen in fatty acids can be used to assess the geographical origin and authenticity of food products [2]. The aim of this presentation was to develop a simple, robust, and derivatization-free GC method suitable for the routine analysis of the major FA components and their isotope compositions.

Methods:
All analyses were performed using an Agilent model Hewlett-Packard 6890 gas chromatograph equipped with a flame ionization detector (GC-FID), while for stable isotope analysis an isotope ratio mass spectrometer IsoPrime (GV Instrument) with a combustion interface (GC-C-IRMS) was used. Separation was achieved using a Phenomex ZEBRON FFAP (30 m × 0.25 mm with film thickness of 0.25 μm) capillary column. The temperature program was as follows: 130 oC to 255 oC at rate of 20 oC/min. Helium was used for the carrier gas at a flow rate of 2.6 mL/min.

Results:
The separation of the FA was optimized by adjusting both the initial oven temperature and the temperature ramp. It was noticed that a faster temperature rate resulted in better resolution between palmitic and palmitoleic acid. The linearity of the method was verified between 50 - 200% of the injection volume as prescribed by the method. The results show an acceptable RSD% and mass deviation for all 16 major and minor constituents and confirm the linearity of the method. Method precision was checked in terms of repeatability and reproducibility. When the method was optimized using GC-FID, it was also applied to determine the stable isotope composition of C by GC-C-IRMS.

The stable carbon isotopic values are expressed in the usual notation as the per mill (‰) deviation of the isotope ratio of a sample relative to that of a standard. The 13C values of the 16 main FA vary from -30.9 to -28.6 ‰. The developed method was further tested on milk and oil samples.

Conclusions:
The method for determining the FA profile of different food samples has been optimized for the nitroterephthalic acid phase. Results showed that the method has acceptable selectivity, linearity precision, detection and quantification limits, robustness and stability. The method is suitable for the routine determination of the amount and isotope composition of FA in vegetables and food of animal origin with a distinct FA profile.

Novel Aspect:
Determining the fatty acid profile of different food samples without the need for derivatisation. The method can be used for food nutritional labelling, authentication, traceability and quality control.

Acknowledgment:
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References:
444 - DETECTING FOOD FRAUD: DETERMINATION OF A POTATO’S VARIETY AND ITS GEOGRAPHICAL ORIGIN IN ONE ANALYSIS VIA UPLC-IMS-QTOF

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Keywords: Food fraud, Metabolomics, Lipid Profiling, potato variety, geographical origin

Introduction:
In recent years, consumers increasingly claim for regional potatoes of certain varieties. Suitably labelled products are considered to be of higher quality and prices what might result in increased rates of food fraud. While regulators enhance consumer protection, existing analytical methods cannot ensure compliance of food properties and its labelling sufficiently. Therefore, a Metabolomics-based approach was developed to evaluate potatoes’ authenticity.

Methods:
About 200 organic and conventionally produced potato samples of known variety were collected from various German regions in 2016 and 2017. Samples were cryomilled, lyophilized and extracted with a Liquid-Liquid-Extraction protocol including Chloroform, Methanol and water. The analysis of the nonpolar extracts was performed by an UPLC-IMS-QToF system. Obtained data sets were evaluated via multivariate data analysis using commercial and open source programs.

Results:
PCA and OPLS-DA analysis were carried out to differentiate between the varieties Belana, Gunda, Wega and Queen Anne. Additionally, samples were distinguished according to their growing regions. The structural elucidation of biomarkers was performed considering the retention time, precise molecular mass, MS/MS fragmentation data, isotopic patterns and ion mobility spectra provided by the UPLC-IMS-QToF analysis platform. A set of more than 60 lipid biomarkers was identified which allows a reliable discrimination of the four potato varieties on the one hand and their cultivation region on the other. Characteristic substances, predominantly tri- and diglycerides, were found to differentiate potatoes originating from Thuringia, North Rhine-Westphalia, Bavaria, Schleswig-Holstein and other German production areas. These generic markers withstand impacts like different farming methods and multiple harvesting periods. Furthermore, the reliability of the model was tested by analyzing mixtures of varieties and geographical origins.

Conclusions:
The results demonstrate that this metabolomics-based approach in conjunction with a comprehensive biomarker database has potential as a future screening tool for the investigation of labelled potato tubers regarding their variety and their German cultivation region to prevent food fraud. This tool may be used to preserve consumer interests and support regulators’ efforts to keep pace with fraudsters.

Novel Aspect:
This approach determines a potato’s variety and its cultivation region within one analysis whereas methods like electrophoresis and isotope analysis require multiple tests.

References:
Introduction:
Chlorogenic acids (CGAs) are a large class of esters formed between quinic acid and hydroxycinnamic acids frequently present in plants as a complex mixture of positional and geometric (cis/trans) isomers, however not easily and univocally identified [1]. Additionally, the UV light exposure is proven to convert the trans double bond to cis and structure and activity relationship for these cis/trans isomers have not been the subject of detailed investigations in spite of the need to deepen the knowledge on these biologically active polyphenols [2,3].

Methods:
Separation of analytes was achieved by UHPLC system equipped with a C18 column coupled to a Triple Quad MS, and, in order to discriminate isomers for dicaffeoylquinic acids (diCQA), a targeted MS3 mode (m/z 515.0 + 353.0 on negative mode) experiment was used.
Ad hoc UV light exposure experiments were performed on standard solutions and green coffee extracts.

Results:
Results confirmed that geometrical isomerism does not influence the fragmentation pattern, as previously reported in the literature [2], and an identification of the positional isomers is possible by means of an accurate chromatographic separation. Different behavior of light exposed green coffee extracts were evaluated, showing how differences in chlorogenic acids profile of different species could lead to different mixture of isomers after UV treatment.

Conclusions:
This work showed that UV irradiation of diCQA samples produce three positional isomers, and separation of this isomers could be efficient with the use of a LC-MS/MS method. Differences in chlorogenic acids profile of various green coffee samples were showed and these findings could represent an important step in the detection and discrimination of isomeric plant metabolites.

Novel Aspect:
UV irradiation experiment on green coffee extracts of different species with characterization of isomers of di-acylchlorogenic acids.

References
1342 - COMPARATIVE PROTEOMIC ANALYSIS OF THE METABOLIC FRACTIONS COMPOSITION IN MODERN AND OLD WHEAT GENOTYPES

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Keywords: wheat, old genotypes, label free quantification, high resolution mass spectrometry

Introduction
Most of the modern wheat genotypes are derived from old wheats and have appreciable properties in terms of grain yield[1]. Wheat derived products plays an important role in many adverse reactions, such as celiac disease (CD), non-celiac wheat sensitivity (NCWS) and allergies. This has led to an increasing interest for the old wheat genotypes which are generally considered better tolerated than the modern ones, but without any scientific evidence.

Methods
Metabolic fractions were extracted from flours using 0.4 M NaCl, 0.067 M K2HPO4, pH 7.6 for 15 at 20°C and a final centrifugation at 12,000 x g for 15 min. The extraction was repeated twice. 50 µg of each protein extract were reduced, alkylated, digested and analysed by high resolution mass spectrometry. MS data were processed by PEAKSQ and searched against a protein database, including only entries of Triticum, Oryza, Hordeum, Avena, Secale, Maize and Brachypodium.

Results
The aim of the present work is the qualitative and quantitative comparison by a proteomic approach of the metabolic protein fractions extracted from the mature kernel of two old Sicilian durum wheats landraces (Russello and Timiliarestebianche), and Simeto, an improved durum wheat variety, widely spread in Italy and other Mediterranean countries, chosen as representative of the most widely commercial cultivars. The qualitative comparison of the protein composition revealed a remarkable similarity between old and modern cultivars. In particular, for each cultivar about 450 proteins were identified. The quantitative evaluation of the identified proteins detected that some protein including avenin, NLTPs and β amylase, are over-expressed in the old genotypes compared to Simeto. On the other hand, differential expression is also observed in the comparison of the two old cultivars. In particular α-amylase inhibitor 0.28 is over expressed in Timiliawheareas serpin Z2A is over expressed in Russello.

Conclusions
This study represents the first exhaustive qualitative and quantitative characterization of the metabolic protein fractions of the old Sicilian landraces Russello and Timiliarestebianche and its comparison with a modern cultivar.

Novel Aspect
This comparison would help to understand the relationship occurring between protein profile of old wheat varieties and potential benefits for human consumption.

References
Introduction:
Food authenticity is nowadays highly relevant since food fraud of expansive foods is a common problem. Differential proteomics approaches are powerful tools in this regard. Proteomics needs a tailored sample preparation for every kind of food. Especially foods with high content of lipids like nuts are challenging. In this study different sample preparation methods suitable for downstream LC-MS/MS analysis were tested for obtaining proteome profiles of the walnut.

Methods:
In the 1st approach nutmeat was grinded and defatted with hexane. Proteins were extracted by boiling in a sodium deoxycholate (SDC) containing buffer and then proteolytically digested with trypsin. In the 2nd approach grinded nutmeat was boiled in SDC containing buffer for protein extraction. Proteins were precipitated with acetonitrile (ACN) and then digested with trypsin. Peptides were analyzed by LC-MS/MS and raw data was processed with Proteome Discoverer 2.0.

Results:
The two protein extraction methodologies were compared regarding the quantity of the extracted protein amount in relation to the weighed portion of nutmeat and the number of unambiguously identified proteins and peptides respectively. Protein amounts were determined with a BCA assay and the recovery per weighed in mg of nutmeat was calculated. The protein recovery was approx. 38% higher if the nutmeat was defatted first. Nevertheless the protein yields of both methods were sufficient for LC-MS/MS analysis. Although the ACN precipitation procedure yielded less protein, it performed superior in terms of the number of peptide and protein identification. Approximately 3.5 times more peptides and about six times more proteins were identified by the 2nd approach using the ACN precipitation. Most abundant in all samples were seed storage proteins like legumin B-like and 11S globulin seed storage protein 2-like with up to 1000 peptide spectrum matches. The data suggests that the walnut nutmeat proteome has a large dynamic range.

Conclusions:
Using ACN for precipitation of extracted proteins shows advantages over first extracting lipids with hexane for walnut sample preparation for proteomics applications. To overcome problems associated with large dynamic range proteomes 2-D LC approaches will be used to build spectral peptide libraries in the future and data independent acquisition will be used for reliable quantification to generate reference protein barcodes as fingerprints for authentic foods.

Novel Aspect:
This study presents evidence that defatting of walnut nutmeat prior to protein extraction isn’t beneficial for differential proteomics applications.
Keywords: virgin olive oil; protected designations of origin; LC-MS, GC-MS; chemometrics.

Introduction:
Product labeling has gained considerable attention over the last years, as a means to both provide product-specific information and reduce the quality uncertainty faced by consumers. Implementation of Protected Designations of Origin (PDOs) is one of the most prominent differentiation strategies used in olive oil market. They are often perceived as valuable tools that promote specific attributes of the oil linked to its geographical provenance.

Methods:
126 oil samples from 6 Mediterranean PDOs were analyzed by LC-MS and GC-MS combined to chemometrics. The extracts were eluted in LC using a C18 (2.1x 100 mm, 1.8 μm) column, with acidified water and acetonitrile and a flow gradient (0.4-0.6 mL/min) at 40°C. The derivatized extracts were injected in GC, using a BR-5 column with a T gradient from 150 to 320°C (4°C/min rate). Both systems were coupled to a Compact™ QTOF MS (Bruker) by an ESI interface for LC and an APCI source for GC.

Results:
Samples from six different Mediterranean PDOs (Meknès and Ouazzane (Morocco), Priego de Córdoba and Baena (Spain), Kalamata (Greece) and Toscana (Italy)) were collected and analyzed. Non-targeted and targeted approaches were used to offer maximum coverage of the olive oil metabolome’s chemical space in a 1st step, and the possible validation of the identified markers afterwards. Data treatment (PCA, PLS-DA) -done by using MetaboScape®- led to a noticeable discrimination among the six evaluated PDOs taking into account the data coming from both platforms. Several compounds such as elenolic acid, luteolin, oleuropein and ligstroside aglycones, and some other tentatively identified substances, were pointed out as possible PDOs distinctive markers.

Conclusions:
-Multi-class LC-MS and GC-MS methodologies together with chemometrics made possible the discrimination among different PDOs, identifying potential origin markers.
-The combined use of non-targeted and targeted approaches can enhance or reinforce the outcomes of any study.
-GC-APCI-Q TOF can preserve the pseudo-molecular ion information, which is a great advantage over the “classical” GC systems and facilitates the identification.

Novel Aspect:
Combination of non-targeted and targeted approaches implying the use of powerful platforms (LC-ESI-Q TOF/GC-APCI-Q TOF) to identify potential “PDOs’ markers”.
Introduction:
Two different untargeted approaches based on Direct Analysis in Real Time (DART) and Liquid Chromatography coupled to High Resolution Mass Spectrometry (LC-HRMS) were applied to the lipid and protein fraction of 50 farmed or wild-type salmons belonging to Salmo salar species to assess the feasibility of MS-based untargeted analysis for the discrimination between the two groups of salmons.

Methods:
Salmon lipid extracts were analyzed by DART coupled to an Orbitrap mass spectrometer. For the proteomics approach, proteins were extracted using Urea buffer systems and subjected to trypsin digestion and sample cleanup. Samples of farmed and wild-type salmon were analyzed by LC-MS/MS on TT6600 triple TOF-MS in DIA and SWATH mode and data evaluation was performed employing different chemometric approaches.

Results:
Lipids or peptides retrieved from DART-HRMS and LC-HRMS spectra, respectively, and exhibiting significant spectral intensity were selected as variables for multivariate statistical analysis. The latter allowed to distinguish clearly wild-type from farmed salmons on the scatterplot based on the first two principal components, accounting for the 77% of the total variance. The subsequent search in the online databases showed that some of the selected variables might belong to the fatty acids class. In particular, three saturated fatty acids (14:0, 16:0, 18:0) had the main discriminating loading for wild-type salmons, whereas unsaturated fatty acids (18:1, 18:2, 18:3) and several oxidized forms arising from them were found to have a higher incidence in farmed salmons, due to their relevant incidence in the feeding mixtures adopted in salmon aquaculture plants [1]. Similarly, samples were clearly distinguishable using proteomics approaches.

Conclusions:
The application of untargeted DART/LC-HRMS approaches, integrated by multivariate statistical analysis, appears to be a promising tool for the discrimination of farmed and wild type salmons. DART/LC-HRMS spectra provide a set of variables able to distinguish salmons on the basis of their different living conditions.

Novel Aspect:
A novel coupling between DART and Mass Spectrometry has been investigated and exploited in the untargeted mode to discriminate between wild-type and farmed salmons.

References
Characterization and quantitation of water-soluble vitamins in wine by liquid chromatography-high resolution and tandem mass spectrometry

Introduction:
Wine is the oldest alcoholic beverage obtained from the fermentation of grapes. Among micronutrients, vitamins play a role in wine production. Some have origin from grapes and/or are formed during fermentation, others may be added for fine-tuning some properties or for avoiding undesired processes.

In this study we set up a simple and reliable method by using liquid chromatography-high resolution (HR) and tandem mass spectrometry (MS) for characterization and quantification of water-soluble vitamins (vitamins B, C and J) in Italian red and white wines. Furthermore, the method is applicable to beers, grapes and grape juices.

Methods:
Preliminary studies by direct infusion have been carried out on vitamin standards using both HRMS (Q-Exactive Hybrid quadrupole-Orbitrap, Thermo Scientific) and an ion trap instrument (LCQ Deca, Thermo Scientific) both equipped with an ESI source in positive mode. MS/MS experiments by using higher-energy collisional dissociation (HCD) in the Orbitrap and low-energy collision induced dissociations (CID) in the ion trap have been carried out to choose the appropriate reactions to be monitored. Successively, real samples have been filtered and injected in the HPLC equipped with a C18 column (Acquity BEH 100 × 2.1mm 1.7µm) and analyzed by HRMS. Analysis have been performed in Full MS-ddMS2; quantification by external standard method.

Results:
Vitamin fragmentation patterns show differences between HCD and CID spectra. The former, acquired in high resolution, generally show a higher number of product ions and ions that in the CID spectra are shown only in MS3. The quantities measured in wine are not relevant in terms of daily vitamin intake, but the study is of oenological interest. Vitamin B12 and pyridoxal were not detected and folic acid was found in trace only in beer samples. Ascorbic acid was detected only in white wines. Vitamin C is a quick-acting and powerful antioxidant that can be added in white winemaking to protect against light and short aeration, preserving freshness and fruity [1]. Also, thiamine is an allowed additive. Some compounds (e.g., pantotenic acid) were in higher amounts in grapes than in wines, indicating that these vitamins are used by yeasts [2]. Others (vitamin J, B2) showed a reverse trend probably because they are not used by yeast alcoholic fermentation [3] and they are released in the medium as the result of yeast lysis. Some are present only in wines (nicotidamide) and are produced by yeast metabolism during fermentation [4,5].

Conclusions
We developed an HPLC-HR-MS/MS method for the determination of water-soluble vitamins in wine and also can be extended to beer, grapes and grape juices. Vitamin concentrations are comparable with those reported in wines by other Authors. Different vitamin profiles were found in the analyzed matrices. Changes between grapes and wines might be attributed to the fermentation process.

References

Novel Aspect:
Quite large-scale screening of water-soluble vitamins in Italian wines. Method fast, sensitive, specific and robust without any sample pretreatment.
Determination of melatonin and related compounds in beverages using liquid chromatography coupled to low and high resolution mass spectrometry

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Determination of melatonin and related compounds in beverages using liquid chromatography coupled to low and high resolution mass spectrometry
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Keywords: Melatonin, related compounds, beverages, triple quadrupole, high resolution mass spectrometry

Introduction:
Melatonin (N-acetyl-5-methoxytryptamine) is an endogenous indolamine hormone, synthesized from tryptophan [1] and it has a significant role in regulation of the circadian rhythm, mitigation of sleeping disorders and jet lag. Melatonin can also be found in a variety of foods including edible plants, fruits and beverages [2] in addition to isomers or related compounds, but their identification is not a straightforward process [3].

Methods:
For the determination of melatonin and related compounds, a simple dilution of the beverage, including different types of beer (alcoholic and non-alcoholic), wine, aloe vera drinks and cherry juice, was performed prior determination applying liquid chromatography (LC) coupled to triple quadrupole tandem mass spectrometry (QqQ-MS/MS) or high resolution mass spectrometry analyzer as Exactive-Orbitrap.

Results:
First, a nontargeted approach based on LC coupled to Exactive-Orbitrap has been used for the reliable identification of melatonin related compounds in beverages. Thus, in addition to melatonin, tryptophan ethyl ester, which has the same molecular formula than melatonin, was detected. All ion fragmentation (AIF) mode was used to get MS/MS spectra and different ions were obtained for both compounds. Then, an analytical method based on LC-QqQ-MS/MS has been validated in terms of selectivity, limit of quantification (0.01 µg/L) and precision, for the determination of melatonin and tryptophan ethyl ester in beverages. It was observed that tryptophan ethyl ester was detected at higher concentrations (from 0.2 to 90 µg/L) than melatonin (<2 µg/L) in the tested samples. In addition, the concentration of tryptophan ethyl ester was higher in alcoholic beers than in non-alcoholic, indicating that the fermentation could affect the concentration of this compound.

Conclusions
Melatonin related compounds, as tryptophan ethyl ester, has been identified in several beverages using Exactive-Orbitrap analyzer and combining the information provided by AIF mode and characteristic fragmentation pattern of the molecule. Because both compounds have similar retention time and common ions, false positives of melatonin could be reported in some beverages due to tryptophan ethyl ester was detected at higher concentrations.

Novel Aspect:
The combination of several MS platforms allows for the reliable identification and determination of melatonin and related compounds in different types of beverages.
References
Introduction:

Confirming food authenticity is important not only for consumers and the food industry but also for government regulators. There is often the need to detect trace levels of contaminants in complex matrices, which requires the use of state-of-the-art mass spectrometers. Sophisticated software tools are needed to quickly process large data sets generated in these studies.

Thermo Scientific™ Sample Profiler is a new cloud-based software application that can be applied in diverse fields such as food authenticity, environmental monitoring, metabolomics, and detection of new designer drugs. Using this application, large data sets can be processed to detect and integrate compound peaks. Further, compound identification is automated by comparing the fragmentation spectra of compounds against mzCloud™, a web-based spectral database.

Using the Sample Profiler application, chemical profiles of samples of known quality and authenticity can be created within minutes. Individual samples of suspect/questionable quality can be readily compared against these profiles. Statistical evaluation of these comparisons helps users identify certain compounds of interest or ‘marker compounds’ that are present in significantly different concentration levels in the two sets – authentic and suspect samples. Thus, the presence or absence of marker compounds helps with the quick verification of authenticity and/or characterization of challenge samples when compared against profiles of samples of known/desired characteristics.

In this study, we used Sample Profiler to distinguish between soy sauce samples that were brewed during manufacturing from those that were not.

Methods:

Sample Preparation

Different brands of soy sauces were purchased locally. Samples were prepared in triplicate by diluting aliquots of soy sauce with water (3:1). Pooled quality control (QC) samples were prepared by combining a fixed amount of each soy sauce into a pool and preparing a set of dilution samples as normal.

Test Method

Analysis was performed by LC-MS with separation achieved by reversed-phase linear gradient (ACN/ water, 0.1% formic acid) with a run time of 10 min. Mass spectrometric analysis was performed on a Thermo Scientific™ Q Exactive™ HF hybrid quadrupole-Orbitrap mass spectrometer operated in positive mode with electrospray ionization. Full scan MS1 (120,000 resolution FWHM at m/z 200) and precursor selected MS/MS (30,000 resolution) data was acquired. Sample injection order was randomized with QC samples injected every 10 injections.

Data Analysis

Mass spectral data for different types of soy sauces, such as those manufactured in different regions, stored in different types of containers, and even processed differently (by brewing or by not brewing), were collected. Data
for blanks and QCs were also obtained. Raw data files were uploaded to Thermo Fisher Cloud and imported into the Sample Profiler application for further processing. The components were detected in samples after the application aligned all the retention times in all samples. Compound identification was done by matching fragmentation (MS2) spectra against the mzCloud spectral database. Sample Profiler aggregated the name, predicted formulae, m/z, RT, and area counts of each compound from all the samples.

Results:

Background

Soy sauce is a popular condiment manufactured and consumed in many Asian countries. Traditionally, soy sauces are prepared by two methods: a) by naturally brewing fermented soya beans with wheat and brine, which can take several months, or b) by using hydrolyzed vegetable protein (HVP), which takes only days. Soy sauces manufactured by the two processes differ in their physical characteristics such as aroma, flavor, and texture. In addition, soy sauces made by acid-hydrolysis of soy proteins have a longer shelf life.

In this study, different brands of soy sauces purchased locally were analyzed to determine the differences in their compositions and identify spurious samples based on our knowledge of genuine products. HRAM data of each sample was collected in triplicate followed by statistical analysis using Sample Profiler, an application hosted in Thermo Fisher Cloud.

Data Processing in the Cloud

Large data sets uploaded to the Data Manager in the Thermo Fisher Cloud can be easily added to projects created in Sample Profiler and processed using suitable processing Methods: Data processing involves aligning the chromatographic peaks, followed by component extraction. The accurate mass information was used to predict elemental composition for detected components. Finally, compounds were identified by matching the predicted formula and MS2 fragmentation data against ChemSpider and mzCloud™ databases, respectively.

Creating Profiles

The application also allows the user to aggregate samples with similar characteristics such as packaging and or manufacturing process which can be pre-defined as “Study Factors” prior to data analysis. These sample subsets that share one or more study factors can be then used to establish a ‘product-profile’. Other samples having same or even different study factors can be compared against chosen profiles to evaluate the extent of similarity.

After all samples including QCs and blanks are processed, two databases of compounds or profiles, a) All Samples and b) All QCs samples, are automatically created by the application. The user can also create additional profiles or subsets of the All Samples profile by partitioning it based on study factors or criteria such as manufacturing region or packaging material used to characterize the samples. For example, in the present study, a database or a profile of compounds present in soy samples that were brewed during manufacture was created.

In the present study, a profile of soy sauces that were brewed during manufacturing was used to determine the quality of soy samples that were not brewed. The data indicated that the chemical compositions of both types of soy sauce are similar in that they contain same compounds in similar concentration range. Yet, some compounds were either not found at all or found in much lower concentrations in soy sauces that were not brewed. Similarly soy samples manufactured in different regions or samples stored in different containers can be compared and statistically distinguished from one another.

The application displays the profile of “Brewed” soy-sauces, in a tabular format which includes details such as compound name, predicted formula, MW, RT, and highest intensity value from all samples for 11,572 compounds found in this subset of soy sauces. The spread in the intensity values found across all samples is shown as distribution plots. The ‘Compoundstable’ can be filtered to show the compound details in any of the profiles created in the project.

Trendline Plots

Sample Profiler also allows for visualization of the trends in compound areas. When trendlines for compound areas across all samples in the profile are plotted, those samples that may have significantly higher or lower concentrations of one or more compounds can be easily identified. These samples can then be investigated further to get a better understanding of the products’ chemical composition.

Comparing Different Types of Samples

Using Sample Profiler, comparisons between different types of soy sauces can be easily set up and data processed at different fold changes and adjusted p-values. In the present study, a profile of brewed soy sauces was created and used for comparing against not-brewed samples. Data analysis performed by setting the fold change value to 8 and adjusted p-value to> 0.001. It was found that of a total of 11,803 compounds, the not-brewed samples were found

1173
to contain 267 compounds in significantly higher abundance, and 1791 compounds in significantly lower abundance than in brewed samples.

Marker Compounds

Once a product’s profile is well understood, certain compounds when present in a product can be labeled as markers of authenticity or contaminant. These marker compounds are not only useful in qualifying study samples but also for setting up targeted data acquisition.

In this study, Sample Profiler was used to search for genistein and glycitein specified by name. Post search, the table was updated with formulae, MW, and RTs of the two compounds along with the total number of samples and QCs in which they were found. After the compounds are added to the table, Sample Profiler will automatically search for their presence in any comparison set up in the project.

The relative distributions of the two marker compounds in the comparison between brewed and not-brewed soy sauces were determined. From the results obtained, brewed soy sauces are understood to have higher abundances of these two marker compounds.

This knowledge can be applied to quickly verify the authenticity of a challenge sample(s) by setting up appropriate comparisons between them and known profiles and studying the relative distributions of marker compounds.

Conclusions

Sample Profiler helps in the creation of chemical profiles of authentic samples and identification of ‘marker compounds’. Marker compounds help with easy verification or characterization of suspect/challenge. The application also allows for visualization of trends in the data in addition to TIC and XIC traces. In the present study, it has been shown that soy sauces manufactured in different regions or packaged differently can be easily differentiated by the Sample Profiler application. Compounds such as glycitein and genistein were found to be present in higher abundance in brewed soy sauces as compared to not-brewed samples.

Novel Aspect:

Sample Profiler, a cloud-based data analysis software that can be process large data sets has been used for establishing food authenticity.

References

Introduction:
Extravirgin olive (EVO) oil aroma and taste influence consumer preferences. Volatile organic compounds (VOCs) release from food within oral cavity is a complex process. Nose-space (NS) analysis should unravel the retro-nasal olfaction processes happened during olive oil tasting. Proton Transfer Reaction-Mass Spectrometry with Time-of-Flight analyser (PTR-ToF-MS) was already successfully applied for NS analysis of apples [2], cereal bars [3], coffee [4,5].

Methods:
In this study 2 Italian EVO oils were used: commercial vs home made (Lago di Garda, TN). Pure oil, oil mixed with tomato sauce, oil on bread prepared for each EVO oil were used for headspace (HS) and NS measurements by PTR-ToF-MS 8000. The samples were presented to 8 assessors in duplicate. For NS sampling a glass nose-piece with silicone rubber tube was fitted into assessors’ nostrils and connected to PTR-ToF-MS. The method for HS analysis was described elsewhere [6].

Results:
26 out of 319 mass peaks of NS dataset displayed signal related to olive oil, tomato or bread aroma. Peaks related to human metabolism were eliminated. The individual profiles differed in peak intensity and duration. However it was possible to distinguish the two types of EVO oils. HS data confirmed diversity of the oils. Home-made EVO oil in all products was characterized by significantly higher concentrations of mass peak 99.081 tentatively identified (t.i.) as (E)-2-hexenal associated to a green-leaf and fruity flavor [1]. Commercial EVO oil was richer in t.i. pentanal, acetic acid, butanal, and several esters which are reported as oxidation products of olive oil [7]. The samples of olive oil on bread showed the best performance (higher signals intensities) for all panellists due to an increased release surface area, prolonged presence in mouth, increased interaction with saliva and mucosal surfaces. Adding tomato sauce to olive oil decreased the olive oil aroma release in NS and contaminated it with tomato aroma.

Conclusions
NS analysis performed by PTR-ToF-MS discriminated different EVO olive oils. HS and NS data were consistent. The next steps are the optimization of sample preparations, tasting protocol, and the improvement of PTR-ToF-MS sensitivity by applying an ion funnel.

The optimized methods will be applied to study different EVO olive oils within the project “VIOLIN” aiming to the valorisation and promotion of Italian EVO oils.

Novel Aspect:
For the first time NS analysis was applied for olive oil profiling taking into consideration individual perception and the combination with different food matrices.

References

Acknowledgment
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Introduction:
Aroma of coffee beans (Coffea arabica) contributes to its quality evaluation and its origin tracing. The part of coffee aroma such as volatile aldehydes is linked directly to volatile organic compounds (VOCs) naturally occurring in green coffee beans as well as to roasting process. Fast and direct volatile aldehydes detection in green beans, during coffee roasting, and in roasted beans by a selected ion flow tube mass spectrometer (SIFT-MS) may possibly be a way for characterization of coffee origins.

Methods:
VOCs of coffee beans of 5 geographical origins and 3 batches each were measured by SIFT-MS (SYFT VOICE200 ultra, Syft Ltd, New Zealand). 5 green coffee beans were placed in a 22 mL glass vial, incubated at 37°C for 30 min, and measured for 1 min by an autosampler coupled to SIFT-MS. Single bean was placed in a vial and roasted in the oven at 211°C for 20 min. SIFT-MS monitored roasting through the oven ventilation. Then vials were closed and measured in the same way as green beans.

Results:
Twenty one aldehydes, two alcohols, and two methoxypyrazines were monitored in green beans along with an unspecific monoterpenes peak. Some typical compounds such as furans, pyridine, and pyrazine were added to the list of monitored compounds for the online monitoring of coffee bean roasting and the screening of roasted coffee beans. During roasting two types of evolution curve of VOC emissions were observed: the peak of emission at the beginning (2-nonenal, benzaldehyde, and 2-pentylfuran) and at the end of the roasting (hexanal, 2-hexenal, and others).

The special attention was paid for the possibility of aldehydes to discriminate the coffee bean origin. In green beans origin was characterized by the significant differences in the concentrations of five aldehydes such as benzaldehyde, hexanal, 2-methyl-2-propanal, 2-methylbutanal, and pentanal. The concentration of aldehydes in roasted beans was significantly different from green ones but no significant difference according to their origin was observed.

Conclusions:
The technique was successful applied for screening of green and roasted coffee beans and for online monitoring of the coffee bean roasting. The main differences in aldehyde content in coffee beans were found in green beans rather than in roasted ones.

Novel Aspect:
For the first time the analysis of green and roasted coffee beans and online monitoring of VOC emission during coffee roasting was performed by SIFT-MS. Moreover for the first time SIFT-MS was coupled with a multipurpose GC automatic sampler.
Introduction:
Food fraud is becoming an increasingly critical topic. For expensive foods like truffle, incorrect labeling or contamination of premium products with inferior products are common. Truffles are scarcely researched and thus detection of food fraud challenging. Here, protein extraction methods were tested and a LC-MS/MS based differential proteomics approach was developed for determining reference LC-MS/MS data for future validation of truffle quality.

Methods:
Homogenates from authentic European and Asian truffle samples were obtained by picosecond infrared-laser (PIRL) ablation or by lyophilization and grinding of truffles. Protein extracts were boiled in presence of sodium deoxycholate and enzymatically degraded by trypsin. Peptides were analyzed by LC-MS/MS in data-dependent acquisition mode to generate spectral libraries and, for obtaining reference LC-MS/MS data, in data-independent acquisition mode.

Results:
The quantitative comparison of homogenates from PIRL ablation and classical homogenization of truffle showed an at least twofold change in relative amount for 15 % of extracted proteins. There was no preference towards a certain method. In the differential proteomic analysis of truffles by measurements in DIA mode, reference LC-MS/MS data were generated for the samples available at the moment. By hierarchical clustering analysis and principal component analysis samples from European truffles could be well distinguished from Asian truffles.

Conclusions:
The quantitative differential proteomics approach generated reference LC-MS/MS data by which truffles of different origin can be distinguished. Therefore this approach is a future tool for food fraud detection.

Novel Aspect:
This is the first study presenting LC-MS/MS-based data as reference for validation of truffle quality.
Introduction: The largest seafood fraud investigation in the world to date found that 33% of seafood samples are mislabeled in the U.S. Seafood fraud hurts consumers and also raises serious preservation concerns for our oceans. DNA-based analytical techniques are commonly used to determine the species that belongs to a given sample. However, the long analysis time complicates its applicability to be enforced. Here, we present a protein based method that identifies the most common commercially available salmon species in minutes using a simple protein extraction protocol coupled to PaperSpray® and top-down proteomics. The high mass accuracy and resolution of the benchtop quadrupole orbitrap MS allows not only the classification of proteins with high protein sequence homology, but also species dependent amino acid substitutions. The use of PaperSpray, a direct ionization technique, enabled us to simplify the mass spectrometric analysis and subsequent identification of the target proteins.

Methods: Seven reference samples from commercial species and 50 commercial foodstuffs were included in the work. Protein extraction was carried out by homogenizing 1 g of muscle. Water soluble proteins were centrifuged, the supernatant heated at 70 °C for 5 min and centrifuged again. Soluble proteins then were cleaned using automated microSPE devices. The analytes were then spotted onto the PaperSpray cassette and placed into the Prosolia Velox™ 360 PaperSpray® system coupled to a Thermo Scientific Q Exactive HF mass spectrometer. PaperSpray technology relies on cellulose paper and ions are generated directly from paper when an applied high voltage induces electrospray from the sharp tip of the wet paper. Proteins migrate through the paper and are directly ionized into the mass spectrometer. Extraction solvent used was 95/5/0.01 methanol/100 mM ammonium acetate/formic acid. Full MS spectra of intact thermostable proteins were analyzed using Thermo Scientific Biopharma Finder 2.0 software for molecular mass determination and the protein identities were confirmed by MSMS.

Results: The main goal of this work was to develop a simple and fast strategy that allows the authentication of fish species and be widely applied to enforce the guidelines from the U.S. Food and Drug Administration. The work was divided in two phases; primarily a discovery phase to develop the analytical workflow and to choose the best protein biomarker from standardized samples. This phase was followed by a validation phase to determine whether a commercial sample is mislabeled or not. During the discovery phase, a two-step workflow was created. First, the fraction of thermo-stable proteins from the tissue was purified because most of the commercial foodstuff has already been cooked. The second step, consisted of an ultrafast top-down proteomic approach of the purified proteins. Samples were cleaned up using reverse phase micro-spe cartridges. Proteins were eluted using 50% acetonitrile enabling easy spotting into the paperspray cassette while significantly reducing the drying time before being the proteins are electrosprayed into the mass spec. The overall sample preparation workflows allows for automated sample preparation and clean-up of the samples before been introduced in the MS system without impacting the throughput. A 1 min acquisition was performed and the proteins were then analyzed doing a top-down msx in the HCD cell of a benchtop quadrupole orbitrap mass spectrometer. For all the samples, an 11 kDa protein was the most abundant protein and identified as a parvoalbumin (PRVB). This protein is highly conserved between different species. However, slightly mass differences in the mass of the PRVBs corresponding to amino acid substitutions allow for unambiguous systematic discrimination of the different species. Further validation on
commercial samples demonstrated that over 10% of the samples were mislabeled. We believe that this workflow coupled to the PaperSpray technology platform could represent the ultimate payoff to reliable and widely used top down proteomics to trace and authenticate food.

Conclusions
In this work, we coupled PaperSpray ionization with high resolution accurate mass (HRAM) Orbitrap technology for authentication of fish products. PaperSpray demonstrated that qualitative analysis of proteins is possible without time consuming sample preparation and advance chromatography techniques. This work also demonstrates the potential use of PaperSpray coupled to mass spectrometry as an emerging approach to protect customer rights and preventing fraudulent practices. And that can be useful to food industries that are seeking the opportunity to assure their food products labeling.

Novel Aspect:
Fish traceability in minutes using a high throughput PaperSpray-Orbitrap MS platform and top-down proteomics.
A Novel Robust Probe for the Rapid, On-Line Characterisation of Food Samples using Rapid Evaporative Ionisation Mass Spectrometry

Keywords: Ambient ionization method, Rapid Evaporative Ionization Mass Spectrometry (REIMS), Food authenticity,

Introduction:
There has been a significant increase of the incidence rate of food fraud cases related to products originating from uncontrolled origins. Rapid Evaporative Ionization Mass Spectrometry (REIMS) has been used for the analysis of various meat samples and has shown to be capable of the fast identification of diverse tissue types based on their lipid fingerprints. We aimed to test the feasibility of REIMS combined with our robust sampling probe for different food products.

Methods:
Authentic mangalica pork and carp fish samples from 6 different suppliers based in Hungary were sourced. All samples were analysed using a custom built bipolar sampling probe. The aerosol generated by the thermal ablation of the samples was introduced into the REIMS source of a Xevo G2-XS ToF MS and spectra were acquired between 150-1500 m/z. By the application of multivariate algorithms, our goal was to identify the species or location of origin of each sample.

Results:
The original monopolar sampling probe was modified forming a robust bipolar probe to allow rapid on-line sampling of products. The spectra acquired from the samples were first pre-processed (lockmass correction and background subtraction), then subjected to Principal Component Analysis with the results processed using Linear Discriminant Analysis. All classification models were tested on leave-one-sample out cross-validation, and an independent validation set. In the case of the pork study, the cross-validation resulted in >80% accuracy, with 12 out of 15 samples correctly identified from the independent validation set. The most significant peaks in the spectra were identified using tandem MSMS measurements and featured mainly fatty acids, glycerophospholipids and triglycerides. In case of the carp study, the location of origin showed good separation in the first three principal components, and the cross-validation resulted in an 85% correct classification rate.

Conclusions:
The results demonstrate that the lipidomic profiles can be taken in the few seconds timeframe and the profiles show good species- and origin-level specificity. The custom built food probe was found to be suitable for on-line sampling in an industrial environment. REIMS technology was found to be a promising tool for food safety applications providing a reliable and simple method for the rapid characterization of food products.

Novel Aspect:
On site, on-line rapid sample identification with robust sampling probe based on REIMS lipid fingerprints.
CORRELATING CONSUMER SENSORY EXPERIENCE OF APPLE AROMA WITH VOC PROFILES ACQUIRED BY AUTOMATED TD–GC–MS

Gareth Roberts (1) - David Barden (1) - Nick Bukowski (2) - Laura McGregor (3) - Massimo Santoro (1) - Natasha D. Spadafora (4)

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Keywords: automated thermal desorption, apple cultivars, sensory analysis, VOCs

Introduction (400 characters):
The aroma of fruit is regarded as one of the most important quality parameters sensed by consumers, and is influenced by numerous factors including genotype and growth conditions. In this study we determined the VOC profiles of apple cultivars and correlated them to consumers sensory evaluation.

Methods (400 characters):
Apple headspace (cultivars ‘Smitten’ and ‘Granny Smith’) was sampled onto sorbent tubes using a micro-chamber system, with analysis by thermal desorption–gas chromatography–mass spectrometry. Twenty panellists were asked to rate the samples according to nine sensory characteristics, and two-dimensional hierarchical cluster analysis (HCA) was performed to relate these scores to the VOC profile.

Results (900 characters):
A total of 29 VOCs were identified across the samples, of which 20 were esters. ‘Smitten’ was found to have significantly higher hedonic scores on all sensory measurement than ‘Granny Smith’, and significant positive correlations were found between high ‘Taste/Flavour’, ‘Fruitiness’ & ‘Aroma/Smell’ scores and five of the esters identified in the headspace analysis. These compounds are: (with their odour as described in an olfactometry study of apple cultivars1) 2-methylpropyl acetate (pear, apple), n-butyl acetate (sweet, fruity), 2-methylbutyl acetate (fruity, apple), n-pentyl acetate (fruity, banana), and n-hexyl acetate (fruity, pear). In that study, the first three of these compounds were identified as ‘principal odorants’.

Conclusions (400 characters):
We conclude that esters could be used as objective markers for the consumer appreciation of fresh apples, enabling optimum conditions for processing and storage to be identified without recourse to expensive sensory panels in every case. Such tests could also be used as part of routine quality control by the producer and retailer.

Novel Aspect (150 characters):
Detection of trace-level VOCs was only possible because of the large micro-chamber sample volumes and the low thermal desorption split ratios.

Reference
Keywords: Polyphenols; Spanish Paprika; Food Authentication; UHPLC; High resolution mass spectrometry

Introduction:
Paprika is a red powder seasoning with a characteristic flavor obtained from red peppers. Spanish paprikas from La Vera and Murcia have protected designation of origin (PDO), which increases product value but also their production costs, making necessary to properly authenticate them. Polyphenolic contents in these products may be employed as descriptors to achieve their authentication [1].

Methods:
A UHPLC-HRMS (Orbitrap) method was employed to obtain paprika polyphenolic fingerprints. HRMS data was processed with TraceFinder v3.3 software by employing a customized target accurate mass database of 54 polyphenols. Polyphenolic data was considered as a source of potential descriptors for the classification and characterization of paprika by exploratory principal component analysis (PCA).

Results:
111 samples belonging to La Vera and Murcia Spanish PDOs (including sweet, bittersweet and spicy varieties), as well as 15 samples obtained from the Czech Republic were analyzed. The plot of scores obtained after PCA using UHPLC-HRMS polyphenolic profiles revealed patterns that were correlated to sample characteristics such as the production country (Spain, Czech Republic) and regions, allowing a clear differentiation between paprika PDOs and varieties. Based on the PCA plot of loadings, the most remarkable polyphenols and phenolic acids on each type of paprika PDO and variety were identified, making possible to differentiate them according to their different content and distribution of polyphenols. These polyphenols can be studied as possible future biomarkers to guarantee sample authentication and for the prevention of frauds.

Conclusions
The obtained results demonstrate that UHPLC-HRMS polyphenolic profiles obtained by a simple screening with a customized accurate mass database of polyphenols can be employed to achieve the characterization and classification of paprika samples according to their production region to guarantee product authentication. Potential polyphenolic bioactive compounds for paprika varieties were identified.

Novel Aspect
New analytical approach for the characterization and classification of Spanish paprika based on HRMS polyphenolic fingerprinting.

References
Introduction
Turmeric (Curcuma longa) is a plant related to ginger family that has been used for centuries as a remedy in traditional Asian pharmacy due to its antioxidant, anti-inflammatory and antineoplastic activities [1]. Turmeric is also appreciated worldwide as a condiment in cuisine. Curcumin is the most relevant molecule of turmeric, providing both color and pharmacological activity [1]. Related curcuminoids and polyphenols are important as well.

Methods
The UHPLC-HRMS method used an Accela UHPLC system coupled to a Q-Exactive q-Orbitrap mass spectrometer (Thermo Fischer Scientific) equipped with a heated-electrospray ionization (H-ESI) source. Compounds were separated by reversed-phase mode using an elution gradient based on 0.1 % formic acid in water (v/v) and acetonitrile. Chromatograms were acquired in the negative mode in the range 100 to 1000 m/z. Data was analyzed using PLSToolbox (Eigenvector Research).

Results
Here, UHPLC-HRMS was used to analyze commercial turmeric and curry samples. The resulting data consisting on polyphenolic profiles and UHPLC-HRMS fingerprints was treated chemometrically to tackle characterization and authentication issues. Quality controls (QCs) prepared from all the samples were used to assess the instrumental variability. Principal component analysis (PCA) models from both approaches showed that curry and turmeric samples were clearly segregated. QCs appeared in a compact group in the center of the model, showing the good performance and reproducibility of the proposed method and the obtained data. Principal component 1 (PC1) mainly described quantitative patterns, with samples richer in curcuminoids to the right of the plots and those with lower contents to the left. Turmeric samples were distributed according to plant varieties, e.g., Erode in the top right corner, Allepey in the bottom right corner, and Madras in the top left corner.

Conclusions
UHPLC-HRMS resulted in an excellent approach for a feasible determination of curcumin and its derivatives in food samples. The proposed method revealed a great complexity in polyphenolic composition, showing a variety of components to be elucidated. It was believed that minor curcuminoids could be relevant descriptors for classification and authentication purposes using chemometric Methods:

Novel Aspect
Comprehensive UHPLC-ESI-HRMS characterization of polyphenols and curcuminoids and classification of turmeric products by PCA.

References
Introduction:

Monoterpenes are among the most important aromatic metabolites to be found in a free and bound form in grapes [1]. Aromatic characteristic of grape spirits can therefore be especially attributed to this class of molecules despite the presence of countless volatile compounds produced during alcoholic fermentation [2,3]. The aim of this study was to describe the terpenic profiles responsible for the varietal aroma of a selection of grape spirits produced from 10 different varieties using an innovative fast GC-MS/MS method.

Methods:

20 mL of sample was diluted to 100 mL with Milli-Q water after the addition of 2-octanol as internal standard. Monoterpenes were extracted by adsorption on a SPE cartridge and eluted with dichloromethane [4,5]. The organic phase was analysed using an Agilent Intuvo 9000 GC system coupled with an Agilent 7000 Series Triple Quadrupole MS operating in dynamic MRM mode. Separation was obtained injecting 2 µl in split mode (1:5) into a DB-Wax UI (20 m ×0.18 mm× 0.18 µm).

Results:

The presented method permitted to qualify and quantify 15 different monoterpenes characteristic of the grape spirits in only 15 minutes. The compounds detected were α-terpineol, β-citronellol, geranic acid, geraniol, HO-diol I, HO-trienol, linalool oxide A, linalool oxide B, linalool oxide C, linalool oxide D, linalool, nerol, rose oxide I, rose oxide II and terpinen-4-ol. All of the measured monoterpenes were found in the 39 grape spirit samples except for HO-diol I and HO-trienol that were detected in only 12 and 30 samples, respectively.

The correlation matrix showed a positive correlation between the amount of linalool oxides, linalool, α-terpineol and HO-diol I. A positive correlation was also observed between β-citronellol, nerol, geraniol and rose oxides.

Principal component analysis (PCA) was used to display the samples in an unsupervised pattern recognition map. The PCA showed that a group of samples was characterised by a high content of linalool oxides and linalool, while another one by a high content of β-citronellol, nerol and rose oxides.

Conclusions:

The proposed method made it possible to obtain a broad monoterpenic profile characteristic of grape spirit products in only 15 minutes. 15 monoterpenes were qualified and quantified in 39 grape spirit samples obtained from different grape varieties. The statistical approach highlighted that there was a positive correlation between the different compounds and allowed to discriminate the most aromatic samples on the basis of their different monoterpenic profile.

Novel Aspect:
The method developed can be proposed for the fast characterisation of grape spirit samples based on their different monoterpene profile.

References:

Introduction:
A very low percentage of mothers around the world practice exclusive breastfeeding. The low breastfeeding rate is the driving force for the infant formula manufacturers to produce formula that simulate human milk as closely as possible. However, the information of bioactive molecules in the NIST *SRMs for human and infant formula milk is limited. This study focuses on the method of structural elucidation of a material based mass spectral library of functional oligosaccharides using NIST reference materials.

Methods:
The human milk, infant formula, goat and buffalo milk underwent sample preparation [1]. The purified oligosaccharides were analyzed on Hydrophilic Liquid Interaction Chromatography-electrospray ionization in Fusion Lumos Orbitrap-based MS using HCD & Fourier transform ion trap (FT-IT) fragmentation techniques. The spectra are extracted from LC-MS raw data and processed using in-house algorithm then analyzed using different tools including NIST Tandem mass spectral library.

Results:
The high mass accuracy both for precursor and product ions from FT-IT and HCD data was sufficient to enable the assignment of oligosaccharides including novel oligosaccharide structures with different adduct types in negative/positive detection mode. Core group (Gal-Glc) with different terminal sugars, Fuc-Gal-Glc eluted prior to Sia-Gal-Glc. The elution profile of DP 5 isomers, such as lacto-N-fucopentaose (I, II, III) and LS-tetrasaccharide (a, b, c) displayed different retention times on HILIC that enhances oligosaccharide identification. A clear distinction in the HILIC elution pattern of isomeric oligosaccharides related to size and polarity confirmed the identifications. The base peak chromatogram of infant formula differs from human milk in terms of their glycan profiles. Infant formula contains fucolactose as well as maltodextrins with degree of polymerization 2-13 whereas human milk has over 70 bioactive oligosaccharides. The spectral library of oligosaccharides in human milk demonstrate to aid in identifying glycans and other compounds present in other biological samples such Asian goat and buffalo milk, which represents a new variety of mass spectral library. Moreover, buffalo milk contains oligosaccharides close to human milk.

Conclusions
The HILIC MS/MS and NIST Tandem MS libraries facilitate the structural assignment of 70 complex oligosaccharides with 470 spectra including newly annotated glycan structures. Infant formula was fortified with maltodextrins and fucolactoses—a concept that can be extended to other varieties of biological fluids such as detection of glycans in other milk and human plasma samples.

Novel Aspect:
A comprehensive structural and tandem MS library of annotated oligosaccharides derived from the NIST milk reference materials: a robust method of detecting other compounds in milk products.

Reference
* SRM (Standard Reference Material)
553 - IKNIFE, A NOVEL AMBIENT MASS SPECTROMETRY APPROACH FOR THE REAL-TIME IDENTIFICATION OF FOOD SAMPLES

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Keywords: Rapid evaporative ionization mass spectrometry, electrosurgical knife, food authenticity, food safety, chemometric data analysis

Introduction: (Limit of 400 characters without spaces)
Rapid Evaporative Ionization Mass Spectrometry (REIMS) is a novel ambient MS method that can be used for the real-time identification of unknown samples, without any sample pretreatment. Such an approach is based on the analysis of the vapor produced by thermal ablation. MS profile represents an univocal fingerprinting, usable for geography evaluation and authenticity assessment of foodstuffs. For this purpose, a database of authentic samples need to be created.

Methods:
The REIMS method was employed for the fast characterization of different food products (pistachio, fish, cheese, olive oil). It operates using an electrosurgical knife (iknife), which creates an aerosol that is evacuated from the sample through a transfer line into the ionization source, where a heated collision surface is located for the thermal ionization process. A multivariate statistical algorithm was validated for the real-time identification process.

Results:
High value products, granted with PDO (Protected Designation of Origin) indication, were analyzed and successfully differentiated, without any geographical mismatching, thus demonstrating the applicability of the new technique in the detection of food fraud. The new technology was also applied to commercially popular and genetically similar Mediterranean Sea fish to obtain fast and accurate speciation results.
Marker compounds, responsible of sample discrimination, were determined and confirmed by conventional chromatographic techniques. These results demonstrated the capability of the iknife to provide in few seconds an holistic profile of the matrix.
Moreover, inter-laboratory studies gave proof of the reproducibility of such MS approach and transferability of the statistical model. Within this context, the present study confirmed also the potentiality of the new technique to be easy-to-use by any operator, even not expert, after the building of a reliable database and a statistical model by an analytical chemist.

Conclusions
Iknife represents a powerful tool in the preservation of food security and safety. It can be also used as a shotgun approach to achieve a comprehensive characterization of a complex sample and, since lipids are normally the most representative detected molecules, it can be successfully employed in lipidomics. Finally, the comparison with chromatography methods made marker identification very reliable, conversely from the tentative assignment of previous works.

Novel Aspect:
Among ambient methods, the iknife has been developed in close combination with a dedicated statistical software, that made the identification process very straightforward.
Introduction
Saffron is a precious spice known as “red gold” obtained from the dried stigmas of the Crocus Sativus L., a plant of the Iridaceae family. Its importance is related to the peculiar quality and sensory properties, in particular aroma, colour and taste that are mainly due to the presence of safranal, crocins and picrocrocin, respectively [1]. Recent studies have highlighted the different beneficial health values beyond its colouring and flavouring abilities [2].

Methods
Forty-two saffron samples of different origin, drying process and age were collected. An UHPLC equipment composed by a Nexera LC20AD XR apparatus (Shimadzu, Tokyo, Japan) coupled with a 4500 Qtrap mass spectrometer (Sciex, Toronto, Canada) equipped with a Turbo V ESI source, was used for analysis. The target analytes were separated using a Kinetex C18 column in 9 minutes. The results were analysed by means of principal component analysis (PCA) using XLSTAT2016.

Results
A preliminary experimental design was applied to optimize the extraction of crocins. UHPLC-MS/MS conditions were set to obtain the best analytical performances in terms of sensitivity and selectivity; the selected experimental conditions allowed to determine all crocin isomers and isoforms in a single run. The crocin content in the samples was significantly different and resulted affected by process, age and origin, with a clear separation between the mild and high thermally processed samples. In particular results indicate that, increasing the drying temperature, a degradation of the crocin isomers with high number of glucose moieties occurs. Principal Component Analysis of all crocin data allowed to discriminate samples based on origin (Italy vs. other countries) and age and to confirm the feasibility of the use of crocin pattern as marker of quality of saffron.

Conclusions
A simple and fast procedure for the simultaneous determination of crocins has been developed by UHPLC-MS/MS. Multivariate analysis was used to distinguish saffron samples with different storage and process and data also confirmed that geographical traceability can be obtained by crocins concentration and isomers pattern.

Novel Aspect
The experimental data confirmed the feasibility to use -cis and -trans isomers of crocins as markers of quality, process and traceability.

Reference
Introduction: Currently there’s no globally harmonised definition for “food fraud”. Food and beverages of high commercial value are subject to fraudulent practice. Whisky is sold as the product of one distillery or as a blend. Analytical methods are required for process control and formulation. Whiskey characteristics are influenced by the cereals used in fermentation, distillation, maturation and blending regimes. Palm oil is also a commodity of socioeconomic importance.

Methods: DART was coupled to a single quadruope MS to produce profile data. Linear X rail module was used for direct sample introduction: The DART source was operated with a helium gas flow of 1.5 L/min at 400°C. The MS was operated in full scan mode (mass range m/z 150-950) in positive and negative polarity; cone voltage 5-30 V; scan rate of 2 Hz. Chemometric models were generated using authentic samples, these models were used for real-time classification of whisky and palm oils.

Results: Within this study we have investigated the potential for the implementation of DART QDa LiveID as solution for the rapid authentication of high value food and beverage samples of both liquid and solid types with minimal sample preparation. Multivariate models for product authenticity have been generated using a collection of authentic reference samples of single malt whiskey and palm oils from different geographical locations. In whisky, ions with m/z between 50–300 were seen to be responsible for the brand level differences whereas ions at between m/z 300-400 drove the differences between the authentic and adulterated samples. In the palm oil, ionized species suspected to be generated from saturated fatty acids; carotenoids; vitamin A; tocopherols; tocotrienols; diglycerides and triglycerides were evident in the full scan spectral data. On the basis of these spectral features, it is possible to generate a multivariate model using a combination of PCA for data dimension reduction and LDA for class discrimination to distinguish between grades of palm oil processing and production type.

Conclusions: DART produces relatively simple mass spectra characterized by M+, [M+H]+ in positive mode and M-, [M+H]- in negative mode. Fragment ions are observed for some compounds, the degree of fragmentation can be influenced by the choice of gas. In both the whiskey and palm oil applications, positive polarity ionization was found to give most abundant spectral features and considered to be the more diagnostic mode for model training.

Novel Aspect: DART QDa with LiveID for rapid characterization and authentication of solid and liquid food and beverages
1007 - EVALUATION OF SOLID-PHASE MICROEXTRACTION FOR THE ISOTOPIC ANALYSIS OF VOLATILE COMPOUNDS IN APPLE

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Keywords: SPME; GC-C-IRMS; apple; flavor compounds; authenticity

Introduction
The use of solid-phase microextraction (SPME) coupled with isotope ratio mass spectrometry (IRMS) for the δ13C and δ2H analysis of flavor compounds from apple fruit and recovery aromas has been evaluated using headspace sampling (HS). The method can be used to differentiate between natural and synthetic apple aroma compounds[1] and it can be easily transferred also to other commodities.

Methods
Factors affecting SPME were optimized[2], and the suitability of coupling SPME and gas chromatography-combustion-IRMS (GC-C-IRMS) [3] for determining δ13C and δ2H values was investigated. To do this, we investigated if HS-SPME extraction and analysis conditions result in isotopic fractionation. This was done by making a comparison of δ13C and δ2H values obtained by HS-SPME by GC-C-IRMS and δ13C and δ2H values determined using bulk analysis of reference material.

Results
Volatile components were extracted in 10 mL SPME vials containing 1 mL of the sample prior to gas chromatographic analyses. Optimal parameters were selected based on DVB/CAR/PDMS coated fibre and headspace sampling mode. Optimized SPME conditions were based on an adsorption time of 20 min at 30 °C and desorption time of 60 s at 250 °C. It is also shown that neither the analyte concentration nor the split/splitless mode affects δ13C values. Finally, we verified that there are no matrix effects from the apple fruit or apple juice medium and that the technological production from apples to apple recovery aromas cause no isotopic fractionation. Therefore, comparison of δ13C values is possible, and the use of cheaper apple samples instead of expensive recovery aromas for creating an apple aroma compound δ13C database is appropriate. Such a database is required to provide reliable results for determining the authenticity of flavorings.

Conclusions
Our results show that by using an optimized SPME method we can obtain highly reproducible results and that SPME is, therefore, an appropriate tool for the isotopic analysis of volatile apple compounds extracted from fruit, juice or recovery aroma. Since many different compounds with different concentration are present in one sample the selection of reference material and appropriate processing and interpretation of the results obtained is crucial.

Novel Aspect
We report for the first time how different HS-SPME extraction parameters and analysis conditions affect the δ13C and δ2H values of apple aroma compounds obtained by GC-C-IRMS.

References
A story on food, faith and fraud – detection of the fakes

Keynote – general introduction of the topic
Food fraud is a form of criminal behaviour, no matter the definition of crime. Its consequences are devastating. The interaction between motivated offenders, and the opportunities presented by victims and lack of control measures favour occurrence of food fraud. Control measures help to counteract fraud opportunities and motivations. Analytical testing is one of those control measures. Traditional measurements have focused on the analysis of one or a few product characteristics. However, nowadays analytical techniques generating detailed analytical fingerprints are used to determine the identity of foods, and many techniques are available. Mass spectrometry methods are an important sub-group of these Methods: Sensitive targeted methods will be presented, for instance for authentication of olive oil grades, as well as various fingerprint approaches. They include for instance broad anomaly methodology for authentication of spices, as well as for the characterization of organic products. Furthermore, MS-based breath analysis will demonstrate the importance of the origin of cocoa beans for a distinct identity of chocolates.

Conclusions
Food fraud is everywhere and its management requires analytical methods for detection of anomalies and confirmation of the authenticity of food products. Mass spectrometry offers a wide range of high value authentication applications.
1313 - FOOD PROTEOMICS: A NOVEL APPROACH TO FOOD AUTHENTICATION USING GLOBAL PROTEIN SIGNATURES AND SPECIES SPECIFIC MARKER PEPTIDES

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Keywords: proteomics, food authenticity, LC-MS/MS, global marker peptides

Introduction:
Food fraud by undeclared blending of meat from different species is a highly relevant topic in food industries as shown by the European horse meat scandal in 2013 [1]. For the detection of fraudulent blending with undeclared species to the earliest stage highly efficient methods for identification and quantification are necessary.

Methods:
Peptide based high performance liquid chromatography-multiple reaction monitoring methods using species specific marker peptides allow the detection of less than 1% horse meat or pork in a beef matrix [2]. Additionally, unspecific marker peptides - present in numerous species - have been discovered. Both specific and unspecific marker peptides have been discovered using information dependant acquisition and subsequent de novo sequencing of tryptic peptides.

Results:
In this study, we investigated the ratios of species-specific markers and global markers for different binary mixtures of beef and pork, beef and horse and pork and horse as well as the amounts of the global markers in different mammalian meat products by means of high resolution-multiple reaction monitoring. Considering the constant ratio of specific and global markers for each species, these ratios can be used to detect alterations of food products. While the amount of species-specific marker peptides decreases in case of food fraud, the amount of global markers remains more or less the same. Thus, a decrease of the ratio can be used to prove the presence of species mixtures even in cases where the substituting species are not known (untargeted food authentication). Furthermore, a linear correlation between this change of ratios and the extent of the alteration offers an approach to quantify the addition of undeclared meat. The results show that for most cases a good linear correlation with correlation coefficients of $R^2 > 0.95$ can be achieved.

Conclusions:
Our data shows that the targeted analysis of species-specific and global markers and the evaluation of their ratio is a promising way of detecting food fraud and additionally relatively quantifying fraudulent blending of meat from undeclared species.

Novel Aspect:
This food authentication method uses unspecific markers instead of species-specific ones allowing the detection of food fraud even when the substituting species are not known.

References:
Introduction: Lignans, phenolic compounds that are present in a number of foodstuffs, possess some biological activities i.e., antioxidant and antiestrogenic[1,2]. The aim of our work was to develop a new analytical method to quantify three lignans, i.e., secoisolariciresinol (SECO), lariciresinol (LARI), matairesinol (MAT) from espresso coffee (EC) by using HPLC-MS/MS Triple Quadrupole.

Methods: Different sample preparation methods, including dilutions, acidic hydrolysis and enzymatic digestions were evaluated and, once validated, the most performing was applied for the quantification of target compounds in coffee samples. The HPLC-MS/MS Triple Quadrupole, equipped with electrospray ionization (ESI) source, operating in negative ionization mode, was used for the analysis. Detection was performed in multiple reaction monitoring (MRM) mode.

Results: Each lignan showed different transitions with different intensities of MS/MS acquisition parameters in MRM mode; the highest product ion abundances for all three compounds were found at 350°C as temperature of the drying gas of ionization source. At this temperature the precursor ions for lignans were the deprotonated molecules \([M-H]^-\) in negative polarity, therefore, those conditions were chosen for lignan monitoring. About the sample preparation process, the most performing was the enzymatic digestion with the Clara-Diastase (mixture of enzymes), because it showed the highest recovery values (93.55 – 97.62 %). The analytical method was characterized by short chromatography run time (the separation of target compounds was obtained within 4 minutes) and high sensitivity (LOD ranging from 2 to 3 µg l\(^{-1}\) and LOQ ranging from 5 to 10 µg l\(^{-1}\)).

SECO and LARI were found in all nine EC samples from 27.9 to 52.0 µg l\(^{-1}\) and from 5.3 to 27.8 µg l\(^{-1}\), respectively, contrary to MAT that it was not possible to detect it in each type of coffee.

Conclusions: A new analytical method for the quantification of three lignans that avoided long clean up and enrichment sample procedures has been developed and validated. The application of our method to EC samples showed that SECO was the lignan present at the highest concentration (27.9 – 52.0 µg l\(^{-1}\)) followed by LARI (5.3 – 27.8 µg l\(^{-1}\)). Therefore, regular consumption of EC can contribute to the dietary intake of lignans.

Novel Aspect: The developing of a new analytical method for the quantification of three lignans in espresso coffee by using HPLC-MS/MS and their determination in different coffee samples.

Keywords: proteomics; old wheat genotypes; gluten; allergens; celiac disease

Introduction
Over the last years, old wheat varieties have gained increasing attention since they have been suggested to present health benefits when compared with modern cultivars of bread and durum wheat and are generally considered better tolerated, even if without any scientific evidence based data [1]. Modern wheats have beneficial properties in terms of yield compared with older varieties, but information about changes in gluten proteins are still scanty.

Methods
In order to investigate the effects of the breeding that took place during the 20th century on gluten composition, a modern and an old Italian durum wheat genotypes were studied by coupling 2D-PAGE of the gliadin fraction and a bottom-up approach consisting of aspecifically designed enzymatic digestion (trypsin plus chymotrypsin), high resolution mass spectrometry analysis and database search using a manually curated database (GluPro V1.0) of gluten proteins [3].

Results
A previous comparison of a set of old and modern Italian durum wheat genotypes [2] showed that 2D-PAGE profiles of the gliadin fractions did not presentsignificantdifferences in relation to the expression of α- and γ-type gliadins. Conversely, they evidenced a drastic decrease in the expression of ω-type gliadins in the modern genotypes, with the highest content of ω–gliadins observed in the old genotype Dauno III and the lowest in modern genotype Saragolla. Particularly, the two genotypes presented differences in both the number andposition of ω-gliadin spots. Here we report the preliminary results about the MS analysis of these spots. Taking into account that the main difficulties in ω-gliadin structural analysis are related to the presence of repeatedpeptide blocks rich of glutamine and proline, and to the very low level of lysine and arginine residues, a mixture of trypsin and chymotrypsin was employed with the aim to obtain an efficient ω-gliadin spots digestion.

Conclusions
The dedicated enzymatic digestion provided a lot of medium-sized peptides, which were analysedby MS/MS. Because proteomic methodsrequire comprehensive and well annotated sequence databases, which are lacking for gluten, more reliableidentifications were obtained searching MS data in a manually curated database of gluten proteins. This approach allowed the identification of some ω-gliadins responsible for the differences observed in 2D-PAGE profiles.

Novel Aspect
Provision of a custom approach together with the use of a curated and appropriate databasewill improve the reliability of gluten protein identification by proteomic analysis.

References
Introduction:
The presence of chemical contaminations in the environment and food can lead to human exposure to various molecules (including pesticides), which could be responsible for adverse effects for the human consumer. Biological monitoring allows improving the exposure assessment, but this usually concerns a limited number of compounds analyzed in a targeted way, while untargeted approaches should provide a wider and more relevant assessment of this exposure.

Methods:
HRMS data were acquired on LTQ-Orbitrap XL (Thermo) and Synapt G2-Si (Waters) mass spectrometers running under both positive and negative electrospray ionization modes. Both spectrometers were fitted with UHPLC systems using generic H2O - MeOH gradient elution on classical C18 columns. Data were treated using various software solutions including MassLynx and Progenesis from Waters, Xcalibur, MetWorks and TraceFinder from Thermo Scientific, XCMS, SIMCA-P.

Results:
A non-targeted UHPLC-ESI-HRMS exposomics workflow has been developed[1] and applied to the analysis of Human cohorturine samples. After HRMS detection, data can be preprocessed in two ways: (i) focusing on a list of suspected metabolites generated from selected contaminants (e.g. pesticides), or (ii) starting from all features for identification of discriminant metabolites (including both endogenous and xenobiotics metabolites). From the same dataset, the first approach allows untargeted exposure assessment whereas the second can highlight possible impact of these exposures on the health status. Through few examples, this lecture will give illustrations of how untargeted HRMS allows characterisation of pesticide metabolites as well as other chemicals, representing markers which enable classifying various population groups[2]. Other complementary strategies can also be implemented in order to get more toxicological relevance, by focusing e.g. on toxic reactive metabolites formed in vitro.

Conclusions:
The combination of untargeted and suspect screening approaches, constitutes an efficient tool for exposomics studies, and could be used as an upstream multi-exposure assessment step for food and environmental exposure evaluation. The relevance and limitations of these untargeted or semi-targeted approaches for better assessment of food safety in the context of multi-exposure to contaminants will be discussed.

Novel Aspect:
Untargeted and suspect screening data acquisition strategies for evidencing potentially new biomarkers of exposure to chemical contaminants

References
Supplementation of animal feeds with vitamin D2 (VitD2) is less effective than vitamin D3 (VitD3) at increasing serum 25-hydroxyvitamin D [25(OH)D; status marker] of beef cattle and pigs [1,2]. A faster rate of degradation of VitD2 may explain this finding, and measurement of the serum 24,25-dihydroxy catabolic markers, 24,25(OH)2D2 and 24,25(OH)2D3, by LC-MS/MS would test this hypothesis.

Sera were obtained from animal feeding trials in which beef cattle received diets containing 0 IU/kg VitD or 4000 IU/kg as VitD2 or VitD3, and pigs received diets containing 2000 IU/kg as VitD2 or VitD3 (n=8/10 per group). [1,2] Sera were analysed with a validated LC-MS/MS method for 24,25(OH)2D3, 25(OH)D3 and 25(OH)D2 in 150 µL serum [3] which was extended to include the analyte 24,25(OH)2D2.

The majority of the 30 beef sera analysed had concentrations of 24,25(OH)2D2 (90%) or 24,25(OH)2D3 (77%) below the LOQs (0.68 and 1.09 nmol/L, respectively). By contrast, 7 out of 8 pigs receiving VitD2 had quantifiable 24,25(OH)2D2, ranging from 1.85-4.77 nmol/L, and 24,25(OH)2D3 concentrations in pigs from both treatment groups ranged from 2.6-24.2 nmol/L. These differential 24,25(OH)2Dfindings were despite the fact that mean±SD serum 25(OH)D was higher in beef cattle compared to pigs (122±37 and 53±18 nmol/L, respectively), which suggests a slower 24-hydroxylation rate in beef cattle. In relation to catabolism of the vitamers amongst pigs, the mean±SD serum 24,25(OH)2D3 of pork receiving VitD3 was 21.0±2.8 nmol/L with no detected 24,25(OH)2D2, and the mean±SD serum 24,25(OH)2D3 and 24,25(OH)2D2 of pork receiving VitD2 was 4.5±1.8 nmol/L and 2.3±1.3 nmol/L, respectively.

Differences in serum 24,25(OH)2D2 and 24,25(OH)2D3 concentrations of beef cattle and pigs shows that regulation of the vitamin D degradation pathway is not the same in these two species. Amongst pigs, the catabolic rate of 25(OH)D2 is lower than that of 25(OH)D3, suggesting that different rates of catabolism do not account for the difference in efficacy of VitD2 and VitD3 feed supplementation.

Most 24,25-dihydroxyvitamin D methods measure only 24,25(OH)2D3. Including 24,25(OH)2D2 allowed direct comparison of vitamin D2 and D3 metabolism.

References:
NOVEL IDENTIFICATION OF 3-EPI-25-HYDROXYVITAMIN D3 IN PORK AND BEEF LONGISSIMUS THORACIS STEAKS CONFIRMS THE PRESENCE OF THIS METABOLITE IN THE HUMAN FOOD CHAIN

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Keywords: 3-epi-25(OH)D3, 25-hydroxyvitamin D3, beef, pork, LC-MS/MS

Introduction: (Limit of 400 characters)
The source of the C3 epimer (3-epi-25(OH)D3) of 25-hydroxyvitamin D3 (25(OH)D3) in human serum may arise, in part, from exogenous intake [1]. While pork and beef contain 25(OH)D3 [2,3], it is not known if pork and beef supply 3-epi-25(OH)D3 to the human diet. Measurement of 3-epi-25(OH)D3 in pork and beef and in the corresponding serum of domestic animals may provide clues to the epimer's origin.

Methods: (Limit of 400 characters)
A UPLC-MS/MS method in serum [4] was adapted for the analysis of 25(OH)D3 in Longissimus thoracis steaks (LT). The pentafluorophenyl (PFP) column was able to separate isobaric 3-epi-25(OH)D3 from 25(OH)D3 in both matrices. Quantification of 3-epi-25(OH)D3 in LT was achieved using 25(OH)D3-d6 as the IS and applying a correction factor to adjust for different ionization efficiencies of the epimers.

Results: (Limit 900 characters)
The LOD for 3-epi-25(OH)D3 in LT was 0.008 ng/g, and the RSD% of minced beef control (n=13 over 7 days) was 30%. The concentrations of 3-epi-25(OH)D3 in LT (n=18 pork, n=19 beef) ranged from 0.016-0.670 ng/g. There was no significant difference between mean 3-epi-25(OH)D3 concentrations in pork and beef (P>0.05), and the overall mean+SD was 0.102±0.070 ng/g. Thus, a typical 150g pork or beef steak would provide 15.0 ng 3-epi-25(OH)D3. In serum collected from the same animals, the mean+SD 3-epi-25(OH)D3 was higher in porcine (2.9±0.9 ng/mL) compared to bovine serum (1.9±0.7 ng/mL). 3-epi-25(OH)D3 was positively correlated to 25(OH)D3 in both LT (R=0.570) and serum (R=0.444). The %epimer (ie. 3-epi-25(OH)D3/(3-epi-25(OH)D3+25(OH)D3)*100) was equivalent in matched LT-serum pairs (P>0.05), and the mean+SD% epimer in beef LT was 6.2±3.5%, compared to 4.0±1.8% in pork LT.

Conclusions: (Limit of 400 characters)
This is the first known evidence of 3-epi-25(OH)D3 in the human food chain originating from pork and beef LT steaks. The %epimer in LT was the same as that in the circulation of both species, which suggests epimerisation occurs endogenously within the animals. The difference in %epimer content in pork compared to beef highlights species-related differences in the rate of epimerisation.

Novel Aspect: (Limit of 150 characters)
The PFP column commonly used to separate 25(OH)D3 epimers in serum was used to identify 3-epi-25(OH)D3 in pork and beef LT steaks.

References:
ARTIFICIAL INTELLIGENCE ENABLES THE DETECTION OF PATHOGENS IN FOOD AND OTHER COMPLEX BIOLOGICAL MATRICES BY MASS SPECTROMETRIC ANALYSIS OF RNA MODIFICATIONS

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Keywords: machine learning; pathogen detection; biomarker panels; food safety; medical diagnostics.

Introduction
We have recently demonstrated that a variety of microorganisms and viruses can be accurately differentiated according to their specific RNA post-transcriptional modifications (PTMs) observed by MS analysis [1]. We have now employed machine learning to establish a classification and identification platform that capitalizes on comprehensive PTM profiles to enable pathogen detection not only in cell cultures, but also in actual biological samples and materials.

Methods
Comprehensive PTM profiles were obtained from total RNA extracts treated with exonucleases to generate mononucleotide mixtures, which were analyzed by direct infusion nanospray MS on either an Orbitrap Velos (Thermo) or a Synapt G2 (Waters) instrument [2]. A non-redundant database of RNA modifications created in house was employed to perform data interpretation and generate final datasets used for training machine-learning algorithms written in Python.

Results
Machine learning constitutes an ideal approach for establishing a classification/identification platform in which the unique differentiating feature consists of an entire panel of biomarkers, including identities and corresponding abundances, rather than an individual biomarker. In our case, each unique panel consists of covalent ribonucleotide variants (i.e., PTMs) identified according to their characteristic masses, fragmentation patterns, and arrival time distributions. In this report, we show that the PTM profile of a typical microorganism – comprising on average ~30 PTMs – can be unambiguously retraced to its source. We demonstrate the ability of either supervised or unsupervised algorithms to properly classify labeled samples and recognize unlabeled (“unknown”) ones. Among the different systems included in the study, our classification/identification platform correctly differentiated milk contaminated by S. typhimurium from uncontaminated samples, as well as mosquitoes infected by Zika virus from healthy controls.

Conclusions
We demonstrated that different machine learning algorithms enable the correct identification of bacterial/viral pathogens in samples containing high backgrounds of foreign RNA, which were afforded, for example, by milk somatic cells or mosquito tissues. The ability to identify pathogens in their typical biological matrices and without analytical separations has an enormous potential for a broad range of food safety and medical diagnostic applications.

Novel Aspect
Demonstration of the ability of artificial intelligence to support pathogen identification through the analysis of large biomarker panels.

References
Introduction
Oxidative stress (OS) is a risk factors for human health, and several stress markers have been proposed for the evaluation of OS, and many of them are available for this purpose. The induction of organ function disorders can lead to age-related decline in biological functions in human [1]. The aim of work is to create suitable methods for identification of oxidative stress markers by means of UHPLC-MS/MS techniques applied in human samples, in particular for Oxysterols and Isoprostanes [2-3].

Methods
In this project, methods for extraction from various tissue has optimized for little amounts of samples (for example sperm and plasma) or for analyte enrichment (urine). After extraction, a clean-up procedure has performed in the samples using SPE methods for each type of analytes. For each of them a an UHPLC-MS/MS experiments was performed, being careful to take every possible parameter into account, using different source (ESI or APCI) and optimizing every instrument parameter.

Results
For the analytes different experiments was performed, for the oxysterols the best condition for analyst was obtained through using of APCI source operating in positive ion scan, in according with literature [4], using C18 column for the separation of analytes. In other hand, isoprostanes were more responsive by using of ESI source operating in positive ion mode; for the liquid chromatographic separation a C18 column was used. SPE clean up methods were optimized for the selected classes of analytes: for Isoprostanes SPE step was used to concentrate the OS markers present in urine before injection in the auto sampler. The extraction protocols of Oxysterols has been optimized, starting from bovine sperm procedures [5]: the protocols were miniaturized to allow the use of small sample amount. The developed methods were tested for different biological samples (human).

Conclusions
UHPLC-ESI(APCI)-MS/MS methods for the detection and quantitation of OS markers were optimized. These markers were detected and quantified in different human biological matrices both for oxysterols and Isoprostanes; both groups of samples were taken from subjects that followed controlled feedings, adapted to modify the levels of oxidative stress.

Novel Aspect
New analytical methods for detection and quantitation of OS markers were developed and validated, increasing sensitivity and reliability in the different matrices.

References
COMPREHENSIVE CHARACTERIZATION OF MINIMAS, A NATURAL COMPLEX FOOD SUPPLEMENT, BY DIFFERENT MASS SPECTROMETRY TECHNIQUES.

Enrico Flamini (1) - Giada Fodaroni (2) - Sara Tamimi (2) - Stella Bedont (2) - Denise Decarli (2) - Michela Burico (2) - Anna Gaetano (2) - Luisa Mattoli (2) - Pietro Traldi (3)

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Keywords: MiniMas, mass spectrometry, food supplement

Introduction
Food supplements are intended to support, maintain or optimize physiological parameters, therefore the organism’s homeostasis. The complex mechanisms that contribute to the maintenance of homeostasis have brought researchers to move from a reductionist to a holistic vision of physiology, biology and medicine. In this panorama, natural complex food supplements play a key role as all the compounds presents forms a natural complex system ready to interact at various level with the complexity of human organism[1].

Methods
To reach the global characterization of Minimas, different chromatographic methods based on mass spectrometry have been used. Volatile organic compounds have been analysed by means GC-QqQ and Head Space sampling. Organic polar compounds have been analysed by means UHPLC-qToF. The metal part of inorganic compounds have been analysed by means ICP-MS.

Results
The quality assurance of all natural complex food supplements is a very important goal particularly that of Minimas, a food supplement that helps to maintain the homeostasis of blood pressure and cardiac functions. The old fashion of analyze only few marker compounds is not enough, as the activity of Minimas, as well that of all natural complex products, cannot be referred to the presence of few compounds. All the compounds of the product are important as they express an effective complex natural system. Therefore, a comprehensive product characterization is necessary to assure batch compliance and efficacy through the time. In this work, plant dried-extracts ingredients of Minimas and Minimas itself have been analyzed by means of GC-QqQ and UHPLC-qToF Methods: Inorganics have been characterized by means of ICP-MS. All the data collected have been matched with an in-house library containing more than 1000 natural compounds providing the identification of several compounds, that than have been quantified[2].

Conclusions
The potency of the developed analytical approach applied to the natural complex food supplement Minimas led to its comprehensive characterization, getting a quali-quantitative identity card of its complexity. The analysis performed by different mass spectrometry techniques permits to check the presence of all Minimas ingredients, opening new perspective to the routine quality control of natural complex food supplements.

Novel Aspect
A complete mass spectrometry based analytical platform is available and it represents a very important tool to get the broader compositional knowledge of all natural complex products, included Minimas.

References
946 - THE SYNERGISM OF MALDI LIFT-TOF/TOF MS AND ESI–Q-TOF MS INSTRUMENTS TO DISCOVER NEW ISOFORMS OF ALLERGENS FROM HAZELNUT OLEOSIN EXTRACT.

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Keywords: MALDI-TOF/TOF, ESI-Q-TOF, hazelnut allergens, allergens

Introduction:
Oleosins are proteins associated to oil bodies of seeds and investigated because demonstrated to be responsible of severe allergic reaction in allergic patients [1]. During the characterization of hazelnut oleosins, selected by immunoreaction with the sera of allergic Italian children, the analyses with two MS instruments improved our capacity of data interpretation and allowed to identified a new isoform probably more reactive than the two oleosins already known.

Methods:
A protocol for the extraction of oil bodies associated proteins was optimized and SDS-PAGE was performed. The PMF method (“in gel” trypsin digestion, peptide collection and MS analysis) was applied to the bands at 14 and 17 kDa. All protein bands were identified by both MALDI-TOF/TOF and LC-MS/MS analyses. MALDI-TOF/TOF spectra were manually interpreted, if necessary, and results were used to refine LC-MS/MS interpretation.

Results:
The electrophoresis profile of the oil bodies associated proteins showed two most abundant bands. MALDI TOF/TOF analysis was performed on bands at 14 and 17 kDa from SDS-PAGE of hazelnut extract, where 100% of protein content resulted to be oleosins Cora12 and 13, already known as allergens. The LC-MS/MS analysis confirmed the same results. However, the manual checking of major signals of the MALDI-TOF spectra of the band at 17kDa revealed that only minor signals identified the oleosin Cor a 12 and that at least two high intensity signals were without any protein attribution. The fragmentation spectra from LIFT-TOF/TOF of two major picks were analyzed using MS-Tag Protein Prospector software in identity mode and didn’t give any protein identification. When the software was set in homology mode with Cor a 12 as template, a new N-terminal was revealed indicating the presence a new isoform not present in the NCBI database.

Conclusions
In protein identification the dependence of MS techniques from the DNA and protein database is very strong and new isoforms are rarely identified. In this work a new isoform of oleosins from hazelnut was identified combining manual and software-assisted interpretation of MALDI LIFT-TOF/TOF spectra.

Novel Aspect:
Sometimes old practices like to have look at spectra and try to assign almost all signals by manual interpretation of MS/MS spectra allow to really discover something new that normally miss out on automatic data interpretation.

References
Introduction:
Pyrrolizidine alkaloids (PAs) are secondary plant metabolites that are supposed to be carcinogenic and genotoxic. They occur mainly in plants of the Boraginaceae, Asteraceae and Fabaceae families. Plant food and beverage, phytopharmaceuticals or even animal feed can easily be contaminated with PAs and enter the food chain. Currently there are discussions on possibleregulatory measures caused by the presence of PAs in honey, tea, herbal infusions and food supplements. Existing methods include laborious sample preparation, e.g. solid-liquid extraction followed by solid phase extraction for clean-up.
Here we report an on-line SPE UHPLC-MS/MS method, which overcomes the difficulties of combining

Methods:
Tea samples were extracted twice with 0.05M sulfuric acid by sonication. Before centrifugation the pH of the extract was adjusted with ammonium hydroxide. These samples were put into the autosampler and transferred to the on-line SPE column using an aqueous solution. After washing the sample was eluted and trapped into a loop. By switching the loop the eluted sample was transferred to the analytical column. A binary gradient separated the PAs for quantification. Due to this hardware set-up UHPLC with high backpressure and on-line SPE which is pressure limited were successfully combined. By careful fine-tuning of the SPE elution and the chromatographic conditions the separation of critical peak pairs could be maintained.

Results:
By using the reported instrument set-up, analysis and thus the quantification of 16 PAs (Indicine, Trichodesmine, Senkirkine, Senecivernine, Senecyphilline, Senechionine, Retrosine, Monocrotaline, Lycopsamine, Lasiocarpine, Jacobine, Intermedine, Heliotrine, Europine, Erucifoline, Echimidine) and 14 of their related N-Oxides (Echimidine-N-oxide, Lasiocarpine N-oxide, Jacobine N-oxide, Intermedine N-oxide, Heliotrine N-oxide, Erucifoline-N-Oxide, Europine -N-Oxide, Indicine N-oxide, Senecivernine N-oxide, Senecyphilline N-oxide, Senechionine N-oxide, Retrosine N-oxide, Monocrotaline-N-oxide, Lycopsamine N-oxid) could be performed. Calibration curves showed good precision and accuracy and even in a complex matrix like tea we were able to easily quantify the PAs in at least the range of 10 to 300 µg/kg. This is consistent to established methods using manual sample preparation, but less time consuming and less tedious. For all analytes, linear weighted regression resulting in $r^2 > 0.99$ could be achieved, with S/N > 10 for LLOQ levels.

Conclusions
A method for high-sensitivity analysis was successfully developed for PA analysis in plant material. The manual sample preparation could be reduced to a minimum as the set up of on-line SPE followed by UHPLC-MS/MS saves additional clean-up steps without compromising the performance of the assay.

Novel Aspect:
On-line SPE of Pyrrolizidine alkaloids coupled to an UHPLC-MS/MS system which overcomes the problems with the different pressure ranges.
Astride Franks Kamgang Nzekoue (1) - Elisa Vittori (2) - Sauro Vittori (2) - Giovanni Caprioli (2) - Gianni Sagratini (2)

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Keywords: Cheese shelf-life, free fatty acids, hexanal, food packaging, HS-SPME-GC-MS.

Introduction:
This study aims to develop a quantitative HS-SPME-GC-MS method to monitor concomitantly the evolution of short and medium chain free fatty acids and hexanal during cheese storage. Indeed, food storage is associated with the production of these markers whose amounts determine the taste and the spoilage level of cheese [1,2]. Therefore, their analysis can be useful in the evaluation of dairy product shelf-life [3].

Methods:
Hexanal, butanoic, isovaleric, hexanoic, octanoic and decanoic acids were analyzed by optimizing the GC-MS conditions in SIM mode. Moreover, different salting-out agents and SPME-fibers were compared to improve their extractions. This optimized method was validated and applied on two cheese samples (Grana Padano and Fresh Asiago) to compare the efficacy of two packaging systems: a polycoupled packaging named Ideabrill® and a HDPE film.

Results:
The trapping abilities of 4 fiber types (100 µm PDMS, 75 µm CAR/PDMS, 50/30 µm DVB/CAR/PDMS and 85 µm PA) was evaluated and followed by the comparison of 4 salts (NaCl, Na2SO4, NaH2PO4 and Na2CO3) on the extraction of FFAs and hexanal. The 75 µm CAR/PDMS fiber and the use of NaH2PO4 as salting-out agent resulted to be the most appropriate conditions for the extraction of analytes. A progressive increase of hexanal and FFAs during storage was noted confirming that these compounds are sensitive markers of cheese deterioration. All along storage, the concentrations of these markers became higher in the HDPE film. After 12 weeks of study for Grana Padano (GP) and 4 weeks for Fresh Asiago (FA), the Ideabrill® packaging allowed a lower increment of FFAs (11.6-fold increase for GP and 5.9 for FA) in comparison with the HDPE film (21.5-fold increase for GP and 7.4 for FA). The amounts of hexanal were also lower in the Ideabrill® packaging (0.58 against 1.04 mg/kg in GP and 0.38 against 0.55 mg/kg in FA).

Conclusions
By slowing down the increment of FFAs and hexanal, the Ideabrill® packaging has proved to be more effective than a common HDPE film in the preservation of the cheeses studied. This method can represent an important solution in the food industry sector, whose growth requires the optimization of fast and performing analytical methods that could help for example in shelf life studies, quality control or active packaging development.

Novel Aspect:
Hexanal and FFAs are good chemicals markers of cheese shelf-life. This is the first GC-MS method reported to quantify simultaneously these markers in cheese.

References
Non-targeted Characterization of the Degradation Products of Soybean Oil After Frying Process by HS-SPME-GC-MS and LLE-LC-HRMS

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Non-targeted Characterization of the Degradation Products of Soybean Oil After Frying Process by HS-SPME-GC-MS and LLE-LC-HRMS

Keywords: non-targeted analysis, degradation products, soybean oil, thermal processing

Introduction
Soybean oil has a maximum total production currently in edible oils and mostly used in the world. Furthermore, a high temperature thermal processing, such as frying and stir-frying have been proceeded in traditional Chinese cooking Methods: The degradation compounds of soybean oil after thermal processing might cause the damage of human health like cancers [1]. Hence, to develop an efficiency analytical method for identification of degradation compounds of soybean oil after high temperature processing is important in food safety and toxicology. In this study, HS-SPME-GC-MS and LLE-LC-HRMS methods were developed for characterization of components produced in soybean oil before and after fried. Multivariate analysis has been widely used in non-targeted analysis in recent years. Principal components analysis (PCA) was utilized to assist the identification of the biomarkers used for distinguishing the fried and un-fried soybean oil.

Methods
The manual SPME holder and three kinds of SPME fibers including 65μm Polydimethylsiloxane/Divinylbenzene (PDMS/DVB), 75μm Carboxen/Polydimethylsiloxane (CAR/PDMS) and 50/30μm Divinylbenzene/Carboxen/Polydimethylsiloxane (DVB/CAR/PDMS) were obtained from Supelco (Bellefonte, PA, USA). These coated fibers were conditioned according to manufacturer’s recommendation before the first use. 4 mL of soybean oil sample was placed in a 15 mL brown sample vial then sealed with a PTFE septum cap. Subsequently, the oil sample was heated to 175℃ in oil bath with stirring bar at 300 rpm for imitating the heating processing while unheated oil at 50℃. Optimum incubation time was 3 min, and then extracted via headspace for 30 min at 175℃. After 30 min, the SPME fiber was desorbed in the GC injector. The separation of volatile analytes were performed with a TSQ Quantum GC system (Thermo Scientific, San Jose, CA, USA). Compounds were separated on a DB-5MS capillary column (30 m×0.25 mm i.d.,0.25 μm film thickness ). The injector was set at 270℃ for 5 min. Ultra-high purity helium was used as the carrier gas at flow rate of 1 mL/min. The column temperature program was set as following: initial temperature 50℃ for 2 min, subsequently raised to 300℃ at the rate of 15℃/min, then held at 300℃ for 5 min at splitless mode. The GC/MS transfer line temperature was set at 275℃. Mass spectrums were obtained with full scan and mass range was from m/z 50 to 550. Library search was operated on NIST MS search 2.0.

Results
In order to extract all volatile compounds in soybean oil, the oil samples were incubated at 175℃ for 3 min. After incubation, the volatile components were extracted by CAR/PDMS, DVB/CAR/PDMS and PDMS/DVB SPME fibers at 175℃ for 30 min as heated oil. Un-heated oil was processed at 50℃. After 30 min, the SPME fiber was desorbed in the GC injector. The separation of volatile analytes were performed with a TSQ Quantum GC system (Thermo Scientific, San Jose, CA, USA). Compounds were separated on a DB-5MS capillary column (30 m×0.25 mm i.d.,0.25 μm film thickness ). The injector was set at 270℃ for 5 min. Ultra-high purity helium was used as the carrier gas at flow rate of 1 mL/min. The column temperature program was set as following: initial temperature 50℃ for 2 min, subsequently raised to 300℃ at the rate of 15℃/min, then held at 300℃ for 5 min at splitless mode. The GC/MS transfer line temperature was set at 275℃. Mass spectrums were obtained with full scan and mass range was from m/z 50 to 550. Library search was operated on NIST MS search 2.0.

In order to extract all volatile compounds in soybean oil, the oil samples were incubated at 175℃ for 3 min. After incubation, the volatile components were extracted by CAR/PDMS, DVB/CAR/PDMS and PDMS/DVB SPME fibers at 175℃ for 30 min as heated oil. Un-heated oil was processed at 50℃. The adsorbed extracts were desorbed in GC linear under 270℃ for 5 min then analyzed by GC-MS/MS. For comparing the volatile compounds in standard soybean oil, brand A and B soybean oil, statistic technology was used for handling the GC-MS/MS data. PCA is most widely multivariate statistical method used to highlight similarities and differences. In this study, the obviously difference between heated and un-heated soybean oil was found by using PCA under 95% confidence interval. The volcano plot was used to discover the potential biomarkers for distinguishing the heated and un-heated soybean oil.
The potential biomarkers was characterized by NIST database matching in the end. The matching results showed 1-Dodecene, 1-Undecanol, 1-Tridecanol, Hexanal, Tetrhydrofuran and 1-Tetradecanol were potential biomarkers in standard soybean oil; Nonanal, Dodecane, Benzaldehyde, 1-Octene, 1-Tridecanol and 1-Undecanol were potential biomarkers in brand A; Trimethylene oxide, Trichloromethane, Acetic acid, Tetrhydrofuran and 1-Tridecanol were potential biomarkers in brand B. In these compounds, Tetrhydrofuran, Trichloromethane were possible carcinogens. The biomarkers found in this study could be used for differentiation between the fresh soybean oil and recovered soybean oil.

Conclusions
This study concentrates on finding the possible biomarkers between fried and un-fried soybean oil by using HS-SPME-GC-MS and LLE-LC-HRMS. Use three kinds of fibers to make the data completed. We used heated oil to imitate fried oil. The experimental results indicated that there are significant differences between heated and unheated soybean oil in standard and two brands of oil. PCA will be employed to assist the classification and identification of biomarkers between fried and un-fried soybean oil. According to PCA and volcano plots, we can find a possible biomarkers by NIST. In the end, we find some possible biomarkers and google for their carcinogenicity. The experimental progress and results could assist us to distinguish fresh oil or not.

Novel Aspect
HS-SPME-GC-MS and LLE-LC-HRMS combined with PCA was developed and used to characterize the biomarkers for soybean oil adulterating.

References
Bioactive compounds of brazilian green coffee beans collected in different levels of maturation by ESI-LTQ-ORBITRAP

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Keywords: Conilon coffee; chlorogenic acids; caffeine; trigonelline

Introduction:
Harvesting coffee beans at different cultivating conditions may influence their chemical composition and the quality of the beverage [1,2]. Considering the economic importance of conilon coffee, the objective of the study was to identify the presence of metabolites in inedited conilon coffee clones and to compare them with known species, using a high resolution technique, as well as to verify if different maturation times can affect the composition of the coffee.

Methods:
Green coffee beans were collected in Espirito Santo, Brazil (3 cultivars of Arabica coffee, 3 clones of conilon coffee and one cultivar of Robusta Tropical). The samples were harvested in 3 different levels of ripeness (60%, 80% and 100% of mature) and the extracts were prepared with methanol. The extracts were injected into a mass spectrometer (LTQ-Orbitrap Elite) equipped with an electrospray ionization (ESI) source, operating in positive and negative ion modes.

Results:
A total of 21 coffee bean extracts were characterized in positive and negative ion modes by ESI-MS and tandem MS. Coffee bean metabolites, such as caffeine, trigonelline, and some sugars were observed in positive and a wide range of polyphenols such as chlorogenic acids in negative ion mode. It was found that coffee bean species Arabica, Robusta and clones 101,105 and 108 showed similar metabolomic profiles but distinct relative abundances. In positive ion mode, trigonelline was the most abundant compound with higher concentrations in Arabica samples and in clones 105 and 108. No trend was observed regarding to the maturation of this compound. However, the content of caffeine was higher in Robusta coffee and also in clones 105 and 108, increasing with maturation. In negative ion mode, caffeoylquinic acid was the most abundant compound with higher concentrations in Robusta coffee and in clones 105 and 108. The polyphenols and chlorogenic acids detected showed constant concentrations during the maturation in almost the samples.

Conclusions:
Among the 3 clones studied, it was possible to conclude that clone 105 and 108 were similar to Arabica coffee (better quality) and Robusta (higher productivity), being promising for a good quality. Trigonelline and caffeoylquinic acid were the main compounds detected in coffee beans. It was observed that maturation may affect some metabolites and it is important to monitor these changes because these compounds are scent and flavor precursors of the final beverage.

Novel Aspect:
To understand the conilon coffee quality, as well as the effects of different harvesting conditions, which could impact the composition and quality of the beverage.

References:
700 - DEVELOPMENT OF A LC-MS/MS METHOD FOR THE DETECTION OF SPECIES-SPECIFIC MUSCLE PEPTIDES IN PAPS

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Keywords: PAPs, peptides, proteins, LC-MS/MS, BSE

Introduction
Ruminant PAPs (Processed Animals Proteins) were forbidden in feed since 2001 [1], while non ruminant PAP were reauthorized in aquaculture in 2013 [2]. A PCR method is used in official controls: it permits to discriminate between ruminant and non-ruminant materials [3]. Mass-spectrometry can be helpful in identifying peptides from species-specific muscular proteins, since milk is an allowed ingredient in feed and PCR is not able to discriminate the origin of DNA.

Methods
High resolution mass spectrometry was applied to identify species-specific peptides from bovine muscular proteins. Then, a LC-MS/MS method was developed in order to detect these peptides in animal feed samples eventually contaminated/adulterated with bovine PAPs. Sample preparation consists of a proteins extraction step, followed by an accurate extract purification by means of SDS-PAGE and by an enzymatic proteins digestion in order to obtain the peptides [4].

Results
Three peptides, TR-12, TR-14 and YK-16, have been identified as markers of bovine PAPs presence by means of high resolution mass spectrometry. They derived from three bovine skeletal muscle proteins, Desmin, Vimentin and Myoglobin respectively. Proteins’ specificity has been carefully evaluated, excluding all proteins also contained in milk, colostrum and in other sources. Then, an accurate selection has been executed on peptides found, excluding all peptides not species-specific and with no adequate physical/chemical properties.

A LC-MS/MS method for the simultaneous detection of these peptides in animal feed samples was developed. This method is able to detect a contamination of animal feed with bovine PAP 0.1% w/w. Validation as qualitative method is in progress. The method was also applied to a real sample resulted non-compliant for ruminant DNA at PCR analysis and not reporting milk or milk by-products as ingredients: peptide TR-12 has been correctly identified.

Conclusions
The bovine peptides identified appear to be excellent biomarkers for detection of bovine PAP in animal feed. Their presence is an indisputable signature of bovine PAP, as they were selected from bovine meat protein sequences, and no possible cross reactivity appear to exist with dairy products. This could be of primary importance, as it will allow official and in house laboratories to detect unquestionably the presence of bovine PAP in feed.

Novel Aspect
A new approach to detect bovine PAP contamination was developed. It permits to discriminate between milk and bovine PAP addiction in feed, unlike currently official Methods:

References
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Keywords: ESIFT-ICR, NMR, metabolomics, White Celery, Red Pepper

Introduction
Untargeted analyses of complex biological mixtures such as vegetable extracts is a rapidly expanding field. Combined use of Fourier transform ion cyclotron resonance mass spectrometry (FT-ICR MS) and nuclear magnetic resonance (NMR) spectroscopy allows to increase metabolome coverage devoid of authentic standards[1] and is applied here to characterize white Celery of Sperlonga (WCS) and “Cornetto diPontecorvo” red pepper (CPRP) samples.

Methods
Both vegetables are endowed with Protected Designation of Origin and Protected Geographical Indication mark. Extracts from fruit (peel, pulp and seeds of CPRP) and plant (heart, stalk and leaves of WCS) were obtained by the Bligh-Dyer method. High resolution mass analyses were conducted with an ESI source coupled to a 4.7 T FT-ICR (BioApex, Bruker Daltonics) mass spectrometer; NMR spectra were recorded on a Bruker AVANCE 600 spectrometer equipped with a multinuclear z-gradient 5 mm probe head.

Results
Use of both NMR spectroscopy and FT-ICR MS has allowed a comprehensive metabolite profile of pulp, peel and seeds hydroalcoholic and organic extracts of CPRP grown in open field (OF) and in greenhouse (GH), revealing the presence of sugars, amino acids, organic acids, polyphenols, flavonoids. The very low content of biogenic amines and mycotoxins verified quality and freshness of the vegetables. Interestingly, the main features of CPRP are preserved in either OF or GH cultivation Methods:[2] The same experimental approach applied to hydroalcoholic extracts of WCS revealed that leaves and stalks present a similar composition, including choline, sugars, polyalcohols, amino acids, phenols, glycosylated compounds, free organic acids, including chlorogenic and ascorbic acids. In organic extracts, major peaks correspond to phtalides, as senkyunolide, responsible for organoleptic properties, chlorophyll a, linoleic and linolenic fatty acids, the latter more abundant in leaves.

Conclusions
The high sensitivity, resolution and mass accuracy of FT-ICR MS and the powerful quantitative and structural characterization allowed by NMR spectroscopy have provided a detailed metabolic description of different vegetable extracts. The large variety of secondary metabolites reflects the expression of biosynthetic pathways influenced by the environment.

Novel Aspect
These data may support efforts in preserving biodiversity and in developing new cultivation strategies.

References
Introduction:
The name "Grappa" is reserved exclusively for the grape pomace distillate obtained from raw materials produced in Italy and distilled and bottled in plants located in Italy (CE Regulation n. 110/2008). This research project aimed to investigate different conditions (wood type and alcohol percentage during aging) in the production of two high quality distillates.

Methods:
Grappa produced from a white marc (Prosecco) and a red blend (Cabernet Sauvignon/Merlot), at 55% and 68% Ethanol each, were aged over 6 month in 225 L, middle toasted cherry and oak barrels, which were selected on the basis of previous findings [1,2]. After one, three, and six month, samples were taken, volatiles isolated by SPE and analyzed by GC/MS. In particular, the evolution of variety aroma compounds from the marc and volatiles released from the woods, was monitored.

Results:
The analysis of the different grappa samples before ageing allowed the identification of more than 50 volatiles, mainly higher alcohols, aldehydes, acids, ethyl esters and acetates. The main differences between the two types of samples was, as expected, the higher abundance of variety aroma compounds, mainly terpenes, in the white marc grappa. Samples analyzed after one month showed already a slight increase of some phenolic compounds from the wood, but due to the low levels the impact on the overall aroma can be neglected. After three month the aroma of the grappa was already characterized by a wood aged aroma, and the concentration of phenolic compounds increased significantly.

Conclusions:
At the end of the process the different distillates showed characteristic profiles due to the different varieties and the wood type used. Main volatiles detected in the samples markedly characterized the organoleptic profile of the final products.

Novel Aspect:
This is a systematic GC/MS investigation of the impact of wood type and alcohol percentage on the aroma profile of grappa aimed to make new products.

References
HYDROGEN/DEUTERIUM EXCHANGE IS AN EFFECTIVE METHOD FOR THE STRUCTURAL RESOLUTION OF ISOMERIC PROANTHOCYANIDINS IN WINE AND OTHER DIETARY SOURCES

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Keywords: isotopic exchange; high resolution mass spectrometry; proanthocyanidins, wine

Introduction:
Proanthocyanidins(PAC) show high degree of isomerism, which makes it difficult to confirm the identification of new congeners. A novel class of PACwith a macro-cyclic structure has been recently found in wine[1, 2]. Hydrogen/deuterium isotopic exchange (HDX) combined to high resolution MS is discussed for the disambiguation of such cyclic B-type class from other isomeric PAC present in wine and other significant dietary sources.

Methods:
HPLC-HDX-HRMS and HPLC-HRMS/MS were applied to an SPE purified and concentrated Lagrein (a red wine from South Tyrol, Italy), a peanut skin extract and a cranberry extract. For SPE purification a published methodology was followed [3]. The instrumental method was adapted from a published report [1].

Results:
A series of 4-, 5- and 6-mers PAC were tentatively identified by HPLC-HRMS/MS in wine and (in lower extent) in cranberries. Their MS/MS fragmentations and retention time profiles showed remarkable differences with non-cyclic B-type analogues (already known and studied): a) beside the expected -2H difference, much lower degree of MS/MS fragmentations and very different retention time distributions distinguished the cyclic B-type PAC from the more abundant non-cyclic B-type PAC; b) tentative cyclic analogues eluted faster and showed fewer (often only one) isomeric peaks. However, cyclic PAC could not theoretically be distinguished from the isomeric non-cyclic A-type PAC. HPLC-HDX-HRMS was applied with this aim: the analysis of a peanut skin extract provided evidences that upon HDX the isomeric A-type PAC exchange one proton less than the cyclic B-types. By means of this approach, several cyclic B-type 4-, 5- and 6-mer PAC were confirmed in wine, whereas mostly non-cyclic A-type were confirmed in peanut skin.

Conclusions:
Isotopic exchange HPLC-HRMS proved to be a powerful tool, complementary to NMR and other techniques, to investigate the macromolecular structure of proanthocyanidins from several plant-derived foods. In fact, a whole series of novel cyclic B-type 4-, 5- and 6-mer proanthocyanidins could be distinguished from their isomeric non-cyclic A-type analogues by isotopic exchange mass spectrometry only.

Novel Aspect:
A mass spectrometric method based on hydrogen/deuterium exchange for the resolution of novel cyclic isomeric proanthocyanidins in wine and plant-derived foods is presented.

References
1394 - METABOLITES FROM CAFESTOL - A COFFEE DITERPENE- BY ZEBRAFISH TANK MODEL AND LC-MS/MS

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Keywords: mass spectrometry, metabolism, coffee diterpene, cafestol, in silico

Introduction: Coffee bean has a kaurane diterpene, cafestol [1], that acts as anti-inflammatory and anticancer (among others) [2], although being hypercholesterolemic [3]. It remains partially intact in the intestinal tract, and little is known about its metabolites. To understand what we really drink in the coffee beverage and its impact on health, this work was developed in an in vivo Zebrafish (Danio renio) water tank model [4] with LC-MS/MS looking for cafestol metabolites.

Methods: The study was performed in 3 aquariums, 18 zebrafishes/aquarium and cafestol added at 1 mg ml-1. The excretion water was submitted to SPE and then investigated in a Thermo Scientific Q Exactive Plus Orbitrap LC-MS/MS. The metabolites were characterized by at least 3 precursor ions. To corroborate the results and help rationalizing the fragmentation pathways observed, we compared the metabolites found with in silico analyses.

Results: Cafestol has a furan moiety linked to the ring A, a -CH2OH (C17) and a tertiary OH both linked to C-16 at ring D of the diterpene moiety. All typical metabolic transformations were searched in the excretion water which, by tandem analyses, showed five cafestol metabolites with logical fragmentations. The most intense was proposed to be a partial epoxidation of the furan with a hydroxy group at C2 (m/z 349.20095). In other ion metabolites, we propose the intact furan with an aldehyde on C17 (m/z 337.17797); a -COOH at C17 (m/z 331.19038); a -OH at C2 besides -COOH at C17 (m/z 347.18585); and acetylation at -CH2OH (m/z 359.22224). These showed that the preferential sites of metabolization were at carbons 2, 17, 18 and 19, compatible with in silico analyses with SMARTCyp and XenoSite. Although epoxidation was theoretically predicted, hydroxylation at C2 was not in the same metabolite.

Conclusions: Four metabolites observed in the water tank model of Zebrafish associated to LC-MS/MS are correlated to cytochrome P450 and one is a phase II metabolite. All transformations occurred at C- 2, C-17, C-18 or C-19 of the diterpene moiety, in agreement with in silico analyses.

Novel Aspect: This work contributes to a better understand of hot coffee beverage metabolites and helped on the fragmentation pathways of a complex structure like the diterpene cafestol, as also its metabolites.

References:

Sensitive and Specific Allergen Screening Analysis Using LC-MS/MS

Authors
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Allergens in food can result in severe or fatal reactions. At present, there is no known cure for an allergenic reaction and the only thing a person can do is to avoid the potential cause eg nuts, milk etc. Food allergens are of increasing interest due to food allergy recalls that have doubled in recent years. To help safeguard consumers from food related allergies, warning labels on packaging and in restaurants are a must these days to allow people to make a decision on what they eat. However, food testing is also important and as a result, it is vital to have a robust and specific analytical method to reliably identify and quantitate allergens that may be present. This work presents data from a method that has been highly characterized and verified to determine several different types of allergens using a tryptic digest and LC-MS/MS analysis to measure allergen peptides with a high degree of flexibility, specificity but also with high sensitivity.

Samples were trypticly digested and the resulting extracts analyzed using LC-MS/MS. Peptides that were identified to be associated with a specific allergen were measured using electrospray ionization and scheduled MRM data acquisition. In this presentation a comparison between traditional methods and the LC-MS/MS will be described. Important considerations for method development will be discussed. The ability of the method to identify allergens and to quantify them in food samples will be presented and discussed. The method results from several different food matrices will be presented to demonstrate the potential of this method.

Novel Aspect
This work demonstrates the process for identification of the correct peptide for allergen identification.
922 - LC-MS/MS: A USEFUL TOOL TO INVESTIGATE MARINE LIPOPHILIC TOXINS IN MUSSELS

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Keywords: Marine Lipophilic Toxins, LC-MS/MS, mussels, phytoplankton, profiles

Introduction:
Marine Lipophilic Toxins (MLTs) are compounds produced by marine microalgae. They often accumulate in mussels representing a risk for human health. MLTs include okadaic acid OA, dinophysistoxins DTXs, pectenotoxins PTXs, yessotoxins YTXs and azaspiracids AZAs and are regulated within Europe [1, 2]. The Istituto Zooprofilattico Sperimentale Umbria e Marche “Togo Rosati” is responsible for their monitoring in mussels harvested in the Marche Coast (Italy).

Methods:
The official LC-MS/MS method [3] was adopted to evaluate MLTs contamination in mussels bred and collected along the coast, biweekly or monthly, since March 2012 until December 2017. Mass experiments were performed using a hybrid triple-quadrupole/linear ion trap 3200 Q TRAP spectrometer (AB Sciex, Darmstadt, Germany) in Multiple Reaction Monitoring (MRM) mode. Two transitions were selected for each molecule to allow reliable quantification and identification.

Results:
The data collected about MLTs in shellfish samples (M. Galloprovincialis) showed different toxin profiles over the years. OA and YTXs were the only groups present in the analysed mussels. The concentrations of both of them usually increase sharply in autumn-winter, starting from the Northern areas of the coast and afterwards extending southern. Until 2013 YTXs were predominant reaching concentrations that exceed the regulatory limit (MRL) of 1 mg YTX equivalents Kg-1, subsequently increased to 3.75 mg YTX equivalents Kg-1 [4]. In 2015 and 2017, OA was the most abundant toxin measured in the samples with levels four/five times the regulatory limit (160 μg OA equivalents Kg-1), causing closures of local mussel farms. The different toxins profiles characterizing mussels in the years are very likely a result of variable climatic and environmental conditions such as temperature, rainfall, salinity, that may influence the blooms of different phytoplanktonic species.

Conclusions:
The LC-MS/MS method is suitable to monitor MLTs contamination in shellfish. The analytical technique used allows to investigate the profiles in terms of abundance and variety. The hypothetical correlation between climatic and environmental parameters and the observed levels could be useful in the prediction of mussels contamination, therefore a efficient tool to evaluate this threat for public health.

Novel Aspect:
Mass spectrometry applied to MLTs analysis provided detailed information on mussels contamination, enabling the study of temporal trends and toxin profiles.

References
EU-RL-MB- AECOSAN, EU-Harmonised Standard Operating Procedure for determination of Lipophilic marine biotoxins in molluscs by LC-MS/MS, vers.5 (2015)

This work was funded by the Italian Health Ministry - Project Code: RC0072016
Recent advances in mass spectrometry have led to the development of new analytical methods applicable in food chemistry. Target and non-target mass spectrometry methods have been applied for the detection of known and unknown chemical contaminants, including pesticides and antibiotics residues in food. Mass spectrometry-based omics sciences have introduced high-throughput methods permitting the assessment of food authenticity and detection of adulteration. In this work, we focus mainly on mass spectrometry-based approaches such as metabolomics and lipidomics for studying small molecules in different food commodities (wine, honey, and olive oil) as well as LC-MS/MS and GC-MS/MS techniques for the detection of chemical contaminants at low concentration level.
MALDI-TOF MASS SPECTROMETRY IDENTIFICATION AND DETECTION OF RELEVANT PATHOGENS IN BEEF CATTLE

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Keywords: MALDI-TOF, Mycobacterium, Brucella, Salmonella, Beef Cattle.

Introduction:
Mycobacterium bovis, Brucella abortus and Salmonella species account for significant economic losses in cattle production worldwide, besides being public health threats. We have established MALDI-TOF (Matrix Assisted Laser Desorption Ionization – Time-of-Flight) mass spectrometry with modified protein extraction methods and improved the presently available reference spectra libraries, in order to effectively detect those pathogens from bovine samples.

Methods:
Bacterial isolation from bovine tissue and lesions followed microbiological standard Methods: Heat inactivated mycobacteria from solid media were mechanically disrupted as designed herein. Brucella and Salmonella were grown in liquid media and processed as described [1]. Mass spectra acquired with alpha-cyano-4-hydroxycinnamic acid on Autoflex III Smartbeam underwent microorganism identification carried out on MALDI Biotyper software (Bruker Daltonik).

Results:
Analysis of mycobacterial isolates revealed that sixty-three classified as Mycobacterium bovis, two as Gordonia sputi and one as M. nonchromogenicum. These results were confirmed by Polymerase Chain Reaction (PCR) specific for M. bovis and 16S rDNA sequencing (100% concordance). Brucella abortus, B. suis, B. ovis and B. canis mass spectra profiles, generated as reference strains, were clearly different and allowed field samples identification. Amongst twelve isolates with gender specific PCR positive, only two were confirmed as Brucella by MALDI-TOF, indicating possible contaminations during cultivation. We also tested if Brucella fingerprint could be detected directly from tissue lesion homogenates, lacking any microbiological culture, which was successfully demonstrated.

Ninety-six isolates from bovine carcasses were biochemically tested for Salmonella and species were identified by MALDI Biotyper analysis. Four isolates showing inconclusive results in biochemistry actually belonged to Citrobacter and Proteus genera [2].

Conclusions:
The cell processing method established here provides reliable Mycobacterium identification at species level, with scores that safely allow M. bovis and M. tuberculosis distinction of isolates from solid media culture. We have discriminated Brucella species and could detect this pathogen directly from bovine tissue lesions, skipping bacterial cultivation step. In addition, MALDI-TOF enabled Salmonella identification at species level with serovar indicative.

Novel Aspect:
Distinction of Mycobacterium Tuberculosis Complex members at species level and detection of Brucella directly from infected tissue by MALDI-TOF mass spectrometry.

References:
Introduction:
Nutritional status of infants and children has great impacts on subsequent health and disease over an entire lifetime.Supplying appropriate nutrition by well-balanced infant formula is especially important considering that it is often the major, or even sole, source of nutrients at particular growth stages of an infant. It is a basic requirement for balanced nutrition and reliable nutritional studies to provide correct information on nutrients in foods and food supplements based on accurate measurements of their contents. Therefore, it is also important to develop a certified reference material (CRM) for the determination of elemental contents in infant formula or similar matrix. For this purpose, KRISS CRM 108-02-004 has been developed. The mass fractions of eight nutrient elements were certified by isotope dilution inductively coupled plasma mass spectrometry (ID ICPMS) and those of four monoisotopic elements were assigned as information values from the consensus values of expert laboratories. The present CRM is expected to provide a robust means for quality control and analytical method validation in the determination of various elements in infant formula or similar products.

Methods:
Raw material of infant formula obtained from a manufacturer was spiked with six hazardous elements, Al, As, Cd, Hg, Sn and Pb, considering future expansion of the present CRM for hazardous element analysis as well as nutrient elements developed in this study. The spiked material was made into a paste form and blended thoroughly to make homogenous mixture. Then, the paste was freeze-dried, pulverized into powders, sieved to obtain particles between 50 to 250 μm, homogenized using V-mixer, bottled in 15 g each, and finally sterilized using 15 kGy 60Co γ-ray irradiation. Twelve bottles from the batch of CRM were systematically selected and used for certification of nutrient elements. For certification of Ca, Cl, Cu, Fe, Mg, Se and Zn, a primary ratio method, ID ICPMS, was used. Monoisotopic element, I, Mn, Na and P, were analyzed using conventional field laboratory Methods: Two expert laboratories in food analysis also participated in the analysis and their consensus values were given as information values instead of certified values.

Results:
Mass fractions of eight elements certified by ID ICPMS showed excellent between-bottle homogeneity with relative standard deviation between 0.2 and 1.1 %. Expanded uncertainties of certified values were 1-4 % as shown in Table 1.

<table>
<thead>
<tr>
<th>Elements</th>
<th>Certified Values±U. (mg/kg)</th>
<th>k (95% level of confidence)</th>
<th>Homogeneity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mg</td>
<td>582.7 ± 8.1</td>
<td>2.0</td>
<td>0.27</td>
</tr>
<tr>
<td>Cl</td>
<td>3678 ± 117</td>
<td>2.0</td>
<td>0.43</td>
</tr>
<tr>
<td>K</td>
<td>6084 ± 76</td>
<td>2.0</td>
<td>0.24</td>
</tr>
<tr>
<td>Ca</td>
<td>4904 ± 63</td>
<td>2.0</td>
<td>0.42</td>
</tr>
<tr>
<td>Elements</td>
<td>Information Values ± U. (mg/kg)</td>
<td>k (95% level of confidence)</td>
<td></td>
</tr>
<tr>
<td>----------</td>
<td>---------------------------------</td>
<td>-----------------------------</td>
<td></td>
</tr>
<tr>
<td>Na</td>
<td>1377 ± 48</td>
<td>2.0</td>
<td></td>
</tr>
<tr>
<td>P</td>
<td>3280 ± 140</td>
<td>2.0</td>
<td></td>
</tr>
<tr>
<td>Mn</td>
<td>0.61 ± 0.10</td>
<td>2.0</td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>1.55 ± 0.18</td>
<td>2.0</td>
<td></td>
</tr>
</tbody>
</table>

Conclusions
An infant formula CRM for element analysis was developed. Eight nutrient elements were certified, while 4 monoisotopic elements were given as information values. With additional certification including six hazardous elements, it will be the infant formula CRM with comprehensive coverage of analyte elements.

Novel Aspect:
A new CRM for nutrient element analysis in infant formula certified with 8 elements with information values for 4 monoisotopic elements.

For information please contact: scientific@imsc2018.it
PGG (Penta-O-galloyl beta-D-glucose) is currently used as a functional ingredient in health functional foods. This ingredient was approved by the Korean Ministry of Food and Drug Safety (MFDS) as a functional ingredient with the health benefit of the blood sugar level rising after eating. In this study, an analytical method based on High performance liquid chromatography (HPLC) with positive ion electrospray ionization (ESI) coupled to tandem mass spectrometry detection (LC-MS/MS) was developed for the determination of PGG in health functional foods. Performance characteristics such as selectivity, linearity, recovery, repeatability, and intermediate precision were examined. This validated method was successfully applied to the determination of PGG in commercial functional foods.
Analysis of Signature Lipid Biomarkers to Determine Fusarium Contamination in Melon

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Keywords: Lipid fingerprinting, biomarkers lipid, Fusarium equiseti, Cucumis melo L., ESI-MS.

Introduction
The cultivation of melon (Cucumis melo L.) has become a socio-economic activity of the Brazilian semi-arid. This crop suffers serious damage due to phytopathogens, such as fungi of the genus Fusarium which cause melon rot, a disease that leads to total crop loss [1]. The MS has a technique that provides fast, efficient and reliable results to lipid fingerprinting has been for applications like clinical pathogen rapid and identification taxonomic classification of microbial collections [2,3].

Methods
The Fusarium equiseti was cultured in PDA for 14 days at 30°C (8 replicates). The pellet and melons were cut in 1x1 cm pieces. Lipids were extracted using CHCl3/MeOH, according to Bligh Dyer method [4]. ESI-(+)-MS spectra were obtained using Xevo G2-XS Qtof Mass Spectrometer, covering the mass range from m/z 500 to 1000 Da. Peak picking and alignment were performed using MassLynx software and Multivariate analysis by MarkerLink (Waters).

Results
Fusarium equiseti was isolated from contaminated melons in the state of Ceará, Brazil. The lipid analyzes were obtained by ESI-(+)-MS, the exact masses and a fragmentation pattern allowed the use of the LIPID MAPS database and the injections in replicates allowed to perform multivariate analyzes showed characteristic ions of Fusarium equiseti, which are not present in the uninfected melon and which are found in the melon when it is infected by the phytopathogen. Based on the analyzes of PCA and O-PLS, the ions m/z 782.5 and m/z 784.5, these ions were identified as phosphatidylcholines (PC). Also observed the ions m/z 736.6 and m/z 783.5, identified as glycerolipids (GL), in the LIPID MAPS database. In all the replicates of the fungus samples, these ions were characteristic and were not found in the melon samples.

Conclusions
ESI-MS fingerprinting of lipid was able to classify if the melon is contaminated or uncontaminated by Fusarium equiseti. The PCA and O-PSL analyzes for these lipids, the most evident discrimination was for Fusarium equiseti, where highlighted ions which were attributed as GL and PC. Our preliminary results showed the potential the MS as a tool able for screening of major biomolecules participating in host-pathogen relationships.

Novel Aspect
To use the high efficient screening as quality control for melons, detecting contamination in the initial stage and avoiding losses of the lots during transportation.

References
Introduction:
Ancient Apple varieties can present different composition compared to commercial ones [1-6]. For this reason they can be valuable starting material for the development of new nutraceutical ingredients. The aim of this study was to evaluate vitamin C and secondary metabolite contents of nine Ancient Apple of Friuli Venezia Giulia region and compare with commercially available fruits for the possible development of new Apple-based nutraceutical products.

Methods:
Peels and pulps were separated, dried and extracted in aqueous methanol in ultrasound bath. Hydroxycinnamic acids, catechin, procyanidins, flavonoids, dihydrochalcone and triterpene derivatives composition were determined using LC-DAD-ESI-MSn, LC-ESI-QTOF and LC-APCI-MSn. A targeted metabolomic approach was used to describe the different composition of the ancient varieties compared to commercial ones.

Results:
Ancient apple variety showed significant differences compared to commercial varieties with high levels of vitamin C in pulps. Furthermore pulps and peels present higher amount of procyanidins, catechins and hydroxycinnamic acids. Different pattern in triterpene contents was also observed showing ancient varieties presenting theannurcoic acid, while ursolic and oleanolic characterize the commercial ones. With targeted MS data PLS-DA model was generated and the ancient varieties of FVG region appear to be clustered mainly on the basis of triterpene and phenolic contents.

Conclusions:
Ancient apple can be used for nutraceutical production due to secondary metabolite contents. LC-MS based metabolomics approaches may be useful for description and valorization of these fruits composition. Further studies are needed to optimize extract production from these fruits. The use of ancient apple in nutraceuticals can be useful to protect these fruits threatened by intensive cultivation, underlying the richness of Italian biodiversity.

Novel Aspect:
This work point out the usefulness of LC-MS for the characterization of food ingredients for nutraceutical applications and show the valorization of apple biodiversity.

References


For information please contact: scientific@imsc2018.it
Putting Pigment to Paper. The Quantitation of Bioactive Anthocyanins in Fruits and Nutraceuticals with Paper Spray Ionization Mass Spectrometry

Keywords: anthocyanins, quantitation, inhibitor, paper spray, mass spectrometry

Introduction: (Limit of 400 characters)
Ubiquitous in edible plants and fruits, flavonoids constitute part of the human diet. Anthocyanins are a class of coloured flavonoids that have been recognized for their pharmacological properties and have recently been shown to be able to inhibit the replication of the influenza virus. This presentation describes the application of paper spray ionization mass spectrometry to quantify anthocyanins in raw fruits and nutraceuticals to ensure their bioavailability.

Methods: (Limit of 400 characters)
Stock solutions of cyanidin 3-sambubioside, cyanidin-3,5-diglucoside with malvidin-3,5-diglucoside as an internal calibration standard, were prepared. These solutions and those prepared for three nutraceutical formulations and freeze-dried raw fruit and stems, were analysed on a JEOL AccuTOF mass spectrometer equipped with a prototype paperspray ion source. Anthocyanins detected in the fruit and nutraceutical samples were identified and quantified.

Results: (Limit 900 characters)
The component cyanidinglucosides, including cyanidin-3-sambubioside, cyanidin-3-glucoside, cyanidin-3,5-diglucoside, cyanidin-3-sambubioside-5-glucoside and the aglyconecyanidin were detected in all fruits and nutraceuticals. Solutions of cyanidin-3,5-diglucoside, containing malvidin-3,5-diglucoside as an internal standard at a fixed concentration, established a calibration plot used to quantify the total anthocyanin content (TAC) in the fruit and nutraceutical formulations. Wide 5-fold variations in anthocyanin concentration were detected in the nutraceutical formulations from different suppliers ranging from 1050 to 5430 mg/100g, assessed in replicate measurements over an extended timeframe. These concentrations compared with 500 to 2370 mg/100g measured in the dried stems and fruit. The results demonstrated that anthocyanin levels vary considerably in different elderberry crops, and across various cultivars, and care is needed in the production and storage of nutraceuticals to maintain those levels.

Conclusions (Limit of 400 characters)
The ability to rapidly identify and quantitate, over a wide range of concentrations, anthocyanins in food and therapeutic products is important to gauge their medicinal benefits. Sensitive, yet mild, analysis is required given their susceptibility to degradation and transformation. Paperspray ionization has been used to successfully detect and quantify anthocyanin levels in fruit extracts and three commercially available nutraceutical formulations.

Novel Aspect: (Limit of 150 characters)
Paperspray mass spectrometry is a sensitive and reproducible means to quantify anthocyanins in fruit and therapeutic products and provides a rapid means with which to do so.

References

Introduction:
The genre Azorella belongs to Apiaceae family. The Apiaceae includes different plants commonly used as infusion in South America in folk medicine to treat various diseases such as bronchitis, cold, rheumatism and neuralgia [1]. The aim of this study is to investigate the potential antioxidant properties and the phytochemical profile of the aerial parts of Azorellaglabra (A. glabra) Wedd, an endemic uninvestigated plant from Bolivia.

Methods:
The aerial parts of A. glabra were subjected to maceration, solid-liquid, and then to liquid/liquid extraction using different solvents. The polyphenol content, radical-scavenging activity by using DPPH, ABTS, NO and O2− radicals, FRAP and inhibition of lipid peroxidation were determined; and these data were used to calculate Relative Antioxidant Capacity Index (RACI) [2, 3]. The identification and quantification of compounds were performed by LC-ESI-MS/MS.

Results:
The ethanol, n-hexane, chloroform, ethyl acetate, n-butanol and water samples were analyzed for their antioxidant activity by different assays. The ethyl acetate fraction reported the highest RACI value (1.17). For this reason, the LC-ESI-MS/MS analysis was focused on the ethyl acetate fraction. The tentative identification and the quantification of compounds present in the ethyl acetate fraction were reached through accurate mass measurements, MS/MS fragmentation patterns and retention times of standards. This fraction revealed the presence of 15 polyphenols and the pentacyclicterpenoidoleanolic acid identified for the first time in A. glabra. The most abundant present polyphenols are the orientin and 3,5-di-O-caffeoylquinic acid. These health-promoting compounds have medical and nutraceutical applications [4, 5].

Conclusions:
Ethyl acetate fractions of Azorellaglabra extract possessed the best antioxidant capacity. The polyphenolic compounds, in particular the abundant orientin and 3,5-di-O-caffeoylquinic acid, may explain the health benefits of the infusions used in folk medicine.

Novel Aspect:
This research has evidenced for the first time that A. glabra can represent an important natural source for the pharmaceutical, cosmeceutical and nutraceutical applications.

References:
MICROWAVE-ASSISTED EXTRACTION OF OLIVE LEAVES TO OBTAIN PHENOLIC COMPOUNDS WITH MODULATING ACTIVITY OF AMPK

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University of Granada, Analytical Chemistry, Granada (1) - University of Extremadura, Analytical Chemistry, Badajoz (2)

Keywords: Microwave-assisted extraction, phenolic compounds, olive leaves, HPLC-ESI-TOF-MS, AMPK

Introduction:
Phenolic compounds present in olive leaves possess beneficial effects to prevent chronic diseases [1]–[6]. Concretely, some of them provide the capacity to modulate AMPk activation, so they are proposed as therapeutic target in diseases associated with inflammation (obesity and diabetes)[7]. Microwave-Assisted Extraction (MAE) has demonstrated a great potential for obtaining extracts with higher content in these bioactive compounds.

Methods:
An experimental design Box-Behnken has been performed in which temperature (50-150ºC), solvent composition (15-80 % EtOH) and extraction time (5-20 min) have been evaluated to optimize the recovery of phenolic compounds that modulate the AMPk activation using MAE. The resulting extracts were injected into the HPLC-ESI-TOF-MS and phenolic compounds were analyzed following the method of Talhaoui et al. [8].

Results:
A total of 47 phenolic compounds were identified in olive leaves extracts. Among them, a total of 6 compounds, that have demonstrated the capacity to activate AMPk[7], were quantified (hydroxyoleuropein isomer A, rutin, luteolinrutinoside, luteolin glucoside isomer A, hydroxyoleuropein isomer B and verbascoside). Thus, the total concentration of these phenolic compounds ranged from 0.060 mg g⁻¹ to 1.418 mg g⁻¹ depending on the different MAE conditions. The Box-Behnken analysis at 100 ºC, 5 min and 15% ethanol were the conditions that showed the highest concentration of phenolic compounds related to AMPK activation.

Conclusions:
MAE has demonstrated to be a useful technique to improve the recovery of phenolic compounds able to modulate the activity of AMPk from olive leaves. Thus, 15 experimental conditions were assayed in the MAE experimental design in which temperature, solvent composition and extraction time were evaluated. The results showed that 100ºC, 5 min and 15% ethanol were the optimal conditions to extract this kind of phenolic compounds.

Novel Aspect:
These extract conditions could be used to obtain extracts for in vivo analysis and evaluate their possible anti-inflammatory effect (related to diseases such as the obesity and diabetes).

References
Development of isotope dilution-liquid chromatography/tandem mass spectrometry for the accurate determination of trans- and cis-vitamin K1 isomers in food

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Keywords: trans- and cis- Vitamin K1, Food, Isotope dilution mass spectrometry, LC/MS

Introduction
Vitamin K1 exists mostly in the trans form in most of foods such as vegetables and unfortified dairy products. However, infant formula and cereals fortified with synthetic vitamin K1 contains both trans- and cis-isomers. To evaluate the nutritional status of general foods (fortified or not), trans- and cis-vitamin K1 must be measured individually because only the trans-isomer has biological activity [1]

Methods
Isotope-dilution mass spectrometry based on LC/MS (ID-LC/MS) for the accurate determination of trans- and cis-vitamin K1 was developed in this study. A C30 column was used to separate and individually quantify the trans- and cis-vitamin K1 isomers. Vitamin K1-d7 was used as an internal standard. The matrix effect profiles were observed by post-column infusion experiment to optimize sample preparation methods and selecting ionization mode for LC/MS [2].

Results
Trans- and cis-vitamin K1 were well separated with the C30 column. The matrix effect profiles showed that APCI was less susceptible to matrix effects than ESI. Therefore, APCI was used to minimize the difference in the ionization efficiency of the target analytes and their isotope analogues. The validity of the method was evaluated by measuring gravimetrically fortified samples of infant formula, kimchi cabbage, and spinach. The measurement results (after subtraction of pre-existing levels) agreed with the fortified values within their uncertainties. The method showed good repeatability and reproducibility (less than 2 % of relative standard deviation of multiple measurements) for both trans- and cis-vitamin K1 in infant formula, with relative expanded uncertainties of approximately 5 %. For unfortified vegetables, its repeatability and reproducibility were less than 2 % for trans- vitamin K1, but around 6 % for cis-vitamin K1 as its abundance in unfortified food is much less than trans- vitamin K1.

Conclusions
An ID-LC/MS method was established as a candidate reference method for the accurate determination of trans- and cis-vitamin K1 in food. The validity test indicates that the method is of higher-order metrological quality in respect of its repeatability, reproducibly, and measurement uncertainty.

Novel Aspect
Compared to currently available methods, this study developed an ID-LC/MS method for the accurate determination of individual trans- and cis-vitamin K1 in food samples.
References
MEDICINAL EXTREMOPHILE PLANTS: VALUABLE SOURCE OF "GRAS" AND HEALTH PROMOTING BIOMOLECULES WITH MEDICAL, NUTRACEUTICAL AND FOOD APPLICATIONS

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Keywords: Extremophile plants, biological activities, bioactive molecules, functional food, nutraceutical.

Introduction:
Extremophile plants have to deal with frequent changes in salinity/water level by developing adaptive responses including powerful biomolecule synthesis. These plants are traditionally used for medicinal and nutritional purposes. Currently, an increasing interest is granted to these species thanks to their richness on bioactive compounds, which display potent pharmacological activities, and represent key-compounds in preventing diseases and ageing processes.

Methods:
The present research leads to the utilization of extremophile plants as a new source of healthy products. This contribution focuses on the ethno-pharmacological uses of spontaneous species in traditional medicine, reviews our recent investigations on chemical composition (CPC, TOF-LC/MS, RMN), biological activities (in vitro and in vivo) and potential uses after formulation as functional foods, nutraceuticals and cosmetic products by several industries.

Results:
Results showed an appreciable total phenolic content in organ extracts. The estimation of antioxidant capacities using ORAC method and a cell based-assay (WS1) showed that organ fractions exhibit highest antioxidant activities that exceed synthetic phenolics (BHT and BHA) used in Food industry (Ksouri et al., 2012; Oueslati et al., 2012). Moreover, these plants exhibit important pharmacological capacities against emergent diseases. For instance, L. guyonionum showed an appreciable anti-amyloidogenic ability as compared to curcumin. S. fruticosa fraction displayed the utmost anti-inflammatory activity, inhibiting nitric oxide (NO) release, by 67% in lipopolysaccharide (LPS)-stimulated RAW 264.7 macrophages. Besides, T. gallica showed the highest anticancer activity against human lung carcinoma (A-549) and colon adenocarcinoma cell lines (DLD-1, Caco-2 and HT-29). Then, the identification of the main compounds by HPLC-DAD-ESI-MS were assessed and some molecules were purified thank to CPC apparatus.

Conclusion:
These findings demonstrate the remarkable potentiality of these edible halophyte/xerophyte as valuable source of antioxidants which exhibit original and interesting antioxidant, anti-inflammatory and anticancer capacities which can be used as functional foods and to develop nutraceutical formulation to prevent some diseases.

Novel Aspect:
Clinical trials, photo-protective assays, and formulation are planned thanks to our recent Projects (CMCU, SATREPS, ARIMNET2 and PRIMA) linked to industrial applications.

References:
INTRODUCTION

In the present work, a novel identification approach was developed for the determination of bioactives in foodstuffs by Liquid Chromatography (LC) coupled to mass spectrometry (MS). Non-polar lipids were the analytes of interest, mainly due to their regular LC profile under reversed phase conditions. Marine organisms centralized special attention because of their high content of ω-3 fatty acids, whose nutraceutical activity was widely studied in the last decades.

METHODS

A dual-filter MS/LRI library was built by analyzing more than 30 real-world samples, including vegetable oils, milk and dairy products, sea organisms and food supplements. An odd carbon chain number triacylglycerol series from trinonain to trinonadecanoin was chosen as the basis of the LRI scale, being these compounds rarely present in real samples and an ultra high performance LC (UHPLC) method, able to maximize lipid separation, was developed.

RESULTS

The MS/LRI contains more than 200 lipid species, including mono-, di- and triglycerides (MG, DG and TG), as well as sterols and sterol esters.

An LRI variability of ± 15 units was obtained for all the compounds, also by changing experimental conditions (column dimension, flow rate, gradient steepness, instrumental setup, temperature), demonstrating the usability of the LRI database at both intra- and interlaboratory levels.

The MS spectrum was essential to discriminate between compounds falling within the same LRI window (completely or partially co-eluted). Under the employed MS conditions, a highly informative fragmentation pattern was obtained. For example, for a TG species, MG and DG fragments were highlighted, so that each MG fragment correspond only to one fatty acid and each DG fragment is related to their combination. The molecule related ion represents the final confirmation of the right fatty acid combination in the TG species.

CONCLUSIONS

The novel dual-filter library represents a powerful tool to achieve an univocal identification of non-polar lipids. Further efforts will be required to extend it to other lipid species, such as fatty acids and phospholipids, which represent important components in the clinical application field.

Advancements in software, able to perform an automatic identification on the basis of both LRI and MS data, was essential and has been successfully implementing.

NEW ASPECT:

The complementarity between MS and LRI data was exploited for the first time in LC, allowing to obtain for an unknown sample a very narrow list of candidates for each LC-MS peak.
Introduction:
The identification of molecular biomarkers is critical for diagnosis, treatment and for establishing a fundamental understanding of the pathophysiology and underlying biochemistry of inborn errors of metabolism. We show that, using the free electron laser FELIX and a modified Bruker ion trap, infrared ion spectroscopy (IR-IS) is a powerful technique for identifying metabolites. We also show that with our HPLC we can now investigate complex body fluid samples.

Methods:
The experiments were performed on a modified quadrupole ion trap mass spectrometer \(^1\) (Bruker, AmaZon Speed ETD) coupled to the infrared beam line of FELIX FEL. The FEL produces infrared radiation as 5-10 μmacropulses at a 10 Hz repetition rate having 40-100 mJ pulse energy and a bandwidth of ∼0.4% of the center frequency. After one macropulse dissociation occurs. An infrared vibrational spectrum is created by relating the parent and fragment ion intensities.

Results:
In a first example \(^2\), we characterize and distinguish two isobaric biomarkers of inborn errors of metabolism, glutaric acid for glutaric aciduria and ethylmalonic acid for short-chain acyl-CoA dehydrogenase deficiency. We show that the metabolites can be identified using an IR spectrum of a reference compound, but also in a reference-free approach by comparing the spectra against quantum-chemically calculated spectra.

In a second example \(^3\), we show preliminary results from a project in which we attempt to identify two new biomarkers for phenylketonuria (PKU). Using an untargeted metabolomics strategy based on UPLC-qTOF mass spectrometry, several new m/z features were detected in PKU patients. However, their corresponding molecular structures could not be unequivocally assigned based only on retention time, mass and MS/MS fragmentation. Here we show our recent progress using infrared ion spectroscopy in combination with theoretical predictions and synthetic reference standards.

Conclusions
Infrared Ion Spectroscopy (IR-IS) is a powerful technique for identifying and distinguishing many kinds of molecules. Recently we have shown that the combination of liquid chromatography – mass spectrometry with IR light of FELIX creates a unique pathway to identify metabolites in complex samples. We also show that when reference standards are unavailable we can fall back on quantum-chemically calculated IR spectra for the identification of molecules.

Novel Aspect:
Combining Liquid-Chromatography-Mass Spectrometry and the infrared light from FELIX we are able to identify biomarkers from complex body fluids.

References

For information please contact: scientific@imsc2018.it
Keywords: infrared ion spectroscopy, electron transfer reduction, metal-ligand complexes, redox pairs

Introduction: Transition metal organometallic complexes are known for their catalytic activation of small molecules. The catalytic activity is typically based on the ability of the metal to shuttle between different oxidation states. Here we present a novel method to spectroscopically investigate both members of a redox pair – a metal(II)-ligand complex and its charge-reduced metal(I)-ligand counterpart – in the complete isolation of the gas phase.

Methods: Infrared ion spectra were recorded using the tunable infrared free electron laser FELIX in combination with two tandem mass spectrometers, a home built Fourier-Transform Ion Cyclotron Resonance (FTICR) instrument and a Bruker AmaZon Speed ETD quadrupole ion trap (QIT) instrument [1]. The electron transfer dissociation (ETD) option of the QIT can also be used for reducing the charge of metal(II) complexes via an electron transfer reduction (ETR) reaction with the fluoranthene radical anion [2] as electron donor.

Results: Here we demonstrate that isolated metal(I)ligand complexes can be prepared through nondissociative gas-phase one-electron reduction of the corresponding metal (II) ligand complexes. The structure of both the precursor and the charge-reduced ion, i.e. both members of the redox pair, were established with infrared ion spectroscopy in combination with quantum-chemical calculations. Several metal-ligand systems have been studied, including Ni-cyclam which is especially interesting for electrochemical reduction of CO2 [3].

Conclusions: A varied set of Metal(I) ligand complexes have been generated via an electron transfer reduction reaction from the associated metal(II) complexes. The structure of these complexes in both oxidation states have been characterized via infrared ion spectroscopy using the FELIX free electron laser.

Novel Aspect: Electron transfer reduction reactions of metal (II) ligand complexes in the gas phase characterized by infrared ion spectroscopy.

References

919 - PHOTOCHEMISTRY OF GLYOXYLATE EMBEDDED IN SODIUM CHLORIDE CLUSTERS, A LABORATORY MODEL FOR TROPOSPHERIC SEA-SALT AEROSOLS

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Keywords: marine aerosols, glyoxylate, photodissociation, CO2•-

Introduction
As the ocean covers more than 70% of the earth’s surface, marine aerosols [1] are highly relevant for atmospheric processes. Photochemical processing of organic matter can be initiated by sunlight, where reactive species can be produced. In the present study, sodium chloride clusters serve as laboratory model for sea salt clusters. Doped with the atmospherically relevant glyoxylic acid [2], the photochemistry is investigated experimentally and supported with theory[3].

Methods
The clusters are produced with an Electrospray Ionization Source, and are then guided into a Fourier Transform Ion Cyclotron Resonance Mass Spectrometer. After mass selection of a specific ion, the clusters get irradiated with a tunable laser system and photodissociation spectra are recorded. Via the intensity of the fragments, photodissociation cross sections are calculated. Theoretical calculations support the results.

Results
Photodissociation spectra from 225-400 nm of [NanCln-2(C2HO3)]+, n=5-11, reveal the fragmentation pathway, where glyoxylate is photolysed into HCO• and a stable carbon dioxide radicalanion. This is interesting, as the latter species is known to be metastable in free space, which means that the salt environment stabilizes the molecular anion. Also important is that this happens not only in the deeper UV, but also in the actinic region from 300-400 nm, where sunlight reaches the troposphere. Excited state calculations show that the S0 and S1 state are well separated for the isolated glyoxylate anion, which renders internal conversion between these states impossible. As soon as glyoxylate interacts with an ionlike H+ or Na+, the potential energy surfaces show a conical intersection, which can be reached with the energy provided from the excitation into S1. After funneling into the electronic ground state, the dissociating HCO• may react in the ground state and transfer a hydrogen atom to form formate, which is another observed photochemical fragment.

Conclusions
Photodissociation spectra of sodium iodide clusters of different size doped with a single glyoxylate reveal a fragmentation pathway, where a carbon dioxide anion radical is stabilized in the salt cluster. Quantum chemical calculations show that dissociation along the C–C bond occurs after reaching the S1/S0 conical intersection, while this conical intersection is absent in free glyoxylate ions.

Novel Aspect
The photochemistry of glyoxylate embedded in sodium chloride clusters is investigated experimentally, and the results are supported with theoretical calculations.

References
318 - COUNTER CATION-DEPENDENT ACTION SPECTROSCOPY OF IR-797 AND IR-806 CYANINE DYES

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Keywords: Cyanine dyes (IR-797, IR-806); Mass Spectrometry; Action Spectroscopy; NIR; Dipole-induced absorption shift

Introduction:
Cyanine dyes form a large class of molecules absorbing in the near-infrared range. Though their aqueous phase absorption properties have been extensively studied [1], little is known about their gas phase optical characteristics. We measured gas phase photodissociation action spectra of IR-797, a heptamethine cyanine dye, and one of its doubly sulfonated derivative IR-806. This revealed absorption shifts depending on the sulfonate presence and counter cation.

Methods:
Gas phase cyanines were produced using electrospray ionization, and transferred into a triple quadrupole mass spectrometer (Xevo TQ-S micro, Waters). Ions selected in charge and counter cation state were irradiated after the first mass selecting quadrupole with tunable femtosecond laser pulses from 710 nm to 900 nm (80 MHz, 140 fs, 25 nJ/pulse, Coherent Chameleon Ultra II). Photofragments were then analyzed by the last quadrupole.

Results:
Photofragmentation yield was recorded as a function of the laser wavelength resulting in NIR photodissociation action spectrum. Maximum photodissociation of [IR-797]+ is observed at 760 nm, which is blue-shifted compared to the maximum absorption in the aqueous phase, and corresponds to the S0-S1 transition. The addition of sulfonated substituents on the nitrogen atoms leads to an increase of the maximum photodissociation yield to 780 nm for negative [IR-806]- ions and doubly protonated [IR-806,2H]+ ions. This is understood in terms of the presence of two sulfonate-counter cation dipoles in the vicinity of the cyanine chromophore [2]. We demonstrated this effect by increasing the strength of the dipole. Changing the counter cation from H+ to Na+ further shifts the absorption peak to 790 nm.

Power dependence revealed that two photons are necessary to induce fragmentation in these dyes. It thus implies sequential absorption of the photons to the first excited state S1 followed by intramolecular relaxation.

Conclusions
Photodissociation action spectroscopy of gas phase IR-797 and IR-806 cyanine ions reported shifts in the absorption to the S1 excited state. These shifts find explanation in the close environment of the chromophore composed of sulfonate-counter cation dipoles. Control of absorption is possible by control over the counter cation through mass selection. This can have consequences for aqueous phase absorption in the presence of ion pairs [3].

Novel Aspect:
We performed counter cation-dependent action spectroscopy on cyanine dyes using a triple quadrupole instrument coupled to a tunable high-repetition rate femtosecond laser.

References
Introduction:
Even though gas phase studies are essential for the understanding of structure-property relationships in oligomer metal porphyrins – in particular concerning their optical and chemical properties, there is only little information on isolated porphyrin ions in gas phase. Therefore we are presently investigating anionic metal porphyrin oligomers (ligand: meso-Tetra(4-sulfonatophenyl)porphine=TPPS) with different metal centers M= Cu, Zn, Pd, Mn & Fe [1,2,3].

Methods:
A combination of an ion mobility drift cell for determination of ion mobilities and isomer separation, quadrupole mass filters and time-of-flight photoelectron spectrometer allows isomer and mass resolved photoelectron spectroscopy (PES) of electrosprayed analytes. Additionally we use a high resolution orbitrap mass spectrometer to identify monomer and oligomer porphyrin ions of the same m/z, which coexist in gas phase and for CID measurements.

Results:
IMS measurements in combination with quantum chemical calculations have shown for [M(II)TPPS] dimers with M= Pd, Cu & Zn two different structure types [1]. They are independent of the metal center and depend strongly on charge state. Dimers in low charge states with a correspondingly larger number of sodium counterions tend to form stacked, cofacial structures. In higher charge states, the stacks form coplanar structures. For the trivalent metal centers Mn and Fe a third “daisy-chain” like structure is observed with bonds between the sulfonic acid group of one porphyrin unit and the metal center of another [2].

Photoelectron spectra upon Soret (around 420 nm) and Q band (around 550 nm) excitation of [PdTPPS]4-reveal different electron loss channels, which were determined to correspond to direct detachment from the S1 and/or T1 state as well as tunneling emission from these two states. The first triplet state lifetime of isolated [PdTPPS]4- is longer than 10 μs [3]. Compared to [PdTPPS]4-the photoelectron spectra of [ZnTPPS]4-look quite different.

Conclusions:
We found different structure types depending on the total charge state and the valence of the metal center. The stacked, cofacial structure is reminiscent of H-aggregate motifs observed in solution [4].

Photoelectron spectra obtained upon excitation of the Soret and Q bands of mass selected porphyrin anions are a powerful tool to understand photoelectron detachment mechanisms. They show a strong influence of the metal center on the relaxation dynamics.

Novel Aspect:
The determination of isomer and mass selected porphyrin gas phase structures is new as is the measurement of delayed electron detachment processes via isomer selective PES.
References:
Introduction: (398)
The basicity of aromatic aldehydes was widely studied. Protonation of benzaldehyde is directed to oxygen yielding a carboxonium ion with bivalent behavior as oxonium and carbenium ion.[1] In substituted benzaldehydes, the prevalence of the carbenium or oxonium character depends on the substituents and their position. The aim of this work is to characterize the structure of protonated hydroxybenzaldehydes, identified as key inhibitors in biomass hydrolysates.

Methods: (397)
Protonated ortho, meta, and para-hydroxybenzaldehydes (IBZH+) were generated in the gas phase by electrospray ionization of hydro-alcoholic solutions, (10^-5 M) of the select isomer, containing 2% acetic acid. Their structure was probed by combining IRMPD spectroscopy in the X-H spectral region with DFT calculations at the B3LYP/6-311++G(2df2p) level. As further inquiry, IRMPD spectra of both p-IBZH+ and its fragment ion were recorded in the fingerprint region.

Results: (856)
Similar to protonated aliphatic aldehydes, protonation at the formyl oxygen atom of benzaldehydes leading to the formation of carboxonium ions yields two distinct isomers, depending on the relative orientation of the proton that can stay either in cis or trans with respect to hydrogen on the adjacent carbon[2,3]. In this context, the 3200-3700 cm^-1 spectral region results ideal to distinguish the characteristic O-H stretch frequency of either cis and trans carboxonium isomers ions due to the different O-H bond properties of the two isomers. Comparison between IRMPD spectra and DFT calculations suggests that in the case of both p-IBZH+ and m-IBZH+ only cis conformers are present in the ionic population analyzed. In the case of o-IBZH+, IRMPD spectroscopy points to a mixture of one trans and two cis conformers. The value of energy barrier for the cis-trans isomerization calculated for each IBZH+ appears to be a measure of the degree of π-electron delocalization in the considered system.

Conclusions (391)
The present study shows that protonation of hydroxybenzaldehydes is directed only to the formyl oxygen atom to generate a mixture of carboxonium ions, with exclusively cis conformation. Only in the case of protonated o-IBZH+, the cis carboxonium ion isomer may overcome the high rotational barrier, and gives partially the more stable trans conformer. Moreover, the IRMPD spectra of ap-IBZH+ fragment formed by loss of CO corresponds to para-protonated phenol.

Novel Aspect: (139)
This work is the first gas-phase experimental study of IBZH+ and reports for the first time an IR spectrum of gaseous protonated phenol in the fingerprint region.

References

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Introduction:
Pyridine has been extensively investigated by means of REMPI and MATI spectroscopy [1,2]. In contrast, only little information can be found about the vibronic structure of the methyl and halo pyridines in their first electronically excited and ionic ground state. Hence, we focused on analyzing the electronic and geometrical structure of the first excited state S1 (1π* ← n) and ionic ground state D0 of the picolines [3], 3-fluoro and 3-chloropyridine.

Methods:
The molecules were investigated by means of REMPI and MATI spectroscopy. The measurements were done with two dye lasers and a home-built ToFMS. In REMPI mode, the spectra were obtained by scanning the excitation laser. In MATI mode, the excitation laser was kept resonant with an eigenstate and the ionization laser was scanned. The immediately formed ions were separated from the Rydberg neutrals by a spoiling field. Afterwards, the Rydberg molecules were field ionized.

Results:
The excitation energy of the picolines, 3-fluoro and 3-chloropyridine have been determined with a very high accuracy. The influence of the position of the methyl group and the halogen atoms has been identified. All derivatives experience an inplane geometry distortion along the eigenvector of the vibration 6a upon S1 excitation. Except for 3-chloropyridine, this vibration has the highest contribution to the electronic transition. Furthermore, an outofplane geometry distortion along the eigenvector of low frequency vibrations, which have a varying intensity among the derivatives, has been observed. The adiabatic ionization energy of the picolines, 3-fluoro and 3-chloropyridine has been determined by means of MATI spectroscopy. The picolines and 3-fluoro pyridine experience a geometry distortion along the eigenvector of the vibration 8b upon ionization via the S1 state. In contrast, the corresponding MATI spectrum of 3-chloro pyridine is dominated by low frequency vibrations.

Conclusions:
The ionization energy of the pyridine derivatives were measured with the highest accuracy reported so far. In addition, the ionic structure of the molecules has been determined. The MATI spectra via the active vibrations in the S1 state offered the possibility to verify the assignment in the S1 REMPI spectra. This was especially true for low frequency vibrations, which are symmetry forbidden and were not taken into account for the simulations of the vibrational spectra.

Novel Aspect:
The adiabatic ionization energies of the picolines, 3-fluoro and 3-chloro pyridine were measured with a very high accuracy and the ionic geometries were determined.
References:
INTRODUCTION:
Ion spectroscopy has become a powerful method in the modern MS toolkit. Numerous experimental setups make use of ion traps to irradiate molecules, thus accessing absorption of ions in the gas phase. We used here a triple quadrupole instrument to measure photodissociation action spectra of IR-797, a heptamethine cyanine dye, and one of its doubly sulfonated derivative IR-806 [1]. This revealed absorption shifts depending on the sulfonate presence and counter cation.

METHODS:
Gas phase cyanines were produced using electrospray ionization, and transferred into a triple quadrupole mass spectrometer (Xevo TQ-S micro, Waters). Ions selected in charge and counter cation state were irradiated after the first mass selecting quadrupole with tunable femtosecond laser pulses from 710 nm to 900 nm (80 MHz, 140 fs, 25 nJ/pulse, Coherent Chameleon Ultra II). Photofragments were then analyzed by the last quadrupole.

RESULTS:
Photofragmentation yield was recorded as a function of the laser wavelength resulting in NIR photodissociation action spectra. Maximum photodissociation of [IR-797]+ is observed at 760 nm, which is blue-shifted compared to the maximum absorption in the aqueous phase, and corresponds to the S0-S1 transition. The addition of sulfonated substituents on the nitrogen atoms leads to an increase of the maximum photodissociation yield to 780 nm for negative [IR-806]- ions and doubly protonated [IR-806,2H]+ ions. This results from the modification of the geometry of the cyanine chromophore with sulfonate groups in the vicinity. We demonstrated this effect by changing the sulfonate counter cation. Changing the counter cation from H+ to Na+ further modifies the position of the sulfonate substituents around the chromophore, yielding a supplementary absorption shift to 790 nm. Power dependence also revealed that two photons are necessary to fragment, implying sequential absorption to the first excited state S1 followed by intramolecular relaxation.

CONCLUSIONS
Photodissociation action spectroscopy of gas phase IR-797 and IR-806 cyanine ions reported shifts in the absorption to the S1 excited state. These shifts constitute a probe of the change in the geometry of the molecule, where the absorption of the chromophore reveals its close environment composed of sulfonate with or without counter cation [2]. This can have consequences for aqueous phase absorption in the presence of ion pairs [3].

NOVEL ASPECT:
We performed counter cation-dependent action spectroscopy on cyanine dyes using a triple quadrupole instrument coupled to a tunable high-repetition rate femtosecond laser.

REFERENCES
1248 - NEW FRONTIERS FOR CRYOGENIC CHEMISTRY AND OPTICAL SPECTROSCOPY OF MASS-SELECTED IONS

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Keywords: Cryogenic ion spectroscopy, reaction intermediates, catalysis

Introduction: Over the past decade, the structural characterization of mass-selected ions with vibrational and electronic spectroscopies has evolved from a niche technique in the hands of a few specialists to a powerful new capability for routine chemical analysis. We present recent developments in this approach that are designed to capture fragile reaction intermediates, determine their structures, and photoinitiate product formation.

Methods: Vibrational and electronic structure of cold ions generated by ambient ionization sources are obtained using cryogenic RF ion traps (octopole, Paul) coupled to a triple focusing time-of-flight photofragmentation mass spectrometer. This hybrid approach is presently being integrated with a Thermo LTQ Orbitrap XL instrument to provide a general secondary analysis capability. Vibrational and electronic spectra of the cold ions are obtained by photoevaporation of weakly bound mass “tags” (typically He, H2 and N2) or, in the case of bare ions, by two-color, vibrationally mediated photodissociation.

Results:
Two major advantages of the tagging approach are i) high resolution optical spectra can be obtained with the modest power available with table-top laser sources, and ii) the linear spectra of the cold ions can be directly compared with band patterns for candidate structures calculated with widely available electronic structure packages. This scheme has clarified the chemical nature of many molecular ions as well as the docking arrangements of non-covalently bound complexes ranging from polypeptides to unstable reaction intermediates in catalysis. This talk will highlight recent progress in understanding the origin of the strong anharmonicities that are apparent in spectra even when the ions are cooled below 10 K. These effects contribute to broadening and extra features that complicate structural analyses based on fundamentals calculated at the harmonic level. Examples where this occurs typically involve strong hydrogen bonds near charge centers and multi-state Fermi resonances, both of which are common in the vibrational spectra of gas phase peptides. Theoretical markers will be discussed that signal when such complexities are likely to occur. On-going experimental efforts will also be presented involving the development of chemically active ESI ion sources to provide a rational synthetic strategy with which to trap fragile intermediates in the catalytic activation of small molecules. Next-generation opportunities for this rapidly expanding technology will also be noted, such as the prospects for monitoring reaction kinetics in the size-selected solvation regime.

Conclusions: Optical spectra of cold ions yield structures through direct comparison with predictions from electronic structure calculations.

Novel Aspect: Optical spectroscopies of mass selected ions are becoming mainstream capabilities for general chemical analysis.
512 - IR TAGGING PRE-DISSOCIATION SPECTROSCOPY TO REVEAL ISOMERIC COMPOSITION

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Keywords: Cryogenic ion trap, tagging spectroscopy, isomer analysis, tropylium, benzylium

Introduction:
The recent introduction of cryogenic ion traps reaching temperatures of about 4 K opens new possibilities in weak bond ion-neutral attachment, notably the possibility of He/Ne attachment to the ions of interest [1]. The infrared pre-dissociation of these tagged ions can be used in ro-vibrational action spectroscopy. This can be used to infer the isomer composition of the ions.

Methods:
The FELion beamline at FELIX free electron laser facility at Radboud University (Nijmegen, The Netherlands), is equipped with a 22 pole radio-frequency ion trap at 4 K [2]. The ions, produced by electron bombardment are mass selected prior to injection into the trap, where they are tagged with He or Ne. Consecutively tunable FEL light is used to irradiate the tagged ions and obtain a one photon action spectrum (IR pre-dissociation).

Results:
The tropylium (7 carbon-hydrogen aromatic cycle) and benzylium ions (6 carbon-hydrogen aromatic cycle with one hydrogen substituted by methylene CH2 group) are the two lowest-energy isomers of C7H7+. Tropylium and benzylium like structures could also be found in larger polycyclic aromatic hydrocarbon (PAH) species, e.g., in a fragment of 1-methylpyrene C17H11+ (denoted as PyrC7+ and PyrCH2+) as predicted by Rapacioli et al. [3]. In this study, we will demonstrate how the IR pre-dissociation spectroscopy of cold Ne-tagged ions can reveal the isomeric mixture in situations where the IRMPD spectroscopy fails. We applied molecular dynamics simulations (SCC-DFTB level of theory) in order to analyse the difference between the multiphoton and single photon spectra. We also compare our results with isomer mixture ratios that can be obtained from the variation in reactivity of the two isomers.

Conclusions:
We show, that IR pre-dissociation of the weakly bond Ne cluster can be used as a complementary technique to mass spectrometry, providing information on isomeric composition. We identified the most abundant isomer of cationic 1-methylpyrene fragment, produced by low energy electron bombardment of the neutral 1-methylpyrene, to be PyrCH2+ isomer [4].

Novel Aspect:
The single photon dissociation scheme has a great advantage compared to multi-photon ones, in which consecutive heating can lead to isomerisation.

References
797 - VALENCE AND CORE ELECTRON INDUCED PHOTOIONIZATION AND DISSOCIATION DYNAMICS OF THE THIAZOLE MOLECULE

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Keywords: Photoionization, Ionic dissociation, Synchrotron radiation, PEPICO, Thiazole.

Introduction: The thiazole (C3H3NS) molecule belongs to the group of organic compounds called heterocycles, which are present in the formulation of important complex chemical entities, such as carbohydrates, proteins, among others, and stand out for their diverse properties and applications important to several fields. The main goal in this project is to provide reliable fundamental spectroscopic and spectrometric data for this group of molecules in gas phase by using synchrotron radiation, photoelectron photoion coincidence and time of flight mass spectrometry techniques.

Methods: TOF-MS and photoelectron photoion coincidence (PEPICO) techniques have been employed. VUV and soft X-ray synchrotron radiation sources were employed as ionizing agents. Total and partial ion yields (TIY, PIY) and PEPICO spectra were recorded as a function of the incident photon energy, covering from the valence region up to the core electronic shells of the molecule. The resulting photoionization ion products and the possible fragmentation pathways leading to those species are presented and discussed.

Results: For the valence photodissociation studies of thiazole, the PEPICO mass spectra have been recorded covering the range from 10 eV to 21 eV. The high photo-stability of this molecule and its molecular parent ion has been confirmed from our results. It has been mainly attributed to the strong bonding resulted from its closed ring molecular structure. The dominant mechanism corresponds to the consecutive dissociations of the C=N and C=C bonds from the thiazole molecular ion resulting in the fragment C2H2S+ (m/z = 58). The ion yield spectra were recorded by scanning the incident photon energies in the vicinity of the C1s and S1s core edges showing the main spectral features resulting from core electronic excitation transitions from the molecular ground state leading to the core excited and to the corresponding ionic states. In addition, the corresponding mass spectra based on the PEPICO technique recorded at selected photon energies near the core ionization edges provided relevant information leading to the identification of the dissociative photoionization ion products and their corresponding fragmentation pathways.

Conclusions: Time-of-flight mass spectrometry (TOF-MS) used in conjunction with photoelectron photoion coincidence (PEPICO), total and partial ion yield (TIY and PIY) techniques have been proved powerful tools for the analysis of the electronic structure and photodissociation dynamics of the thiazole molecule in gas phase, which resulted in very interesting chemical and physical insights.

References:

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79 - VALIDATION OF AN IRMPD EXPERIMENTAL SETUP AND THEORETICAL METHODS FOR IRMPD ASSIGNMENT

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Keywords: IRMPD; theoretical calculations; intramolecular interaction; ion spectroscopy; bioactive molecules

Introduction
Ion spectroscopy are usually compared to theoretical spectra to determine conformation of gaseous ions. DFT methods, mainly B3LYP, are the most used methods to model IR spectra, but some of them are known to provide inaccurate description of highly anharmonic oscillators.[1,2] We aim to assess alternative methods for describing anharmonic oscillators by comparison with experimental IRMPD spectra.

Methods
The benchmarking and validation of our experimental setup was performed with experimental spectra of protonated biotin and triptophan. Photodissociation was achieved by an OPO/A system in the 2750 – 3800 cm⁻¹ range. Calculations were performed with B3LYP, CAM-B3LYP, M06-2X and MP2 and the basis sets of Pople and Dunning type. Anharmonicity and D3 dispersion corrections were also evaluated.

Results
M06-2X/6-31+G** was the most cost effective level of theory. The increase in the job time, compared to B3LYP, was not significant, while the error (RMSD) highly decreases. Extended basis sets were not as much efficient, when associated to M06-2X. CAM-B3LYP was the better method to describe intramolecular interactions and provided good results with extended Dunning type basis sets. Anharmonic calculations were carried out and, but they showed to be extremely expensive when compared to other approaches. The dispersion correction D3, improved the description of O-H vibrations involved in H-Bond, but worsened the description of N-H stretches. None of the correction methods lowered RMSD as the use of well established scaling factors (sf). The M06-2X/6-31+G** and CAM-B3LYP/aug-cc-pVDZ level of theory was also tested against the fingerprint region, showing a good agreement with experimental data.

Conclusions
Our results suggest that M06-2X/6-31+G(d,p) could be the level of theory to be used in predicting IRMPD spectra. On the other hand, CAM-B3LYP/aug-cc-pVDZ was more efficient in describing long-range interactions, despite requiring more time than the previous level of theory. Between the correction approaches, the use of scaling factors remains as the easiest and more efficient strategy.

Novel Aspect
These are first IRMPD done in Brazil. We show the most used levels of theory to predict IRMPD spectra. We suggest the use of M06-2X/6-31+G**.

References
Introduction:
Peptide sequencing by Electron Transfer Dissociation (ETD) has seen rapid growth especially in top-down applications and for the identification of post-translational modifications (PTM). Understanding its underlying reaction chemistry remains elusive to some extent, in part due the radical-driven dissociation mechanisms and the open-shell product ion structures. Here we apply IR ion spectroscopy to reveal molecular structures of several ETD product ions.

Methods:
Experiments are carried out in a modified ETD enabled quadrupole ion trap MS connected to the IR beamline of the free electron laser FELIX. ETD fragment ions of doubly protonated precursor peptides are formed by ion-ion reactions with the fluoranthene radical anion. Individual ETD fragments are mass isolated and spectroscopically investigated with IRMPD spectroscopy [1]. IR spectra are compared with DFT computed spectra to derive structural information.

Results:
ETD is known to lead mainly to cleavage of the backbone N-Cα bonds in a peptide and results in closed-shell c-type fragments and radical z-type fragments. We have recorded IR spectra for a comprehensive set of c-type ions, mainly to distinguish between possible amide and enol-imine moieties at their C-terminus. Radical z-type ions are also investigated. One of the main questions was whether the radical remains located at the site of cleavage or whether it migrates to a thermodynamically more favorable site. For peptides where ETD occurs at a Cys residue, we observe secondary neutral loss of a thiol radical and spectroscopically establish the formation of a C=C double bond in the so-called w-type fragment ion.

Conclusions
The IR spectra suggest that the c-type ions have an amide moiety at their C-terminal. This may suggest that they are formed via the Utah-Washington mechanism, but post-ETD rearrangement from enol-imine to amide cannot be excluded based on the spectroscopic data. From the spectra of the radical z-ions, we tentatively conclude that radical migration to aromatic side chains does not occur, although other radical migrations have not been exhaustively verified thus far.

Novel Aspect
Systematic investigation of ETD fragment ion structures by IRMPD spectroscopy.

References
Introduction:
Copper and copper oxides are widely investigated for their catalytic properties [1,2]. It was shown that the calcination of copper salts can lead to highly active catalysts [3] and that copper hydride-based catalysts show a distinct reactivity [4]. Here, elementary steps in the calcination of copper formate are investigated in the gas phase by means of infrared multiple photon dissociation of Cun(HCOO)2n+1−, n≤ 4.

Methods:
Cun(HCOO)m− (n≤ 4; m≤9) clusters are prepared using electrospray ionization, transferred into the cell of a Fourier transformation ion cyclotron resonance mass spectrometer and irradiated in the wavelength range of 2500-4475 nm by a tuneable optical parametric oscillator. The decomposition of the ions is investigated by measuring dissociation kinetics at the absorption bands. For theoretical comparison, quantum chemical calculations on the DFT level are used.

Results:
Using action spectroscopy, three intense absorption bands were found in the wavelength range of 3400-3900 nm that shift depending on the size of the cluster and the oxidation state of the copper ions. Using deuterated isotopologues, the character of the vibrations involving C-H/D motion was confirmed and absorptions of C-O combination bands/overtones were identified.

After activating the C-H bonds of the formate ligands with the tuneable laser system, a fragmentation cascade has been observed with heavy secondary fragmentation. Clusters Cun(HCOO)2n+1−, n = 2-4, dissociate into smaller Cun(HCOO)2n+1− species with n = 1-3. For Cu2(HCO2)5−, H atom transfer takes place between two formate ligands, resulting in the elimination of CO2 and HCOOH together with a reduction of the copper centers to +1. For smaller clusters (n = 1,2; m = 2,3), CO2 elimination involves the transfer of a hydrogen atom to copper, leading to copper hydrides. Collisional activation leads to the same fragmentation pathway.

Conclusions
Three intense absorption bands of C-H stretching vibrations were found in the wavelength range of 3400-3900 nm that shift depending on the size of the cluster and the oxidation state of the copper ions. The dissociation of copper formate ions was found to lead towards copper hydrides through a reaction cascade. The results were compared to quantum chemical calculations on the DFT level.

Novel Aspect:
Copper formate clusters are activated with infrared laser light to simulate calcination with defined heating. CO2 elimination leads to copper hydride species.

References
Introduction:
Ion spectroscopy experiments are excellent tools to obtain complementary information in mass spectrometry. We describe the development of the 3D ion trap used for IRMPD spectroscopy and IRLAPS experiments, comparing results already present in literature and different ionization sources. This equipment is the first to perform IRMPD experiments in Brazil and is open to use by external users.

Methods:
Experiments were made in a modified Bruker AmaZon SL3D ion trap mass spectrometer. IRMPD experiments used a Laser Vision OPO/A system pumped by Nd:YAG 1064 nm laser to obtain tunable radiation from ca. 2300 – 4000 cm\(^{-1}\). Crystal optimization were performed when necessary to improve laser power in different wavenumber ranges.

Results:
A LabView interface was developed to annotate the actual laser wavelength to the raw MS data, allowing the direct calculation of the photodissociation efficiency. This interface completely controls the MS events and allows for multiple and variable IRMPD steps by customizing CID events.

IRMPD spectra of species previously reported in literature were obtained to verify the instrument performance using ESI-MS. The spectra for biotin, tryptophan, phosphotyrosine were similar to those previously reported which indicates the good performance of our system.[1-3] IRMPD of the Ag\(^+\)(CH\(_3\)CN\(_2\)) species were obtained at the nitrile stretch region as dissociation at 2350 cm\(^{-1}\) was registered, which is unprecedented given the low output power of the OPO/A at this wavelength. IRMPD spectra of a series of ions were obtained by using other in-house built ion sources like, nanospray, EASI and DESI ion sources.

Conclusions:
We have demonstrated that IRMPD spectra obtained in our new instrument are consistent with previous results of the literature. It was also possible to obtain the vibrational spectra from different ionization sources. The optimization of the OPO crystals positions improved the output power of the pulse energy in different ranges of wavenumber and allowed the IRMPD of nitriles at 2350 cm\(^{-1}\).

Novel Aspect:
The recently assembled instrument is the first to perform IRMPD experiments in Brazil. IRMPD were obtained from different ion sources and at 2350 cm\(^{-1}\).

References
Cold Ion Spectroscopy (CIS) provides accurate spectroscopic fingerprints for stringent validation of the 3D structures of small biomolecules in the gas phase [1-3]. A fundamental question that still remains open is to what extent the gas-phase structures of these biomolecules resemble their native structures in vivo. It was recently revealed that the pharmacological activity of peptide drugs Enkephalins correlates with their gas-phase geometries, but not with the condense-phase structures [4]. These peptide ligands adopt their biologically active geometry only when they dock into hydrophobic pocket of their receptors. Isolation of peptides in the gas phase mimics, to some extent, this hydrophobic environment as it removes strong intermolecular hydrophilic interactions.

In order to test this assumption, we applied conformer-selective multi-laser CIS to record conformer-selective vibrational spectra of individual conformers of three stereoisomers of the opioid pentapeptide [Ala2-Leu5]-Enk, isolated in the gas phase. Together with theoretical calculations, experimental spectra allowed for the identification of 3D structures of six conformers of three stereoisomers (3 for DD, 2 for DL, and 1 for LL). The obtained structures fully support the predictions made previously by FRET experiments in the gas phase. Furthermore, we found an evidence of the kinetically trapped, solution-like structure of one of the DL conformers.

Overall, these data confirm that for a large class of biomolecules there is high relevance of gas-phase studies for biology, as they reflect structural preferences of biologically active geometries.

UV absorption by aromatic residues in cold, gas-phase peptides is extremely sensitive to their structure. Combined with vibrational spectroscopy (IR-UV double-resonance [1] and IR-IR-UV hole-burning [2, 3] techniques) it allows for accurate validation of calculated geometries, while together with high-resolution mass spectrometry (2D UV-MS [4]) it allows for an identification of isoforms of biomolecules. These photofragmentation techniques are applicable, however, only to those peptides that contain at least one aromatic residue.

Alternatively, peptide bonds can be used as chromophores. In solution, peptides and proteins exhibit strong $\pi \rightarrow \pi^*$ transition at ~190 nm and a weak $n \rightarrow \pi^*$ transition at ~215 nm. UV absorption of peptides in the gas phase, however, has never been characterized. We report the first gas-phase UV photofragmentation spectrum of a single peptide bond. The influence of vibrational excitation on the electronic spectrum of cold ions is explored and all-conformer IR-UV gain and conformer-selective IR-IR-UV hole-burning spectra of non-aromatic peptides with up to six amino acids are presented.

2D UV-MS spectroscopy of a model helical peptide reveals that the intensities of the $n \rightarrow \pi^*$ transitions of individual peptide bonds correlate with their local structural preferences. This type of transition is, usually, very weak because of small spatial overlap of the atomic non-bonding orbital of oxygen, $n$, and the molecular delocalized anti-bonding orbital of the peptide bond, $\pi^*$. We suggest that the intensity of the $n \rightarrow \pi^*$ transition depends on the degree of distortion of the O=C-N-H planar orientation due to the local, non-covalent interactions. Since 2D UV-MS spectroscopy is able to separate absorptions of individual peptide bonds, it is a promising structural probe for peptides and proteins.

1179 - PHOTOISOMERIZATION ACTION SPECTROSCOPY OF A RUTHENIUM SULFOXIDE COMPLEX

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Keywords: ion mobility spectrometry; ruthenium; photoisomerization; laser spectroscopy; dynamics

Introduction:
Understanding the excited-state dynamics of photoactive molecules is essential for the design of new-generation photoresponsive materials. One promising family of switching units is based on the reversible linkage isomerism of sulfoxide ligands coordinated to a ruthenium(II) polypyridyl core. In these, the sulfoxide ligands convert between S- and O-bound configurations with quantum yields of up to 79% after metal-to-ligand charge transfer (MLCT) excitation.

Methods:
Photoisomerization action (PISA) spectroscopy was performed using a custom tandem ion mobility mass spectrometer coupled with a tunable laser. In an experiment, electrosprayed isomers were mobility separated in a first drift region, selected with an ion gate, and irradiated with tunable light. Photo-isomers were mobility separated in a second drift region and mass selected with a quadrupole. Resulting PISA spectra plot photo-isomer yield against wavelength.

Results:
The [Ru(bpy)2(bpSO)]2+ complex (bpy is 2,2'-bipyridine, bpSO is a bidentate bis-sulfoxide ligand) displays stepwise isomerization in solution from S,S → S,O → O,O with an overall quantum yield of 42% at 355 nm.[1] In the gas-phase, we observed direct S,S to O,O photoisomerization with minor formation of the S,O isomer.[2] Supporting electronic structure calculations suggested that both nπ* and ππ* excitation resulted in isomerization. The difference in photoisomerization yields between the previous measurements in solution and our gas-phase results was attributed to solvent quenching of vibrational energy before activated molecules could traverse excited-state isomerization barriers.

Conclusions:
Ion mobility mass spectrometry is a powerful technique for separating linkage isomers of ruthenium sulfoxide coordination complexes. Different photoisomerization yields were observed in the gas phase and in solution, indicating that solvation strongly perturbs isomerization process. Future studies will aim to elucidate further details of the photochemical dynamics in these systems.

Novel Aspect:
Ion mobility mass spectrometry coupled with laser spectroscopy characterizes the first example of linkage photoisomerization in a gas-phase coordination complex.

References
1270 - ACTION SPECTROSCOPY OF FLAVINS

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Keywords: flavins, ion spectroscopy, dissociation, isomerization, luminescence

Introduction:
Flavins are a ubiquitous family of redox cofactors which serve as blue light sensors in proteins and enzymes responsible for e.g. DNA repair, phototropism in plants and the perception of magnetic fields by some migratory birds. Little is known about how the optical properties of flavins are affected by micro-environmental perturbations, particularly in their reduced and/or deprotonated forms which act as resting or signaling states in some proteins.

Methods:
Flavin ions are produced by electrospray. Photo-induced dissociation (PID) action spectroscopy was performed using a sector instrument and an electrostatic ion storage ring. Photo-induced isomerization action (PISA) spectroscopy was carried out using tandem ion mobility spectrometry (IMS). Laser induced fluorescence (LIF) was measured in a quadrupole ion trap (QIT).

Results:
Using a multi-faceted approach employing state-of-the-art action spectroscopy experiments and concomitant calculations, we are investigating the photophysics and photochemistry of flavin ions in vacuo, with a focus on Flavin Adenine Dinucleotide (FAD). For FAD mono-anions, where the iso-alloxazine chromophore is in its fully oxidized, neutral form, the gas-phase absorption and fluorescence spectra are very similar to solution-phase measurements [1-3]. The gas-phase Stokes shift is considerably larger than previously reported for other molecular ions, implying significant vibronic effects [2]. Deprotonation of the chromophore leads to a significant red-shift relative to solution due to significant charge transfer character, which also gives a high susceptibility to micro-environmental perturbations [3]. Photo-induced intra-molecular proton transfer is found to be an important step in the photo-degradation of flavins [1,3]. Isomer-selective action spectroscopy has revealed deprotomer-dependent photochemistry [3].

Conclusions:
Intrinsic spectroscopic and photochemical data are established. These gas-phase experiments form a baseline for quantitative comparison to theory and to more complex situations in solution or proteins. The photochemistry of flavins is driven by intra-molecular proton transfer. Time-resolved experiments are needed to disentangle excited-state and statistical processes.

Novel Aspect:
First absorption and fluorescence spectra of flavin ions in vacuo. Photochemistry and micro-environmental sensitivity are strongly dependent on (de)protonation state.

References
PHOTOCHEMISTRY OF Mg+(H2O)N, N=1-5: EXPERIMENT AND THEORY

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Introduction:
There has been a long history of studies [1-4] on hydrated metal ions in the gas phase over the last decades. The study of such cluster systems is important to understand the transition of various properties from single molecules to the bulk. In the ground state these systems are already well understood, but few studies are available on their excited state reactivity. Here, we investigate the photochemistry and spectroscopy of hydrated magnesium ions Mg+(H2O)n, (n=1-5).

Methods:
The experiments were performed on an FT-ICR-MS, using a laser vaporization ion source. Clusters were generated from a target consisting of isotopically enriched 24Mg and supersonic expansion into high vacuum, along with a gas pulse of helium seeded with water vapor. A tunable, pulsed laser system was used to induce photodissociation. Dissociation mass spectrawere recorded in a wavelength range from 240 nm up to 2,000 nm and compared with ab initio calculations.

Results:
Photodissociation spectra for Mg+(H2O)n, n = 1–5 were measured, at 130±20 K and compared to experiments by Misaizu et al.[2]. The detected fragment ions can be grouped into two different channels. The Mg+(H2O)m (m < n) species are produced by the evaporation of one or more water molecules. The other channel, contributing most to the observed fragmentation, is the hydrogen dissociation reaction producing magnesium hydroxide MgOH+(H2O)m. The additional loss of one or more water molecules was also observed in this dissociation channel. For the Mg+(H2O)4,5 clusters, the influence of black-body infrared radiative dissociation was documented. Detailed photofragment branching ratios were evaluated. The main bands in the experimental spectra were assigned to the 3s → 3p transition. Additional bands were observed at higher photon energies, corresponding to the 3s → 3d/4s transitions. Resolved vibrational structure was obtained for Mg+H2O. For this system the possibility of a two photon process in the dissociation was investigated theoretically.

Conclusions:
Photodissociationspectra were measured for Mg+(H2O)1-5. The results are in good agreement with theoretical predictions and earlier experiments[2]. The 3s → 3d/4s dissociation bands were observed for Mg+(H2O)3–5. For Mg+(H2O)1–3, only a single isomer was present, but several isomers contribute to the spectrum of Mg+(H2O)4,5. For all investigated cluster sizes, hydrogen dissociation producing MgOH+(H2O)m was the main observed dissociation channel.

Novel Aspect:
Photodissociation spectra for Mg+(H2O)1-5 were measured with high resolution, far into the UV, in a temperature controlled environment.

References:
Introduction:
Protein unfolding in its initial stages may be reflective of the stability of the entire fold and reveal local sites that may be perturbed. Ion mobility mass spectrometry (IM-MS) provides insight into global structure while UV photodissociation (UVPD) has been shown to be sensitive to secondary structural elements [1,2,3]. In this work we investigate the initial unfolding of three proteins using 213 nm UVPD combined with IM-MS.

Methods:
Modifications were made to a Synapt G2-S [4] to facilitate injection of a laser beam into the trap cell region. Ubiquitin, cytochrome c and myoglobin were sprayed from ammonium acetate by nanoESI; UVPD spectra using 213 nm photons and arrival times of precursor and photofragment ions were recorded at different source conditions by varying the cone voltage affecting the protein conformation prior to UVPD.

Results:
Native structures and how they alter upon gentle in source activation was assessed. Unfolding from compact to extended conformer was induced in the lowest charge state of ubiquitin and UVPD was performed as a function of activation; the fragmentation yield was higher for the extended conformation. Cleavage in the compact structure was enhanced between residues 12-18 compared to residues 33-47 for the extended structure. For cytochrome c UVPD was carried out on a compact, an intermediate and an extended conformation. Again the fragmentation yield increased as the protein increased in CCS with distinct UVPD spectra for each conformation; cleavage at residues Phe10, Thr19 and Val20 was only found in activating conditions. Compact conformations of myoglobin yielded few fragments while a drastic increase was observed after a shift to intermediate and extended structures. The cleavage sites with the highest yield were similar for both native charge states, however fragmentation in the GH loop, H helix and D helix was only found for 9+.

Conclusions:
UVPD-IM-MS discriminates between compact structures and more extended forms. We present differences in terms of fragmentation yield as well as the intensity distribution within the fragmentation pattern. Cleavage sites differ between conformational ensembles revealing areas of local structural changes as a result of progressive in-source activation as well as global changes in terms of ion mobility profiles.

Novel Aspect:
213 nm UVPD-IM-MS is used to identify and locate structural changes upon protein unfolding

References:
PHOTODISSOCIATION SPECTROSCOPY OF M/Z-SELECTED FUNCTIONALISED PROTONATED PYRIDINE IONS: ARE SOME PREDICTIVE PATTERNS EMERGING?

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Keywords: laser spectroscopy, ions, ion trap, protonation, photodissociation

Introduction:
Protonated N-containing aromatics are ubiquitous fundamental molecules but despite their wide-spread prevalence, there are few predictive models that describe their photostability (and fate). Photostability affects photo-induced proton-transfer, electron-transfer and internal conversation. We are targeting small, protonated N-aromatics to better understand photostability to improve this general understanding.

Methods:
A combination of room temperature ion-trap mass spectrometry [1,2], ion-molecule kinetics [3] and tunable UV-Vis pulsed-laser photodissociation is used to measure the photodissociation of m/z selected ions formed from electrospray ionisation. Computational results for excited-states and ground state photodissociation pathways assist in assigning the spectra and rationalising the formation of product ions.

Results:
The UV photodissociation spectra for ethynylpyridineH+ and formylpyridineH+ ions (ortho, meta and para, in each case) are presented for first time. In some cases, the spectra exhibit vibronic detail – these are compared to Franck-Condon simulations – and in all cases multiple photodissociation channels are detected. Ion chemistry kinetics assists with assigning product isomers.

For ethynylpyridiniumH+ ions, UV photodissociation leads to either retention of the N atom in the ring (from C2H2 elimination) or its expulsion (from HCN elimination). It is not clear what mediates this partitioning. UV photodissociation of formylpyridineH+ reveals (at least) three distinct photoproductions and these are tracked within the action spectra, which are rather different for each isomer. These different spectra are likely the result of varying excited-state lifetime from the interplay between “bright” excited states – that dominate the spectra – and “dark” states that dominate the deactivation.

Conclusions:
Photodissociation spectroscopy of mass selected ions reveals important new details about their electronic excitation and fate. Although these target molecules are small, there remains many profound unknowns about the fate of their electronic excited states and mechanisms of deactivation. Our study reveals new information about the excited state energy of these species and some plausible mechanisms for their dissociation from UV excitation.

Novel Aspect: New photodissociation spectra are reported for m/z selected ethynylpyridiniumH+ and formylpyridineH+ ions using ion trap MS combined with tunable UV laser irradiation.

References

For information please contact: scientific@imsc2018.it
Keywords: DNA cation radicals, UV-Vis action spectroscopy, time-dependent DFT calculations, potential energy surfaces

Introduction:
Radical reactions play an important role in several biological processes. An area of particular interest has been the reactions triggered in nucleic acids and their components by electrons, photons, and high-energy particles. Nucleobase radicals have been previously generated by electron transfer in the gas phase. We now report on the generation of nucleic acid cation-radicals and their characterization by UV-Vis photodissociation action spectroscopy.

Methods:
Electron transfer reduction was used to produce nucleobase cation radicals in a linear ion trap from doubly charged ions formed by electrospray. UV-Vis action spectroscopy was carried out in the 210-70 nm range. Structures were obtained by all-electron molecular dynamics calculations followed by DFT and time-dependent DFT for excitation energies and intensities, including vibronic excitations giving thermal absorption spectra.

Results:
Nucleic acid cation radicals of novel type were produced in high yield by electron transfer reduction and dissociation of crown-ether complexes for DNA dimers (AA + 2H), (GG + 2H), (CC + 2H), (GC + 2H), and (CG + 2H). RNA chimeric dimers (A-spacer-A + 2H) were also produced with rigid ring spacers separating the adenosine cation and radical moieties [1]. Action spectroscopy and UV-Vis spectra interpretation revealed that the cation-radicals underwent a conformational collapse associated with hydrogen atom migrations between the nucleobases, forming new isomers. The hydrogen migrations were very facile for adenine and guanine nucleobases, but less so for cytosine. The RNA chimeras with aromatic ring spacers formed zwitterionic structures upon reduction in which the nucleobases maintained the charge and the electron entered the aromatic ring. The formation of zwitterions is consistent with the mechanism and energetics of DNA ionization, as revealed by detailed structure and energy analysis.

Conclusions
Upon electron transfer, single-strand nucleic acids undergo a conformational collapse driven by attractive interactions of nucleobase cations and radicals.

Novel Aspect:
Use of UV-VIS action spectroscopy in combination with electron transfer and TD-DFT calculations for structure assignment of transient DNA cation radicals.

References
Introduction:
Different stability and reactivity of diastereomeric aggregates reflect the number and intensity of the interactions between the chiral species involved. Therefore, investigating diastereomeric aggregates in the gas phase helps to understand the mechanism of chiral recognition through detailed knowledge of the structure of the chiral receptor, as well as of the size- and shape-specific non-covalent interactions in its aggregate with a chiral molecule[1-3].

Methods:
An ESI-MS-IRMPD analysis has been employed to assess the structural features of diastereomeric complexes[4] between an axially chiral receptor (M) and two amino acids, phenylalanine (Phe) and dopa (DOPA), differing only for the aromatic substituents. The results have been interpreted through a computational analysis at the B3LYP/6-31G** level, to assess both the coordination site and the preferential conformation adopted by the complexed guest.

Results:
IRMPD spectra of the [M∙H∙Phe]+ diastereomeric complexes did not exhibit any appreciable difference, in contrast to the significant discrimination exerted by M on the enantiomers of dopa. The comparison between experimental bands and theoretical results indicate that the guest is located out of the cavity, in close proximity of the binaphthyl moiety of M. Furthermore, the computational analysis performed on several rotamers pointed out that the enantiodiscrimination is mostly due to the short range interaction between the hydroxyl groups and the core of the axial chirality. It is worth noting that the structures spectroscopically observed are not the most stable ones.

Conclusions
The IRMPD of the ESI-formed [M∙H∙DOPA]+ diastereoisomers were consistent with an out of cavity structure, not corresponding to the lower energy coordination site. The observed discrimination on the dopa enantiomers is due to the hydroxyl groups, absent in the structure of phenylalanine, that are oriented towards the chiral core of M. Finally, the different spectroscopic signatures indicate the coexistence of several conformers for the diastereomeric aggregates.

Novel Aspect:
Subtle structural differences between non-covalent diastereomeric complexes have been pointed out through IRMPD technique.

References

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Introduction:
Understanding stereochemical effects on the structure of molecular aggregates provides insight into the role of chirality in shaping biological supramolecular systems at the molecular level. Here we focus on 1-amino-2-indanol (AI2H+), a proto-typical cyclic 1,2-amino-alcohol, and reveal how the chirality of the monomer subunits controls the overall structure of its dimers [1].

Methods:
Here, we apply IR-UV double-resonance laser spectroscopy coupled with ESI / cold ion trap mass spectrometry to the protonated homodimers of (1R,2S)-cis- and (1R,2R)-trans-Al2H+ (c-Al2H+, t-Al2H+). The IR-UV double-resonance spectroscopy enables us to measure UV and IR spectra of a single conformer. Such conformer selection is a big advantage of mass-coupled laser spectroscopy [1].

Results:
The conformer-selected UV spectra are measured for both cis- and trans-monomers (c-AlH+, t-AlH+) by IR-UV hole burning spectroscopy. The structural analysis with help of quantum chemical calculations at B3LYP-D3/6-31G++(d,p) level show that c-AlH+ has an intramolecular NH+...O hydrogen bond (H-bond), while t-AlH+ lacks such an interaction. Conformer-selected UV spectra of cis- and trans-AlH+ homodimers are measured and three and two conformers are assigned, respectively. Their structures are analyzed by the comparison between conformer selected IR spectra and theoretical IR spectra. The obtained structures of cis- and trans-dimers are clearly different. The structural difference is rationalized by the presence and absence of the intramolecular H-bond in cis and trans monomer subunits.

Conclusions:
This work demonstrates that the combination of ESI cold ion trap mass spectrometry and laser spectroscopy is a powerful tool to study the structure of molecular aggregates and effect of chirality. The methods in this work will allow us to expand the size of molecular systems to biological supramolecular systems. In the presentation, the application to functional peptides and its complex with chiral ligands will also be shown [2].

Novel Aspect:
The approach developed in this work will provide us a deep understanding of the stereochemistry-based control of biologi-cal supramolecular system.

References
Direct enantiomer-selective Mass Spectrometry of multi-component chiral mixtures

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Keywords: chiral, enantiomeric excess, direct MS, photoionization, instrumentation

Introduction:
Mass spectrometry is chirally blind, it cannot directly distinguish the two enantiomers of chiral molecules. Simultaneous, enantiomer-specific identification of chiral molecules in multi-component mixtures is extremely challenging. Many established techniques for single-component analysis fail to provide selectivity in multi-component mixtures and lack sensitivity for dilute samples.

Methods:
Here we show how enantiomers may be differentiated by Mass-Selected PhotoElectron Circular Dichroism (MS-PECD) using an electron–ion coincidence imaging spectrometer [1-2]. Following an ionizing circular polarized laser pulse, ions and electrons are detected in coincidence on their respective time- and position sensitive detectors. The Mass-Selected PECD reveals that the compound is chiral.

Results:
As proof of concept, vapours containing ~1% of two chiral monoterpene molecules, limonene and camphor, are irradiated by a circularly polarized femtosecond laser, resulting in multiphoton near-threshold ionization with little molecular fragmentation. Large chiral asymmetries (2–4%) are observed in the mass-tagged photoelectron angular distributions. These Mass-Selected PECD asymmetries switch sign according to the handedness (R- or S-) of the enantiomer in the mixture and scale with enantiomeric excess of the chiral molecule [3-5].

Conclusions:
The results demonstrate that direct MS identification of mixtures of chiral molecules is possible using the ultra-sensitive and selective technique of laser-based MS-PECD.

Novel Aspect:
Direct quantitative determination of enantiomeric excess in chiral mixtures is achieved in a table-top instrument using MS-PECD. No prior chiral chromatography or enantiomer-selective clustering is needed.

References
Keywords: Collision-induced dissociation (CID), diketopiperazine (DKP), chiral discrimination, Born-Oppenheimer molecular dynamic (BOMD).

Introduction:
This theoretical studied is inspired by the strong experimental differences observed in the breakdown curves of the collision-induced dissociation of the two diastereoisomers of the diketopiperazine peptide cycloSPhe-SHisH+ (SS) and cycloSPhe-RHisH+ (SR) (unpublished results). The major chiral discrimination is observed in the carbon monoxide and ammonia loss channels. For the former case, the fragmentation efficiency of the SR diastereomer is larger in contrast to what happens in the latter.

Methods:
A comprehensive picture of unimolecular dissociation is obtained by a Born-Oppenheimer molecular dynamic simulation. Internal energy activation through a micro-canonical ensemble and the RM1-D semi-empirical Hamiltonian has been used for this purpose. The method used showed good a balance between accuracy and computational time. All the simulations were carried out coupling VENUS (for dynamics) and MOPAC (for electronic structure calculation) packages.

Results:
Within this level of theory, we are able to reproduce all the main fragmentation channels observed in the CID experiment starting from the geometry determined by infrared multiple photon dissociation spectroscopy. This geometry agrees with the calculated global minimum for both systems and shows that the proton lies in the histidine residue. The main fragmentation products are loss of ammonia, loss of water, loss of CO, loss of formamide or a combination of NH3+CO (these two last products have the same mass), loss of carbamic acid or a combination of isocyanic acid + H2O, loss of HCONH2+CO or a combination of NH3+2CO, the formation of the iminium ion of Phe and the formation of the iminium ion of His. The Histidine residue plays an important role. It consists in carrying the proton to highly energetic proton sites like oxygen and nitrogen atoms in DKP ring (following the proton mobile model). There is a strong difference in the mobility of the proton in the two diastereomers due to the chirality constrain. In the SS case, the proton mobility is lower in comparison to SR case due to the fact the two residues remain on the same side of the DKP ring interacting. Then, the SR system shows, overall, higher fragmentation yield than SS system in agreement with experiments. This is likely due to a lower activation energy.

Conclusions
The strong experimental differences observed in the CID experiments can be rationalized thanks to theoretical calculations which point out the role of the Histidine residue in the proton mobility which is related to differences between the two diastereoisomers.

Novel Aspect:
This chiral discrimination has been noticed before but a clear rationalization was not found. Calculations can help in understanding it from a molecular basis.
A NEW MS-BASED HYPHENATED METHOD FOR ANALYSIS OF HEXURONIC ACID EPIMERS AND SULFATE PATTERNS IN GLYCOSAMINOGLYCAN OLIGOSACCHARIDES BY MSN COUPLED TO INFRARED ION SPECTROSCOPY

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Keywords: Glycosaminoglycan, MS/IR, hexuronic acid epimers, structural characterization, sulfate patterns

Introduction:
We report an original MS-based hyphenated method for the elucidation of sulfate position and the epimerization of hexuronic acids in glycosaminoglycan (GAG) fragments.

Methods: Coupling of mass spectrometry with IRMPD spectroscopy
This method consists of measuring simultaneously the MS/MS spectrum and the gas phase IR spectrum to gain direct structural information. This is possible using a customized MS instrument, modified to allow injection of a tunable IR laser inside the instrument for in situ spectroscopy of trapped ions.

Results:
This approach was tested for the analysis of the hexuronic fragments composing the hyaluronic acid tetrasaccharide [1] and the sulfated GalNAc fragments present in the chondroitin sulfate disaccharides and dermatan sulfate tetrasaccharide [2].

Conclusions
We show that the gas phase IR fingerprint of reference hexuronic acid and sulfated GalNAc monosaccharides are distinct and can be used to detect them in oligosaccharides.

Novel Aspect:
The method presented here is predictive and allows structural elucidation of unknown GAG fragments, even in the absence of referenced standards.

References
Introduction:
Naproxen is one of the most consumed nonsteroidal anti-inflammatory drugs and marketed as S-naproxen since its R-enantiomer is hepatotoxic [1]. Developments of efficient and reliable methods for chiral differentiation and quantification of naproxen are thus essential [2].

Methods:
Recognition and quantification of S/R-naproxen enantiomers with commonly used chiral selectors are studied using tandem mass spectrometry (MS/MS). Structures of the most promising diastereomeric precursors are revealed using collision-induced dissociation (CID), ion-mobility spectrometry (IMS), and density functional theory (DFT) and molecular dynamics (MD).

Results:
Among all studied diastereomeric complexes formed between S/R-naproxen and various chiral selectors [3], including α/β/γ-cyclodextrins, amino acids and their derivatives, glucose, tartaric acid, and vancomycin, a novel binuclear metal bound [(MIIL-His)2(S/R-naproxen)−3H]+ complex (M = Cu, Ni, or Co with Cu being the best) can allow effective absolute configuration identification and enantiomeric excess determination for naproxen using MS/MS. The key candidate structure of [(CuIIL-His)2(S/R-naproxen)−3H]+ revealed from CID, IMS and DFT-MD, has an unusual self-assembled, compact geometry with the two Cu(II) ions bridged closely together by the carboxylate groups of the two histidines. The difference in dissociation efficiency of the two diastereomers is attributed to the σ-π interaction between the NH2 group of one histidine and the naphthyl ring of naproxen in the complex.

Conclusions:
The present report observes and characterizes the diastereomeric complexes of (CuIIHis)2 with aromatic acid, which could contribute to the recognition of chiral aromatic acids, design of catalysts based on binuclear copper-bound complex, as well as the better understanding of metal-ion complexation by His or His-containing ligands.

Novel Aspect:
A novel self-assembled, compact [(CuIIL-His)2(S/R-naproxen)−3H]+ complexes are used for efficient chiral differentiation of naproxen.

References:
Enantioselective reduction from quaternary copper D,L amino acid complexes: origin of the weak chiral effect observed with D/L aspartic and glutamic acids

Jean-Claude Tabet (1)

Keywords: chirality, enantioselective reduction, CID, interpretation

Introduction: Amino acid enantiomers (AAL/AAD) distinction was a challenge solved after the 2000s. A method proposed by G. Cooks (1) was based on (i) electrospray formation of quaternary [2AA’L+AAL/D-H+Cu(II)]+ complex diastereomers (AAL’ as reference, AAL/D, that of unknown chirality) and (ii) their comparative dissociations by CID. Applying this approach and using AA’L=phenylglycine, an enantioselective reduction accompanying expected chiral effects were observed (2).

Methods: Each AA was dissolved with CuCl2 in MeOH/H2O (50:50) within 20 pmol.mL-1. HD exchanges were performed. Experiments were done positive ESI with ion trap, and with LTQ/Orbitrap. Solutions were respectively infused at a flow rate of 160 mL/h and 100µL/h. Resonant excitation (CID) and non-resonant (HCD) were used. The source settings are as follows: capillary voltage, -3.5 kV; heat temperature, 100°C; The image signal was amplified and digitized using 1M data points.

Results: Using the Cooks’ approach (1), the phenylglycine (PhgL)(2) was used as chiral selector in the quaternary complexes. The main cleavage of [2PhgL+AAL/D-H+CuII]+ involved the competitive formal PhgL and [PhgL-H]• losses yielding the [CuII,(PhgL,AAL/D-H)]+ and [CuII,(PhgL,AAL/D)]+ ions, respectively. In fact, the [PhgL-H]• loss is not direct and requires a stepwise pathway through the prompt CO2 release concomitant with CuII reduction. This process was therefore at the origin of the observed enantiomeric reduction. Its extent depends on the one hand, the respective chirality of the partners of the quaternary complex, and on the other hand, the basicity and acidity in the gas phase of the amino acid of unknown configuration. Concerning the thermochemical correlation, singular points appear on the one hand, for very basic and very acidic amino acids (R, K, H) and on the other hand, for D and E which are weakly basic and strongly acidic. Indeed, they exhibit a strong degradation of the enantioselective reduction.

Conclusions: The use of particular reference such as PhgL allows to yield distinction of the chirality of AAL,D by two means either from chiral effects on the diastereomeric quaternary coppered complexe dissociations described by the Cooks works or by the observed enantioselective reduction. The lowering yields of the latter for certains AA, was explained either by the presence of zwitterion for R,H and K or by the particular reactivity of mobilizable proton on D and E in canonic form.

References


Novel Aspect: AA configuration by enantioselective reduction by cleavage of quaternary complexes with PhgL as reference can be explained by considering AA canonic/zwitterion structures
Keywords: ion mobility mass spectrometry, peptides, acids, diastereoisomers

Introduction: The chiral molecules frequently remain undistinguishable using ion mobility mass spectrometry (IM-MS), due to insufficient difference of their collision cross sections at an available mobility resolution of ion mobility drift tubes. The influence of the complexation with organic acids on the ion mobility separation of selected peptide diastereoisomers was evaluated.

Methods: IM-MS measurements were performed on a commercially available (Waters) quadrupole traveling-wave ion mobility time-of-flight spectrometer (Synapt G2-S HDMS). The initial structures of noncovalent complexes obtained with Metropolis Monte Carlo simulations were further optimized at the DFT level of theory. The theoretical cross sections of the optimized associates were calculated using the trajectory method (TM) in the open source software program MOBCAL.

Results: Peptide diastereoisomers containing arginine residue form stable associates with various organic acids in the gas phase. These associates are also stable in conditions of increased pressure of the ion mobility drift cell. Overall the increase of the diastereoisomeric peptide separation after complexation with selected acids was observed. The structures, selectivity, specificity, and the overall effect of complexation with acids on ion-mobility separation of the analyzed diastereoisomers are discussed. Additional relative gas-phase stability experiments were performed to define the association mode between examined peptides and selected organic acids.

Conclusions
The increase of the diastereoisomeric peptide separation after complexation with selected acids was observed. The gas-phase stability studies revealed that the presence of the arginine in a peptide sequence ensures the formation of stable complex in the gas phase. The presence of the available backbone hydrogens in peptide sequence additionally increases the stability of the complexes in the case of diacids.

Novel Aspect: Complexation with the selective acids induces improvement of chiral separation of peptides.
Keywords: Hydrogen atom, N–Cα bond cleavage, fragmentation dynamics. Ab initio molecular dynamics

Introduction
The novel fragmentation technique involving the interaction between peptide ion and hydrogen atom in gas phase, named HAD, has been recently developed. HAD is initiated by hydrogen radical attachment to the peptide ion in the ion trap, and the fragment ions due to N–Cα bond cleavage were selectively observed. The fragmentation process of HAD is not yet fully understood. In this study, the detail processes of HAD were investigated by ab initio calculation.

Methods
HAD experiments were performed using a prototype MALDI QIT-TOF mass spectrometer based on the design of AXIMA Resonance equipped H• source. H• was generated by passing H2 gas through a heated tungsten capillary and introduced into the ion trap. The pressure inside the ion trap chamber was maintained below 5x10⁻² Pa during the reaction. The fragmentation pathway and dynamics were calculated by Gaussian 16 and Car-Parrinello molecular dynamics (CPMD) programs.

Results
To identify the most probable dissociation mechanism of HAD processes, the peptides, AcLys, AcArg, and AcArg-Ala were used as the models. The protonated model peptide was produced by MALDI with CHCA, and then reacted with H• in ion trap. The HAD-MS2 spectrum of [M+H]⁺ showed fragment ions due to N–Cα bond cleavage. The N–Cα bond cleavage was initiated by H• association to carbonyl oxygen in protonated peptide. The TS barrier for the H• attachment was in the range of 0.45 - 0.6 eV. Since the temperature of H• introduced to ion trap was estimated to 2300 K, 10% of H• in ion trap have the kinetic energy higher than 0.6 eV, which can react with protonated peptides, leading to aminoketyl radical intermediate. To investigate the detail mechanism of aminoketyl radical fragmentation, we performed the simulation based on CPMD. According to the result, the aminoketyl radical immediately underwent homolytic N–Cα bond cleavage. The homolytic cleavage resulted in the donation of an electron from the N–Cα bond to the nitrogen atom, producing an c' fragment and z• radical.

Conclusions
The use of hot H• efficiently react with protonated peptide in gas phase, inducing N–Cα bond cleavage. The dissociation rate on N–Cα bond was strongly influenced the temperature of aminoketyl radical. When the temperature was set to be > 500 K, the N–Cα bond occurred within 1 ps. Because the heading of ion trap enhanced yield of fragmentation, the computational results were in good agreement with the HAD-MS2 result of protonated peptides.

Novel Aspect
H• attachment to peptide leads to aminoketyl radical, which immediately underwent homolytic bond cleavage at N–Cα bond.
240 - A QUANTUM CHEMICAL TOOL FOR THE PREDICTION OF MASS SPECTRA AND THE IDENTIFICATION OF FRAGMENTATION PATHWAYS

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Keywords: Quantum Chemical Mass Spectrometry (QCMS2), Density Functional Theory (DFT), inter-side-chain (ISC) interactions, Tandem Mass Spectrometry, Tripeptides

Introduction

A new computational tool for the prediction of mass spectra based on quantum chemical calculations is being developed, called Quantum Chemical Mass Spectrometry for Materials Science (QCMS2). It was benchmarked by predicting the EI fragmentation pathways of a number of organics with a variety of functionalities. The main features in the mass spectra were correctly reproduced and new fragmentation routes proposed by QCMS2 were confirmed by MS/MS experiments [1].

Methods

QCMS2 is based on DFT/B3LYP/6-311+G* calculations and selects fragmentation pathways based on the relative values of bond order, reaction energies (for bond cleavages) and transition state energies (for rearrangements). Known peptide-specific fragmentation mechanisms such as the Mobile Proton Model (MPM) for His, confirmed by quantum chemical calculations, have been implemented into the method and the code omits the breaking of irrelevant bonds [2].

Results

QCMS2 is being applied to predict the fragmentation patterns of tripeptides in ESI CID MS/MS focusing on the influence of inter-side-chain (ISC) interactions on the initial protonation and subsequent fragmentation. The fragmentations of a number of non-cyclic tripeptides consisting of His as the central amino acid (AA) and AAs which allow the formation of strong hydrogen bonds as the peripheral ones, have been studied. Observations such as (1) the fact that the (dis)appearance of ISC interactions in the Lys-His-X (X = Asn, Asp, Gln, Glu and Ser) and Trp-His-X combinations determines the most stable protonation site of the tripeptide, and (2) the fact that in Arg-His-Ser and Asn-His-Ser ISC interactions lead to specific fragmentations, illustrate the importance of strong interactions between AA side chains. The results obtained from QCMS2 are compared to those obtained from conventional methods to demonstrate how many of the peaks not predicted by these latter methods are due to fragmentations involving the side chains and are predicted by QCMS2.

Conclusions

QCMS2 correctly reproduces the main features in the mass spectra of tripeptides, i.e., the traditional backbone cleavages resulting in a-, b2 and y1-ions. Furthermore, due to its taking the fragmentation of the side chain and ISC interactions into account, it also predicts fragments resulting from these and, as a result, convincingly outperforms common tools such as PEAKS, PeptideArt and Prospector.

Novel Aspect

QCMS2 assigns up to twice the number of signals in peptide mass spectra assigned by common mass spectral assignment software, and generates detailed fragmentation routes.

References

Dissociation of ligated copper(II)–peptide complexes, \([\text{Cu}^{2+}(L)M]\) through single electron transfer. \([1]\)Diversity is introduced into the ion chemistry of a peptide radical cation through the possibility of the radical migrating in a number of different ways to corresponding prerequisite radical sites prior to fragmentation; some of these competitive reactions are charge– and radical carrier–specific, and can be perturbed upon varying the nature or location of the radical/charge site, the basicity of the residue at that site, or the peptide sequence, all of which can substantially impact the isomerization barriers for radical or charge mobility and the subsequent reactions. \([2–5]\) The major impetus of this study was to use a simple prototypical system, \([\text{Cu}^{2+}(L)YGW]^{+}\), to track the location of the radical and charge sites, starting from the copper(II)–peptide complex to the predetermined radical sites of the peptide radical cations after a complex permutation of intramolecular hydrogen atom migrations—potentially allowing us to determine whether or not the individual isomerization, rearrangement, or fragmentation pathways would predominate. A wide variety of site-specific radical peptides can be produced efficiently with judicious choice of the metal–ligand–peptide system; \([1–2]\) we used isomeric \(\pi\)-centered \([GGW^{\pi}]^{+}\) (formed directly through dissociation of \([\text{Cu}(\text{amine})GW]^{2+}\) complexes) and \(\alpha\)-centered \([GW^{+}]^{+}\) (generated through side-chain cleavage of the tyrosyl residue of the YGW radical cation) species for our initial infrared multiple photon dissociation (IRMPD) investigations, in conjunction with density functional theory calculations. The IRMPD spectrum of \(GW^{+}\) displays a strikingly different set of product ions than those obtained from \([GGW^{\pi}]^{+}\). It appears that the two isomeric radical cationic GGW structures did not interconvert on the time scale of our IRMPD experiments. Such isomeric \(\pi\)– and \(\alpha\)-centered radical cationic peptides comprise a well-defined prototypical system for dissecting the fundamental parameters that govern the isomerization and dissociation of peptide radical cations, which undergo various rearrangement processes, including hydrogen atom, proton, and electron transfer, in the absence of solvation.

References

Introduction: Cobalamines (Cbls) are important cofactors in various enzymatic processes. Many studies indicate that the reduced form of cobalamin, Cobl(II), reacts reversibly with NO to form a nitrosylcobalamin adduct at a high rate. This evidence is supported by the NO-induced inactivation of Cbl-dependent enzymes and the inhibition of NO actions by Cbl supplementation. [1-2] Here, the intrinsic features for NOx (x=1,2) binding to bare Cbl ions are reassessed by ESI FT-ICR MS.

Methods: Experiments were carried out with a Bruker BioApex 4.7 FT-ICR mass spectrometer coupled with an ESI source. The reduced Cobl(II) and oxidized Cobl(III) forms were formed by ESI of a 10 µM aqueous hydroxycobalamin solution at physiological pH, mass-selected and exposed to NOx (x = 1, 2) in the ICR cell at stationary pressures of 1.0-10 × 10⁻⁸ mbar. Pseudo-first order-rate constants divided by the neutral concentration afford second-order rate constants. [3]

Results: In order to improve the current understanding of NOx (x = 1, 2) scavenging by Cbls, the kinetics and mechanistic behavior of several Cbl species, either in the reduced or oxidized and protonated/sodiated or deprotonated forms, have been explored in the gas phase, lacking any perturbation by solvent or counterions. Some relevant/preliminary evidences may be outlined: i) both sampled neutrals undergo an addition process; ii) for NO2 binding, an overall higher yield is observed with either Cobl(II) or Cobl(III), by a factor of ca. 3 and 10, respectively. The efficiency values span from 4.0 to 18% for Cobl(III) and doubly deprotonated Cobl(III), the only form which undergoes a double addition, whereas (de)protonated and monosodiated Cobl(II) show similar values (~ 8%); iii) the reactivity with NO shows a clear bias for Cobl(II) species; iv) Cobl(III) species undergo also hydrogen atom abstraction by NOx (x = 1, 2).

Conclusions: Gas phase ion-molecule reactions provide mechanistic information relevant to biological functions void of environmental effects. Here, the binding properties of nitrogen oxides to various forms of cobalamin have been addressed, involving either deprotonated “base-on” or protonated “base-off” species. This study confirms preferential NO ligation to Cobl(II) and outlines the influence of the coordination environment of the cobalt ion.

Novel Aspect: Direct determination of reaction patterns and thermal rate constants are provided for NOx (x = 1, 2) binding to naked Cobl(II) and Cobl(III) ions in a solvent free environment.

References
ONE-ELECTRON REDUCTION OF ISOLATED METALLO-SUPRAMOLECULAR AGGREGATES

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Keywords: metallo-supramolecular aggregates, supramolecular chemistry, ESI, ECD, ETD

Introduction:
Are the metal centres in metallo-supramolecular aggregates electronically isolated from each other? Is there an information transfer via ligands or space? How redox-innocent are the ligands? Gaining knowledge on the redox behaviour of metallo-supramolecular aggregates in solution is highly desirable, but rather difficult due to the inherent lability of the system: It is hard to oxidise or reduce only the aggregate of interest – and not affect one of the other compounds of the (desired) manifold of species present in equilibrium. Herein, a means is presented to study the reduction of isolated supramolecular aggregates under well-defined conditions using mass-spectrometry.

Methods:
High-resolution electrospray ionisation (ESI) mass spectra were recorded on a Bruker Apex IV FT-ICR or a Thermo Fisher Scientific LTQ Orbitrap XL mass spectrometer. A series of multiply charged cations typically is observed for metallo-supramolecular aggregates due to abstraction of several anions during the ESI process. After mass-selection of the species of interest, one-electron reduction is performed in the gas phase using the electron capture dissociation (ECD) technique in the FT-ICR or the electron transfer dissociation (ETD) method in the ion trap module of the Orbitrap XL instrument. Reduction products of interest were again mass-selected and fragmented by collision-induced dissociation (CID) or subjected to a second one-electron reduction step.

Results:
During ETD and ECD, a single electron is attached to the metallo-supramolecular aggregate. The resulting reduced species is either characterised by high resolution tandem mass-spectrometry or can even be subjected to another reducing step (ECD/ECD) prior to analysis. The presented examples include the stepwise reduction of a copper(II) helicate revealing otherwise unaccessible mechanistic details of its interconversion to the respective copper(I) congener with different stoichiometry.[1] Single-electron reduction of homoleptic metallosupramolecular squares shows the non-innocence of bipyridine ligands [2] whereas a similar experiment with bimetallic squares [3] enables the differentiation between different reduction sites. The effect of linker length is illustrated by reduction of dinuclear gold macrocycles of various ring sizes.[4]

Conclusions
Both ECD and ETD can be used to generate highly interesting partly-reduced open-shell metallo-supramolecular aggregates. The stability and further reactivity of the reduced species in dependence of type of metal building blocks, bridging and blockings and aggregate size and charge are addressed.

Novel Aspect:
ECD and ETD typically are applied to characterize peptide samples whereas they are used to generate and further study the chemistry of highly unusual reduced metallo-supramolecular aggregates herein.

References
Introduction:
Homogenous gold catalysis is a powerful approach to access high diversity and complexity of molecules.[1] Besides the metal, ligands play a major role to control and orientate the reactivity.[2] Many theoretical works have aimed and succeeded to interpret the particular behavior of ligands in cationic gold complex.[3] In contrast, experimental approaches to evaluate these effects and validate these theoretical models are scarce and would be highly desirable.

Methods:
A collection of gold carbonyl complexes was studied using a modified triple-quadrupole equipped with an ESI source. L-Au-CO+ ions were directly formed in the source and submitted to Collision Induced Dissociation (CID) experiments. From survival yield (SY) breakdown curves, critical energy values were obtained using a kinetic model based on transition state theory (RRKM theory). Results were analyzed using DFT calculations and energy decomposition analysis (EDA).

Results:
Au(I) complexes were successfully formed in the ESI source for 16 L ligands: expected, the greater the capacity of L to enrich gold, the higher the dissociation energy of Au-CO. The SY for this dissociation were recorded for different collision energies during CID experiments. Due to the variety of size and masses of the complexes, a semi-empirical calibration had to be established in order to get an evaluation of the mean internal energies of the complexes prior to decomposition. From there, critical energies were obtained using kinetic modelling. The variety of electronic effects of the studied ligands is reflected by these values: a range of 10.5 kcal/mol is observed between the weakest and the strongest Au-CO bond, as confirmed by DFT calculations. To gain insights into the Au-CO bond nature, an energy decomposition analysis was also performed. The contributions of the orbital and electrostatic interactions and the Pauli repulsion to the bonding were considered. Results are rationalized by considering the influence of each of these terms.

Conclusions:
Bond dissociation energies of 16 gold-carbonyl complexes were obtained using mass spectrometry and kinetic modelling. Depending on the nature of the ligand, a more or less large difference in the BDEs could be observed. These data were assessed as new probes for evaluating ligand effects in gold catalysis. With the help of energy decomposition analysis, a rationalization of the various electronic effects observed for the studied ligands could be achieved.

Novel Aspect:
A new experimental approach, based on mass spectrometry experiments and kinetic modelling, is proposed to evaluate the ligands effects in gold(I) complexes.

ENERGY-RESOLVED COLLISION-INDUCED DISSOCIATION STUDY OF PROTON-BOUND HETRODIMERS OF GUANINE AND CYTOSINE

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Keywords: Proton-bound base pairs; Guanine; Cytosine; ER-CID; DFT

Introduction:
To study the effect of protonation on DNA base pairing, we investigated energy-resolved collision-induced dissociation (ER-CID) of proton-bound heterodimers of guanine (G), guanosine (Gn), cytosine (C), and 1-methylcytosine (1-MeC), and cytidine (Cn), which are likely to be formed by Hoogsteen pairing including an ionic hydrogen bond. The structures, energies, and proton transfer reactions of dimeric conformers were also investigated theoretically.

Methods:
The protonated dimers of [G:C:H]+, [G:1-MeC:H]+, and [Gn:Cn:H]+ were produced by electrospray ionization in the acidified conditions, which were interrogated by ER-CID experiments using LTQ XL (Thermo Fisher) and Xevo TQ (Waters) for multiple and single collision conditions, respectively. For quantum chemical calculations, density functional theory (DFT) at the B3LYP/6-311+G(2d,p) level using Gaussian09 program suite was utilized.

Results:
Recently, an extensive theoretical study[1] and an IR multiple photon dissociation (IRMPD) spectroscopy[2] suggested that the Hoogsteen basepair, i.e. CH•••G, be the dominant conformation for [G:C:H]+ in the gas phase. Based on the previous investigations, we modelled the proton-bound heterodimers of [G:C:H]+, [G:1-MeC:H]+, and [Gn:Cn:H]+ as Hoogsteen base pairs using DFT calculations. ER-CID behaviours of the protonated heterodimers were explored both in multiple and single collision conditions. It was found that dissociation of [G:C:H]+ led to preferential formation of C:H+, while simple consideration of the kinetic method[3,4] predicts preferential protonation on more proton-loving moiety, G. However, CID of the other basepairs of [G:1-MeC:H]+ and [Gn:Cn:H]+ occurred with favored protonation on more proton-loving moiety, yielding 1-MeC:H+ and Gn:H+ as the dominant fragments, respectively.

Conclusions:
DFT and CID study suggested Hoogsteen structures for [G:C:H]+, [G:1-MeC:H]+, and [Gn:Cn:H]+. By varying substituents, alternation of energetics in base-pairing, dissociation, and intermolecular proton transfer reactions were introduced and examined. While the three heterodimers have similar energetic relations, CID of [G:C:H]+ showed a unique behavior, where certain dynamic reasons are likely to be deeply involved.

Novel Aspect:
ER-CID of gas-phase Hoogsteen base pairs occurring by protonation of bases was examined for the first time, and an intriguing dissociation of [G:C:H]+ was revealed.

References
CORRELATION BETWEEN STRUCTURE AND STABILITY OF GAS PHASE OLIGONUCLEOTIDES PROBED BY TUNED COLLISION- AND VUV-MS/MS

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Introduction:
Various MS/MS methods are commonly used to create comprehensive information for bioanalytical applications. However, only little data is available on gas phase and fragmentation behavior of important molecules like double stranded DNA and related clinically relevant analogs. Previously, CID was predominantly used to characterize these kind of samples, leaving other techniques underused.[1]

Methods:
A LTQ Orbitrap XL was used to generate CID and HCD spectra of single and double strand DNA and DNA-LNA hybrids. The Synapt G2-S was used for collisional cross section assessment. As an additional MS/MS method a continuous vacuum ultraviolet lamp with transmission maxima at 125 and 160 nm was used. This approach provided efficient fragmentation along atypical, orthogonal pathways in both ionization modes and sensitivity increase by postionization of neutral losses.

Results:
DNA folding in the gas phase is strongly dependent on the charge level. With the use of IMS and CID data, for this unfolding a clear correlation between the energetic dissociation onset and the reorganization could be determined.[1,2] LNA, as a more rigid structure in comparison to DNA, indicated a complete different behavior concerning unfolding and fragmentation pathways. This was not only found for single strand nucleic acids, but also for the respective DNA-DNA and DNA-LNA duplexes. While DNA dissociates along energy specific pathways, LNA was found to requires higher energy for fragmentation and follow less specific dissociation channels. Additionally, VUV fragmentation was tested for positive and negative ionization. [3] For multiply charged oligonucleotides and proteins a charge reduction following electron ejection followed by dissociation was found. Samples of other categories showed fragmentation into common and atypical fragments. Moreover, for some systems, a strong partition of radiation induced postionization was observed.

Conclusions
Double and single strands of DNA and LNA were selectively fragmented. While DNA showed clear pathways including reorganization, the rigidity of LNA caused multiple dissociation channels and different folding pattern. VUV as new MS/MS method produced charge reduction of multiple charged ions as well as new fragment classes. Also an increase of intensity was observed by postionization of fragment ions.

Novel Aspect:
A comprehensive study of DNA and LNA concerning gas phase organization and dissociation in MS and IMS was achieved. VUV was introduced as a new MS/MS method.

References


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Keywords: protonated water cluster, hydronium ion, cluster distribution, ion mobility spectrometry

Introduction
Protonated water clusters (H2O)nH+ play an important role during many ionization processes, including atmospheric pressure chemical ionization (APCI), and in particular proton transfer reaction (PTR). The cluster size n influences ionization by changes in proton affinity and ion mobility. This is a major issue when calculating concentrations in PTR-MS [1, 2]. Furthermore, transfer into vacuum changes the water cluster distributions, rendering them unknown.

Methods
High Kinetic Energy Ion Mobility Spectrometry (HiKE-IMS) ionizes in a reaction tube operated at reduced pressures (20 mbar) and high reduced field strengths (up to 130 Td) [3], similar to PTR-MS. However, instead of a mass spectrometer, an ion mobility spectrometer operated at the same pressure as the reaction tube is used for ion separation and detection. This allows analyzing the ions under the ionization conditions with anion mobility resolving power of Rp=140 [4].

Results
When monitoring the ion mobility spectrum while sweeping the reduced field strength in both the reaction and the drift region, a shift of the protonated water cluster peak position can be observed at the energies where a change in most prevalent cluster size is expected (30 Td, 60 Td, 90 Td). Significantly below each of these thresholds, a Gaussian peak was measured at the expected mobility of the respective larger water cluster. Near the threshold, the peak starts broadening and a changing ion distribution between the positions of the respective larger and smaller water cluster can be observed. Significantly above each threshold, again a Gaussian peak was observed, this time at the position of the smaller water cluster.

Conclusions
Using HiKE-IMS allows analyzing protonated water clusters under the conditions inside the ionization source. This way, both the prevalent cluster size and the ion mobility can be determined as for example needed for correct concentration calculation in PTR-MS.

Novel Aspect
For the first time, protonated water cluster distributions and ion mobilities have been directly measured under the conditions present during ionization.

Acknowledgements
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References
**Introduction:**
Glyoxal (m.w. 58 g/mol) is a highly reactive molecule associated with some basic biological processes [1], photo-catalysis of CO2 into CH4 on TiO2 or photo-dissociation of anthropogenic and biogenic volatile organic compounds (VOC) [3]. Presence of glyoxal can be monitored using soft chemical ionization mass spectrometry (SCI-MS). Here we discuss the ion chemistry of glyoxal interacting with H3O+, NO+ and O2•+ reagent ions used in SCI-MS and possibility of detection.

**Methods:**
Selected ion flow tube mass spectrometry, SIFT-MS[4], was used to investigate ion-molecule reactions of H3O+, NO+ and O2•+ reagent ions with glyoxal at variable humidity. The ion chemistry under thermal conditions is theoretically described using B3LYP/6-311(p,d) calculations and using numerical modelling of the ion-molecule reaction kinetics.

**Results:**
NO+ reactions lead to formation of C2H2O2(NO)+ and C2H2O2•+ ions, in the humid environment the association reaction is dominant. Charge transfer was observed for O2•+ reactions as well. Additional dissociation observed previously for O2•+ reagent ion was not confirmed [5-6]. The H3O+ reaction proceeds at dry conditions via proton transfer reaction forming C2H3O2+. With increasing concentration of water molecules, formation of m/z 31 (CH2OH+) is becoming dominant. Corresponding hydrated ions were observed as well. Using quantum chemistry calculations, we estimated PA(C2H2O2) = 165.1 kcal/mol characterised the energetics of reaction pathways formingCH2OH+. Reaction kinetics modelling indicates formation of a stable intermediate C2H3O2+.H2O which dissociates to protonated formaldehyde CH2OH+ after interaction with water.

**Conclusions:**
According to theory, protonated formaldehyde is formed from C2H3O2+.H2O by reaction with water resulting into CH2OH+ + HCOOH + H2O products or by a formation of exited C2H3O2+.H2O* after two body association which dissociates to CH2OH+ directly. Finally, protonated formaldehyde hydrate is not the terminating ion at high humidities. Thus, we expect a catalytic effect of CH2OH+.H2O forming a proton bound water dimer CH2OH+.H2O + H2O → CH2O + H3O+.H2O.

**Novel Aspect:**
Understanding of ion chemistry of glyoxal is important for its analysis in gas phase using SCI-MS techniques and its distinguishing from isobaric compounds.

108 - REACTION DYNAMICS OF IONIZED 1-METHYLPYRENE: WHY IS THE 1-METHYLENEPYRENE ION FORMED FROM H ATOM LOSS AND NOT A TROPYLIUM-CONTAINING ION?

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Keywords: polycyclic aromatic hydrocarbon ions, astrochemistry, iPEPICO, collision-induced dissociation

Introduction:
The chemical origins of the Diffuse Interstellar Bands have remained elusive. Polycyclic aromatic hydrocarbons, PAHs have been postulated to be sources of DIBs due to the similarity between the IR emissions of the cations of PAHs and the DIBs. One molecule that has been investigated for its potential role as a DIB source is 1-methylpyrene. Previous work has suggested that, unlike toluene ions, ionized methylpyrene dissociate by H loss from the methyl group [1-5]. This study aims to find out why.

Methods:
iPEPICO experiments were conducted on the VUV beamline at the Swiss Light Source (Paul Scherrer Institut, Villigen, Switzerland). The mass-selected TPES was acquired to establish the IE. RRKM theory was used to determine the 0 K activation energies (E0) and entropies of activation (Δ‡S1000K) by modeling the experimental breakdown diagrams. Reaction pathways were calculated at the UCCSD/6-31G(d)//B3-LYP/6-31G(d) level of theory.

Results: (Limit 900 characters)
The energetics derived from modelling (E0 = 2.84 ± 0.36 eV) is the first experimental value reported for the dehydrogenation of ionized 1-methylpyrene. The loss of H could form either 1-methylenepyrene (A) or a tropylium-motif containing ion (B). The transition state of highest energy in the pathway to B lies ~ 3.20 eV, while that for A is only 2.54 eV, favoring simple H loss from the methyl group, in contrast to that observed for ionized toluene (2.12 eV compared to 2.17 eV [6]). The reason for this flip in relative energies for two the precursors is evident when the geometries for the key TS are compared. In the case of ionized toluene, the TS has equivalent bond angles consistent with a planar 7-membered ring (128.7°). The presence of the three additional rings in 1-methylpyrene prevents the ring from expanding, with the most constrained angle being ∠123 with a value of 125.0°. The presence of geometric constraints prevents the ion from accessing routes of molecular relaxation which would lower the transition state energy.

Conclusions (Limit of 400 characters)
The derived energetics, combined with computationally determined reaction coordinates, allowed for the determination that the dehydrogenation product of 1-methylpyrene cations is the 1-methylenepyrene cation. The tropylium-motif containing fragment ion requires significantly more energy to be formed due to steric hindrance in key transition states caused by the constraints imposed by the three benzene rings. The fHo (1-methylenepyrene cation) was derived to be 945 ± 31 kJ mol⁻¹.

Novel Aspect: (Limit of 150 characters)
The preferential formation of 1-methylenepyrene fragment ions is explained by calculations of the key transition states in the reaction pathway.

References
Keywords: top-down protein characterization, selective fragmentation, ion/ion reactions, aspartic acid effect, ornithine effect, dehydroalanine effect

Introduction:
Strategies for top-down tandem mass spectrometry of protein ions have generally sought to maximize the number of structurally diagnostic product ions. We are interested in developing complementary approaches that tend to lead to a limited number of relatively abundant cleavages that arise from specific/predictable locations.

Methods:
Experiments were conducted using a quadrupole/time-of-flight tandem mass spectrometer (Sciex 5600) that has been modified to conduct ion/ion reaction experiments. Protein precursor ions were subjected to ion/ion proton transfer reactions to bring them into a mass-to-charge region in which aspartic acid and proline cleavages are favored upon ion trap CID.

Results:
Three strategies that provide enzyme-like specificity in protein ion fragmentation are described. These include taking advantage of the known tendencies for cleavages at aspartic acid and proline residues, as well as taking advantage of the ornithine and dehydroalanine effects. We describe an approach that concentrates precursor ion charge states derived from ESI under denaturing conditions into a single charge state via ion parking, ion isolation, proton transfer ion/ion reactions to generate precursor ions within a charge state range where aspartic acid and proline cleavages are most prominent, and use of a broad-band collisional activation technique to fragment all of the charge states simultaneously. This approach is demonstrated using four standard proteins commonly used to test/demonstrate top-down protein characterization approaches. The other two approaches rely on converting existing residues into either ornithine or dehydroalanine. These residues also give rise to highly favored cleavages.

Conclusions:
The ultimate goal is to develop a suite of strategies that provides the analyst with options for multiple selective approaches for deconstructing mass-selected protein ions as complementary approaches to the standard means for maximizing cleavage in non-specific fashion. The present work demonstrates approaches that are selective for aspartic acid residues, proline residues, arginine residues, and several other residues that can be used to generate dehydroalanine.

Novel Aspect:
Novel approaches are described that favor selective cleavages at a variety of residue types in protein ions.
306 - WALK-ON-SPHERE REARRANGEMENT AND RETRO-BINGEL REACTION OF GAS-PHASE FULLERENE MALONATE IONS

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Keywords: APCI, organic fullerene derivatives, retro-Bingel, fragmentation mechanism

Introduction:
C60LX (L=C(COOEt)2; x=4-6) are investigated by APCI with a QIT- and a QTOF-MS to study their chemical stability as radical ions in the gas phase. For uncharged (neutral) fullerene adducts, the positions of the cyclopropane rings on the C60 surface are highly stable. In contrast, under electrochemically reducing conditions in solution an isomerization of the molecules via a walk-on-sphere rearrangement is observed.[1]

Methods:
APCI experiments were performed with a QIT- and a QTOF-MS. The samples were synthesized by a published procedure.[2] C60L6 and C60L5 are isomerically pure (determined by 13C NMR). C60L6 has an octahedral addition pattern with TH symmetry. C60L5 has a quadratic pyramidal addition pattern with C2V symmetry. C60L4 consists of a mix of isomers with varying symmetries.

Results:
For all examined fullerene adducts, radical anion (C60LX•−) and radical cation (C60LX•+) formation is observed applying APCI. Fragmentation experiments with a QIT-MS show the repeated loss of L2 as the main fragmentation pathway for C60LX•− and C60LX•+ (x=4-6).[3] Energy-dependent fragmentation experiments using a QTOF-MS show a decreasing energy-demand for the fragmentation when the number of cyclopropane moieties increases. Furthermore, dissociation of radical anions is less energy demanding than of radical cations, this observation is in agreement with experiments by Kessinger et al. which show isomerization of C60L2 in solution under electrochemically reducing conditions by a walk-on-sphere rearrangement.[1]

Conclusions:
Both C60LX•− and C60LX•+ (x=4-6) show a very selective fragmentation with the loss of L2. This fragmentation can only occur when two diethyl ligands “walk” on the surface towards each other. The walk-on-sphere rearrangement is followed by the formation of a malonate dimer (L2) which is then released from the fullerene.

Novel Aspect:
The charge-induced isomerization of C60LX (L=C(COOEt)2; x=4-6) has been demonstrated unequivocally by observation of the loss of a malonate dimer (L2).

References:
Introduction: 5-hydroxymethyl-2-furaldehyde (5-HMF) is produced in high yields from the acid catalyzed dehydration of carbohydrates. This reaction represents the key process of the cellulose biomass conversion into furan-type platform chemicals.[1] Recently, we have employed mass spectrometric techniques to investigate the acid-catalyzed D-glucose and D-fructose dehydration highlighting the formation in the gas-phase of a protonated 5-HMF isomeric mixed population[2-3].

Methods: Full scan and MSn mass spectra were acquired using a Bruker AmaZon SL ion trap (IT) and a Waters Ultima Q-TOF operating in the positive ion mode. Proton affinity (PA) and gas-phase basicity (GB) of 5-HMF were obtained by using the Wesdemiotis and Fenselau Cooks’s “extended” kinetic method. The PA and GB values as well as the geometries of protonated isomeric structures were fully optimized in the gas-phase at the B3LYP/6-311++G** level of theory.

Results: In order to clarify the stability and structures of protonated 5-HMF isomers, the geometries of six different protomers were optimized at the B3LYP/6-311++G** level of theory and the energetics of their dissociation compared with the experimentally observed mass spectrometric fragmentation. Moreover, the unknown PA and GB of 5-HMF were measured by the extended Cooks’s kinetic method along with theoretical calculations. The experimental PA value of 207.2 ± 3 kcal mol\(^{-1}\) is in excellent agreement with the value of 207.5 kcal mol\(^{-1}\) computed at the B3LYP/6-311++G** level of theory. The whole picture emerging from experimental and theoretical results allowed to attribute the structures of different protonated 5-HMF isomers to the m/z 127 ions generated by the acid catalyzed decomposition of hexose carbohydrates in the gas phase.

Conclusions

The unknown proton affinity and gas phase basicity of 5-HMF were determined by the joint application of Cook's kinetic method and quantum mechanical calculations. Theoretical calculations identify the oxygen atom of the aldehydic group as the most basic site. The formation of less stable protonated 5-HMF isomers as final product of the acid catalyzed decomposition of D-fructose and D-glucose was demonstrated on the basis of energetic considerations.

Novel Aspect: In this work the unexplored gas-phase ion chemistry of protonated 5-HMF, one of the top ten bio-based platform chemicals, was firstly investigated.

References

Selective Conversion of Protonated D-Fructose to 5-HMF and 2-FA: A Nitrogen-Base Assisted Dehydration Process in the Gas-Phase

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Keywords: fructose dehydration, Biomass, 5-HMF, gas phase ion chemistry, computational methods

Introduction: Understanding on a molecular level, the dehydration mechanism of sugar monomers from hemicellulose and cellulose, (e.g. xylose, glucose, fructose) the main constituents of lignocellulosic biomass, to platform molecules such as 5-hydroxymethyl 2-furaldehyde (5-HMF) and 2-furaldehyde (2-FA), can offer the possibility of increasing selectivity and reaction yields in solution for the exploitation of this sustainable source.

Methods: A joined approach based on mass spectrometric techniques (Electrospray Triple Quadrupole Mass Spectrometry (ESI-TQ/MS) and Electrospray Ion Trap Mass Spectrometry (ESI-QIT/MS)) together with theoretical calculations at the B3LYP/6-31 + G(d,p) level of theory was used for the study of the gas phase selective conversion of protonated D-fructose to 5-HMF and to 2-FA.

Results: Our mass spectrometric approach for the gas phase study of acid catalyzed dehydration of xylose, fructose and glucose[1-3] allowed us to structurally characterize reaction intermediates and final products and inequivocally ascertain reaction sequences. Molecular level evidences provided by our mass spectrometric approach highlighted a gas phase pathway for the selective conversion of fructose to 5-HMF through a nitrogen assisted dehydration process.

Conclusions.

Based on mass spectrometric evidences, theoretical calculations allowed a gas-phase mechanism for the selective conversion of protonated D-fructose to 5-HMF and 2-FA assisted by nitrogen-base, to be proposed.

Novel Aspect: In this work the gas phase selective fructose dehydration to 5-HMF and 2 FA assisted by nitrogen bases was firstly investigated.

References

The mechanism of 2-furaldehyde formation from d-xylose dehydration in the gas phase. A tandem mass spectrometric study.

Acid-catalyzed glucose dehydration in the gas phase: a mass spectrometric approach.

A mass spectrometric study of the acid catalyzed d-fructose dehydration in the gas phase.
**299 - SULFUR DIOXIDE UPTAKE BY SODIUM CARBONATE CLUSTER ANIONS IN THE GAS-PHASE**

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Keywords: ion-molecule reactions, gas-phase chemistry, sulfur dioxide, carbon dioxide, intra-cluster reaction

**Introduction:**
Carbonate (CO₃²⁻) and sulfate (SO₄²⁻) species are reactive inorganic components of the fine particulate matter (PM) implicated in several environmental and human health issues. Sulfate aerosols usually arise from the atmospheric oxidation of SO₂, contributing to acid rain and climate change. [1] In this study the reactivity of [(Na₂CO₃)nNaCO₃]- cluster ions (n≥1) was probed towards SO₂ and 13CO₂, as a model of processes occurring at the liquid/gas phase interface.

**Methods:**
The experiments were performed on a LTQ XL linear ion trap (Thermo Fisher Scientific) equipped with an electrospray ionization (ESI) source in negative ion mode. The instrument was in-house modified [2] to allow the introduction of neutral reagent gases (SO₂, 13CO₂) into the ion trap and measure the kinetic rate constants of the observed ion-molecule reactions. [(Na₂CO₃)nNaCO₃]- cluster ions were generated by spraying a 10⁻³ M solution of Na₂CO₃ in H₂O/CH₃CN 1:3.

**Results:**
Cluster ions with the general formula [(Na₂CO₃)nNaCO₃]- were observed in the gas-phase within n≥1. In the presence of SO₂ these carbonate species were quantitatively converted into the corresponding sulfite cluster ions [(Na₂SO₃)nNaSO₃]- through a sequential replacement of each CO₂ moiety with a SO₂ molecule. The SO₂→CO₂ conversion may occur through the direct transfer of O₂⁻ from CO₃²⁻ to a SO₂ molecule, as reported in a previous study identifying the reactions of SO₂ at the surface of a molten carbonate eutectic. [3] The rate constants related to the SO₂ adsorption and CO₂ release were measured by monitoring the signal of the selected carbonate cluster ion as a function of SO₂ concentration. The obtained values were investigated on the basis of n. All the reactions are very fast and efficient. The same experiments were performed also in the presence of 13CO₂. As in the case of SO₂, 13CO₂ is incorporated into the cluster ion structures by replacing CO₂ and leading to [(Na₂13CO₃)nNa13CO₃]- ions with rate constants lower than those obtained for SO₂.

**Conclusions:**
SO₂ molecules were converted into gaseous CO₂ via the reaction of carbonate cluster ions and the consequent formation of sulfite cluster species. Although varying the number n of (Na₂CO₃) moiety of the reactant ion, CO₂ is always efficiently replaced and SO₂ entrapped. The same reactions were observed also in the presence of labeled 13CO₂, but showing rate constants lower than those measured for SO₂.

**Novel Aspect:**
A liquid/gas phase model has been reported to describe the processes involved in the maintenance of the atmospheric SO₂/CO₂ balance or in the SO₂ removal from flue gases.

**References:**
Introduction:
We have been developing a new mass analysis system by coupling a tandem mass spectrometer to a radical beam source. This research is an extension of our previous work: Hydrogen Attachment/Abstraction Dissociation (HAD) for peptide fragmentation[1]. A wide variety of radicals produced in the microwave plasma is introduced to a QIT confining peptide and phospholipid ions to investigate the possibilities of the gas phase reactions for biomolecular analyses.

Methods:
Two types of tuner integrated microwave driven radical sources (CCP [2] and ECR-ICP) have been developed to produce electrically neutral species from any reactive gases such as oxygen or water vapor. These radical sources directly deliver radical beams to a QIT chamber of MALDI QIT-TOF mass spectrometer to investigate the reaction between trapped analyte ions and injected species. In this report, substance P and phospholipids were used as analyte ions.

Results:
Both radical sources can commence hydrogen, nitrogen, oxygen, methane, and water discharges. The CCP source forms the plasma plume which realizes high transport efficiency under a higher gas pressure and high input power condition. Meanwhile the ICP source can ignite and sustain the discharge at a lower pressure and lower input power. The OES results of hydrogen ICP exhibits the high degree of dissociation: prominent atomic lines with faint molecular band spectra. The HAD reaction rates for substance P with hydrogen radicals generated by three types of radical sources are investigated to evaluate the radical source performance. Hydrogen radicals formed in the pure hydrogen and water vapor discharge of CCP source can realize the HAD cleavage similar to the one obtained by a thermal cracking source, while the ICP source did not cleave the precursor ions. On the other hand, when the target ions are phospholipids, the injection of reactive species from the water vapor discharge from both CCP and ICP can realize the double-bond specific fragmentations.

Conclusions:
H attachment pattern for QIT confined C60 ions are found different among thermal cracking, CCP and ICP sources. A series of experiment has shown that the CCP source produces a plasma of a high effective temperature appropriate for peptide analysis that requires higher reaction energy. Meanwhile the ICP source that generates lower effective temperature plasma enables phospholipids analysis with the low energy radicals for double bond specific fragmentation.

Novel Aspect:
The versatile radical sources which have wide range of energy will open new approaches of radical attachment/abduction mass spectrometry.

References:
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Keywords:
Ion-molecule reaction, Photoionization, Planetary science, Absolute cross section, Physical Chemistry

Introduction:
The methyl carbocation is a reactive species whose presence has been detected in gaseous environments fed by high energy sources, such as the interstellar medium, planetary ionospheres and laboratory plasmas. Its reactivity is described only in its ground state while excited species are present and might affect the chemistry. Our established method of photoionization allows to study and quantify effects of internal and collision energy on reactivity.

Methods:
The CERISES setup (a Quad-Oct-Quad guided ion-beam experiment) allows studying the effects of internal energy (vibrational, electronic) on the reactivity of cations with neutral gaseous species. Ions with controlled degree of internal excitation are produced by photoionization at the DESIRS beamline of the SOLEIL synchrotron [2,3]. After mass selection, reaction occurs in a calibrated pressure of target and products are then analysed in mass and counted.

Results:
The CH₃ radicals are formed by pyrolysis in a molecular beam of the nitromethane (CH₃NO₂) precursor molecule[1]. The CH₃⁺ ion is produced via direct photoionization of the CH₃ radicals. This allows to define the average internal energy of the ion. Hence, we have studied the spectroscopy of the cation in order to derive the effective internal energy of the ion at all available photon energies (from 9.8 to 15.5 eV). In a second step, the reactivity of CH₃⁺ with (i) formic acid HC(O)OH (ii) methanol CH₃OH, and (iii) 10 hydrocarbon molecules (ranging from C₁ to C₄ with isomers and different levels of unsaturation) has been studied. When necessary, isotopomers were used to clarify reaction channels. Branching ratios and absolute reaction cross-sections for each systems and channel are obtained and the dependence on the vibrational and electronic excitation as well as kinetic energy (0-20eV, lab) of the parent ion are described. Absolute reaction cross sections are obtained for all the systems allowing to derive a coherent image of the reaction dynamics.

Conclusions:
Strong effects of internal energy (vibrational as well as electronic) are observed in all the considered systems. New reaction channels appear and branching ratios are perturbed, allowing an improved description of the actual chemistry that occurs in terrestrial and astrophysical plasma. Results are analyzed for each system in terms of partition between charge transfer, dissociative charge transfer, and new bond formation.

Novel Aspect:
Access to direct photoionization of radicals, species which are important and difficult to handle, to probe effects of internal energy in ion molecule reactivity.

References:
TWO MOLECULES ARE BETTER THAN ONE: COOPERATIVE EFFECTS OF TWO SULFUR DIOXIDE AND SULFUR DIOXIDE/WATER IN BOND FORMING AND HYDROLYSIS REACTIONS OF POLYVANADATE DIANIONS IN THE GAS PHASE

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Keywords: Polyvanadated doubly-charged anions, sulfur dioxide, bond-forming reactions, hydrolysis, cooperative effects

Introduction:
Vanadium oxides are mostly used as catalysts in many oxidation reactions such as of SO2 to SO3, whereas hydrated vanadium oxides have potential application in Li-ion batteries [1, 2]. Following our interest in the chemistry of SO2, we recently reported an unprecedented bond-forming and hydrolysis reactions of doubly-charged vanadium oxide and hydroxide cluster anions promoted by SO2, leading to singly-charged ions, as a result of a charge separation process [3, 4].

Methods:
The reactions have been studied in the gas phase at room temperature by an in-house modified linear quadrupole ion trap mass spectrometer. NaV3O92- and H2NaV3O102-dianions, prepared by electrospray ionization (ESI) of NaVO3 aqueous solutions, were reacted in the gas-phase with SO2. The rate constant, branching ratio and the efficiency of the reactions have been measured.

Results:
NaV3O92- reacts with sulfur dioxide giving a first stable association product [NaV3O9 ‧ SO2]2-, that further reacts with a second molecule of SO2 forming two singly charged products, NaV2O6 ‧ SO2- and VO3SO2-. The reaction involves formation of new V-O and S-O bonds, by breaking a V-O bond containing a bridging oxygen atom. By contrast, H2NaV3O102-, the hydrated form of NaV3O92-, reacts with only one SO2 also giving the hydrolysis products HOSO2- and HNaV3O9-in addition to those arising from the bond-forming process, H2VO4- and NaV2O6 ‧ SO2-. Both reactions proceed through the formation of long-lived intermediates, where the bonding to the neutral SO2 is favoured with respect to the charge separation, that would arise from an electron transfer to SO2. Instead, the charge separation is the consequence of a chemical reaction leading to the formation of two singly charged products. A common feature is the crucial role played by sulfur dioxide in promoting charge separation processes.

Conclusions:
The sequential addition of two molecules of sulfur dioxide to thermal doubly-charged vanadium oxide anions, NaV3O92-, results in the breaking of the stable V3O9 bone, whereas in the case of the hydroxide H2NaV3O102-dianions one SO2 molecule is sufficient to promote also hydrolysis. These fast and efficient reactions are associated with charge separation processes that result in two singly charged ions by formation of new V-O and S-O bonds.

Novel Aspect:
Cooperativity of two SO2 or SO2/H2O molecules in the bonding to polyvanadated dianions has proved fundamental in addressing bond-forming or hydrolysis reactions.

References:
Introduction:
In recent years, many studies have been carried out for the gas-phase reaction mechanisms of the protonated peptides using mass spectrometry and many of these studies are related with singly-protonated peptides (1). However, there are a few studies related to multiply-protonated peptides in the literature (2,3). Most of these studies are not systematic studies, therefore they are not sufficient enough to better understand the gas-phase reaction mechanisms of peptides. Multiply-protonated peptides can be generated in ESI-MS studies if the peptides contain a basic amino acid residues. In order to determine the amino acid sequence of the peptides correctly and reliably, the gas-phase reaction mechanism of peptides should be studied and understood very well. Multiply-protonated peptides can be easily formed if the peptide sequence consists of basic amino acid residue. As all know, when the proteins undergo enzymatic cleavage (especially with Trypsin), the C-terminal of truncated peptides always contain a basic amino acids such as lysine (K) or arginine (R). Especially, the gas-phase fragmentation mechanisms of multiply-protonated peptides should be investigated in details by MS to elucidate the sequence as well as the function of proteins.

Methods
Mass spectrometry experiments were conducted on a LTQ XL linear ion-trap mass spectrometer (Thermo Finnigan, San Jose, CA, USA) equipped with an electrospray ionization (ESI) source. A 100 pmol L−1 peptide solutions were introduced into the ion source at a flow rate of 5 L/min. The peptide working solutions were prepared in MeOH/dH2O/HCOOH (50:50:1, v/v/v) containing mixture. Full scan MS spectra were acquired in the m/z range 150–1000.

Results
During the study, XXXXXH-NH2 (X = A, C, F, G, L, M, S, T, V, Y) and XYAGFLV-NH2, XYAGFLV-NH2, YAXGFLV-NH2, YAGFLXV-NH2, YAGFLVX-NH2 peptide derivatives were studied. In literature, it has been reported that peptide chain length can effect the fragmentation mechanisms of doubly-protonated peptides using AlaxH (X= 5,6,7,8 and 10). In this work, the peptide chain length is kept constant and the position of basic residues, lysine (K), arginine (R), and histidine (H), is changed within the peptide sequence to probe the effects of position as well as the identity of these amino acid residues on the fragmentation pathways. The effects of location of basic residues (close to the C-terminal and N-terminal) was also studied.

Conclusions
This work presents a detailed investigation of multiply charge ions produced different peptide series. It was shown that, All the peptide series were showed the class 1 ions (b2/y5) considering basic amino acid position starting from N-position (3,4,5,6, and 7)
b2 and y5 ion intensities were changed with respect to position of basic amino acid
The formation of b2 and y5 ion was observed at 4th position at the peptide series of XYAGFLV-NH2, YXAGFLV-NH2, YAXGFLV-NH2, YAGXFLV-NH2, YAGFXLV-NH2, and YAGFLVX-NH2 (where X= K, H and R) not the 3rd position like the peptide series of XAAAAAA-NH2, AXAAAAA-NH2, AAXAAAA-NH2, AAAAXAA-NH2, AAAAAXA-NH2, and AAAAAAX-NH2 (where X= K, H, and R)
b2/y5 (class 1) ion formation was affected by peptide sequences. Especially, the ions (b2/y5) produced from SSSSSSH-NH2

Novel Aspect:
The purpose of the current study is the investigation of the gas-phase fragmentation mechanisms of multiply-protonated peptides to gain peptide sequence correct and reliable.

References:

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HIGHLY CHARGED PROTEIN IONS: THE STRONGEST ACIDS TO DATE

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Keywords: Supercharging, ion-molecule reactions, protein sequencing, Electrospray ionisation, Gas-phase reactions

Introduction:
In electrospray ionisation (ESI), highly charged protein ions (HCPI) can be formed that fragment readily to result in higher sequence coverage than ions with less charge. Recently, we discovered that HCPIs can be formed in such high charge states [1] that they protonate N2, O2 and Ar at room temperature. [2] Here, we report a low pressure ESI source that can be used to form HCPIs in even higher charge states that protonate He; i.e. HPCIs are the most acidic entities known.

Methods:
Ion molecule reactions of HCPI’s with atmospheric gases were conducted on a linear quadrupole ion trap mass spectrometer (LTQ-MS; Thermo Scientific) equipped with an external ESI source. A low-pressure electrospray ionisation source (LP-ESI) was developed using computer aided design and machined using a CNC-mill. Calculated gas-phase values were determined by software written in Python and can be obtained at https://github.com/mgleeming/PredictPrPlus.

Results:
The extent of protein ion charging in ESI is limited by proton-transfer reactions with atmospheric gases (e.g., N2 and O2) surrounding the ESI source. [2] ESI at low pressures should improve protein ion charging by reducing the number of proton-transfer reactions that can occur between protein ions and N2 and O2. Based on the data, it suggests that HCPIs are formed via the ion evaporation model, and not the charge residue model as HCPIs are more than 6 eV less basic than the least basic solution additives.

Using the LP-ESI source, protein ion charge states can be increased by over 40%. For carbonic anhydrase, the charge state improved from 44+ to 58+. Highly charged proteins ions formed by low pressure ESI are sufficiently acidic that He can be protonated in room-temperature ion-molecules reactions; i.e., these ions are the most acidic entities that have been isolated to date.

Conclusions:
Using the LP-ESI for the formation of extremely high charge states should allow for improvements in top-down mass spectrometry because protein ions in high charge states are highly reactive and fragment readily. Thus, it is anticipated that the formation of HCPIs by low pressure ESI and chemical supercharging will significantly improve top-down protein sequence analysis by tandem mass spectrometry.

Novel Aspect:
The limit to protein charging was found to be due to ion molecule reactions between HCPIs and atmospheric gases. Using LP-ESI, protein ions can be formed in higher charge states.

References
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Introduction

The ERC Synergy Nanocosmos project aims to understand the physical and chemical processes leading to the formation of cosmic dust. In the framework of Nanocosmos, the PIRENEA 2 setup, an upgrade of the PIRENEA setup[1], is currently under construction in our laboratory. It combines sources of cosmic dust analogues with cryogenic trapping to study the interaction of relevant species with photons and gas under interstellar conditions.

Methods

The species that are generated from either a molecular aggregation source [2] or a laser vaporization cluster source [3], can be mass-selected and stored in a cryogenic radio-frequency (RF) quadrupole trap to be thermalized at tunable temperatures (~4-300 K). They can then be transferred to a cryogenic ion cyclotron resonance (ICR) cell (~20 K) for high-resolution mass analysis. Interaction with photons and gas can be studied in the quadrupole trap and in the ICR cell.

Results

The different parts of the setup (sources, RF trap and ICR cell) are still in the commissioning phase. In this poster, we will report on the achieved progress. Since polycyclic aromatic hydrocarbons (PAHs) are considered as the smallest in size dust particles, we will show how derivatives of these PAHs, which can be of astrophysical interest, can be produced with our sources including PAH clusters [4] and organometallic complexes made of PAHs and heavy atoms (Fe, Si) [5].

On the other hand, first tests of the performances of the new Fourier Transform ICR mass spectrometer (FTICR-MS) were performed. In particular this setup is equipped with a cryogenic pre-amplifier that was installed in order to significantly increase the sensitivity of ion detection [6].

Conclusions

Ion sources and the FTICR-MS part of PIRENEA 2 were tested. Next step will be to commission the cryogenic RF ion trap and interface all the modules together to get PIRENEA 2 fully operational.

Novel Aspect

The new PIRENEA2 setup opens new perspectives for the study in the laboratory of large molecular species and clusters under interstellar conditions.

References

Mass Spectrometry a New Approach to Study Thunderstorms

Keywords: Mass Spectrometry, Thunderstorms, Separation of Charge, V-EASI

Introduction

Until now in the study of thunderstorms techniques to measure voltage, charge, current, electromagnetic field and visible radiation were employed to understand about their electric aspects[1-5]. Alternatively, herein we present a precursor work in the use of mass spectrometry to understand the molecular aspects involved in thunderstorms. Therefore, physical changes in water were studied simulating some changes in clouds.

Methods

A quadrupole-mass spectrometer (LCMS shimadzu 2010) with Ventury Easy Ambient Sonic-Spray ionization (V-EASI) was used in positive and negative mode with a range of m/z 10-1000 Da to analyze Milli Q water during melting process and warming in the range of 25 °C – 80 °C. Vapor of Milli Q water also measured without any source of ionization during warming in the range of 25 °C – 80 °C and after its boiling point.

Results

During the melting process different rates of variation in ionic current (IC) were observed between 10-50%. In the study of the vapor were observed an increase of the IC directly proportional of the amount of water vapor, the IC was ten times higher in this process. In the study of water warming a rise of two times in the IC only between 25 °C – 60 °C was present in the positive mode, between 60°C – 80°C the IC was constant. On the other hand, in the negative a rise in the whole interval studied was observed and an increase of four times. In all cases, we noted that the species responsible for the rise of IC were water clusters. The rise of charge during the melting is in agreement with other works[6-8], while the charge increase during the range of temperature and liquid-vapor transition is new among the processes of formation of clouds and thunderstorms.

Conclusions

This work presents a new application of Mass Spectrometry, the study of thunderstorms, showing to be efficient to study processes of electrification already studied and also new ones. The most important aspect of this work is that the technique provides information about molecular aspects involved in thunderstorms such as the suggestion that the atmospheric discharge could be a discharge of water clusters.

Novel Aspect

The use of mass spectrometry to study process related to thunderstorms.

References
Introduction:
Sources of ultrafast XUV pulses have brought new possibilities of exploring relaxation dynamics of gas phase molecules following high-energy absorption. We used XUV excitation to produce highly excited states in Polycyclic Aromatic Hydrocarbons (PAHs) cations, thought as important components of interstellar media, and probed their subsequent femtosecond dynamics. This reveals that many-body quantum effects could have consequences in astrochemistry models.

Methods:
We used an XUV pump-IR probe scheme coupled to a time-of-flight setup [1]. Femtosecond XUV pump pulses centered around 23 eV were generated by High-order Harmonics Generation (HHG), and recombined with delayed 800 nm probe pulses to interact with gas phase neutral PAHs of various size (from Naphtalene C10H8 to Hexabenzocoronene C42H18), creating XUV excited cations probed by IR photons. Fragments and dication yields were then measured as a function of the XUV-IR delay.

Results:
In all the studied PAHs, dication yield reveals an instantaneous population of cationic states, followed by an ultrafast decay, ranging from 29 fs for Naphtalene to 59 fs for Hexabenzocoronene. XUV-induced shake-up ionization indeed forms highly correlated cationic states lying just below the second ionization potential, whose relaxation dynamics to lower states is governed by coupled electron and nuclear motion [2]. Addition of a delayed IR photon thus probes this dynamics by second ionization. Increase of this timescale with PAH size is explained by the higher density of shake-up states through which initial population has to decay.
We further observed population of fragmentation channels on the 100 fs timescale (e.g. 119 fs for H loss of Pyrene) in the fragment yields. This longer timescale corresponds to the vibrational energy redistribution in the molecule following the non-adiabatic shake-up states relaxation, leading finally to fragmentation. It reveals the energy spread to nuclear degrees of freedom.

Conclusions
We reported dynamics occurring in small and large PAHs after XUV absorption. Relaxation dynamics of highly excited cations occurs on a similar timescale for all the PAHs, increasing with PAHs size, followed by energy redistribution to vibrations. It demonstrates that relaxation of these highly correlated states through many-body quantum effects is a general mechanism for PAHs. These general trends could be incorporated in the description of interstellar media [3].

Novel Aspect:
Femtosecond XUV sources were used to measure ultrafast relaxation dynamics of astro-relevant PAHs, where electron and nuclei are fully coupled.

References
Keywords: Laboratory astrophysics, polycyclic aromatic hydrocarbons, ion traps, UV processing, gas-phase formation

Introduction:
Polycyclic aromatic hydrocarbons (PAHs) play a key role in the physical and chemical evolution of UV-irradiated astrophysical environments from protoplanetary disks to galaxies. In order to progress on our knowledge of these species, laboratory astrophysics activities have both to address the interaction of PAHs with UV photons, which is key to their stability, and investigate possible formation routes for these large molecules in cosmic environments.

Methods:
The study of the photophysics of isolated PAHs, including ionisation, dissociation and radiative cooling benefits from different setups (molecular jets, ion traps, storage rings) and their coupling with VUV synchrotron radiation [1-5]. PAH formation mechanisms are investigated using gas-phase chemical reactors, in order to identify key reactions [6] or evaluate the impact of various experimental parameters on the production of carbon dust and PAHs [7-10].

Results:
In the recent years, we have been able to better quantify the photophysics of isolated PAHs, including the dynamics of ionisation [11], as well as the rates of radiative cooling [5] and fragmentation [1]. The contribution of PAHs to the formation of H2 has gained further support [12-14]. The detection of the fullerene C60 in cosmic environments has led to considerations on the formation of this species by VUV photoprocessing of large PAHs [15].

On the other hand, activities are ongoing within the Nanocosmos ERC Synergy project [16] to study the formation of carbon dust in conditions that prevail in evolved stars, where this dust is likely formed. More specifically, the Stardust machine [10] aims at mimicking the key steps involved in dust formation around evolved stars. The AROMA setup was designed to support these experiments by analysing the molecular content of the synthesised dust [17]. This analysis, combined with that of meteorite samples, is expected to provide new insights into chemical pathways leading to the formation of cosmic PAHs.

Conclusions:
Based on their photophysical properties, only large PAHs containing ~100 atoms are predicted to survive in the harsh conditions of UV-irradiated regions. On the other hand, these large PAHs do not appear to be formed in gas-phase reactors neither be present in meteorite samples. Understanding this point requires a multidisciplinary approach at the frontier between astronomy, molecular physics, and chemistry. Mass spectrometry remains a key tool in these studies.

Novel Aspect:
In order to identify relevant cosmic PAHs, dedicated laboratory setups are now available to study their photophysics and tackle the chemistry involved in their formation.

References


https://nanocosmos.iff.csic.es/

456 - EXPERIMENTAL STUDY OF HIGH-ENERGY ION/MOLECULE INTERACTIONS RELEVANT TO PLANETARY ATMOSPHERES

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Keywords: ion/molecule interactions, high-energy interactions, ion-induced processes, planetary atmospheres

Introduction:
Atmospheres of Titan and Jupiter have already been investigated by several probes in the past. Atmosphere of Titan is composed of nitrogen and methane and other minor constituents [1], while Jupiter’s atmosphere comprises mainly hydrogen and helium in addition to other less abundant compounds [2]. Constituents of both atmospheres undergo ionization by energetic heavy ions present in magnetospheres of Saturn [3] and Jupiter [4] respectively.

Methods:
The experiments were performed on a modified sector-type mass spectrometer having a quadrupole analyzer attached to a collision chamber located between magnetic and electrostatic sector. The incident ions were formed in EI source, accelerated to \(5 \text{–} 10\) keV, mass-selected by the magnetic sector and directed through the collision chamber. The secondary ions stemming from the neutrals admitted to the collision chamber were analyzed by the quadrupole mass analyzer.

Results:
In this study we focused on projectiles and neutral targets relevant to ionospheres of Titan and Jupiter. When projectiles interact with a neutral target, two processes can occur: ionization and electron transfer. Occurrence of one or the other depends on thermochemistry of the reaction. Objective of this study is to investigate the amount of internal energy deposited in nascent secondary ions which is reflected by the extent of fragmentation of the ionized neutral. It was found out that in the case of atomic projectiles, fragmentation of the ionized neutral strongly depends on the ionization energy of the projectile but depends very little on the center-of-mass collision energy in the collision energy range used. However in the case of molecular projectiles, the extent of fragmentation is generally very low, i.e. only molecular ions are formed, and no apparent dependencies of ionization energies, sizes or chemical structures of ionic projectiles were found.

Conclusions:
For atomic projectiles, it was found that the extent of fragmentation depends on thermochemistry of electron transfer: the higher difference in ionization energies, the higher the extent of fragmentation is. For molecular projectiles the extent of fragmentation seems to be inversely proportional to the size of the projectile.

Novel Aspect:
To our knowledge, processes investigated in this study with the particular sets of projectiles and neutrals have not yet been investigated experimentally.

Acknowledgment:
The study was supported by the Charles University, project GA UK No. 928417.

References:
Introduction:
The chemical origins of the Diffuse Interstellar Bands have remained elusive. Polycyclic aromatic hydrocarbons, PAHs have been postulated to be sources of DIBs due to the similarity between the IR emissions of the cations of PAHs and the DIBs. One molecule that has been investigated for its potential role as a DIB source is 1-methylpyrene. In this study we examine the dissociation of this ion and two other substituted PAH ions, 1-nitropyrene and 9-cyanophenanthrene.

Methods:
iPEPICO experiments were conducted on the VUV beamline at the Swiss Light Source (Paul Scherrer Institut, Villigen, Switzerland). The mass-selected TPES were acquired to establish the IEs of each compound. RRKMtheory was used to determine the 0 K activation energies (E0) and entropies of activation (Δ‡S100K) by modeling the experimental breakdown diagrams. Reaction pathways were calculated at the UCCSD/6-31G(d)//B3-LYP/6-31G(d) level of theory.

Results: (Limit 900 characters)
For ionized 1-methylpyrene, the loss of H could form either 1-methylenepyrene (A) or a tropylium-motif containing ion (B). The transition state of highest energy in the pathway to B lies ~ 3.20 eV, while that for A is only 2.54 eV, favoring simple H loss from the methyl group, in contrast to that observed for ionized toluene. The presence of the three additional rings in 1-methylpyrene prevents the 7-member ring from expanding to relax the structure and lower the TS energy.

For ionized 1-nitropyrene, competition was observed between NO loss (resulting from a nitro-nitroso isomerization in the precursor ion), the loss of NO2 to form a triplet state product ion, and a sequential loss of CO, which was confirmed by D-labelling experiments.

Unlike the previous 2 ions, the –CN group in 9-cyanophenanthrene appears to act only to destabilize the PAH product ions. The –CN group itself is not directly involved in the dissociation, shown to be H and C2H2 loss. The energies required for these reactions (~ 5.5 eV) are higher than observed for unsubstituted PAHs (~ 4.5 eV, regardless of PAH size)[1], an effect traceable to the impact of the electron-withdrawing –CN group on the product ion stability.

Conclusions (Limit of 400 characters)
The substituent on a large PAH like pyrene has been shown to play a variety of roles in the unimolecular dissociation of the ions. It can either drive the reaction mechanism (nitropyrene) or have a predominantly electronic effect (cyanophenanthrene). The larger ring system of pericondensed PAHs can also push the chemistry away from extensive isomerization (methylpyrene).

Novel Aspect: (Limit of 150 characters)
Presented are the first detailed investigations of the unimolecular ion chemistry of 3 substituted PAHs.

References
Introduction:
A serious comparison of one and the same sample preparation on different LDI-MS instruments is usually not possible because each device requires the use of its own, specific target. Therefore, we developed a target system, which enables the use of only one target for different devices. The work was focused on the analysis of small organic molecules selected due to their different desorption/ionization behavior and their potential presence in carbonaceous chondrites.

Methods:
The selected organic molecules (e.g., tryptophan, 2-deoxy-D-ribose and triphenylene) were prepared and spotted onto the target plate (stainless steel or gold) and analysed on three devices with different performance characteristics, namely an Axima TOF2 (LinTOF/curved field RTOF), an ultrafleXtreme (LinTOF/dual stage RTOF) and a Synapt G2 (Qq/dual stage RTOF). The samples were also analysed on the TOF-SIMS 5 mass spectrometer equipped with gridless RTOF.

Results:
The developed target system consisting of modified Bruker target adapter, a standard SS Waters target plate and an Au target plate allowed comparison of data acquired on four MS instruments with different performance characteristics (e.g. different lasers, different laser repetition rates, different ion source vacuum regimes). The data were obtained for all analytes from plain SS and Au target. In positive-ion mode, we were able to detect different types of molecular ions (M+, radical cations, [M+H]+, [M+Na]+, and [M+2Na-H]+ ions) depending on the type of analyte. All sample molecules showed similar desorption/ionization behavior on all instruments in the positive-ion mode independently of the target applied. A deprotonated molecule of tryptophan was also detectable on all instruments with the use of SS and Au target. The use of the Synapt G2 instrument allowed accurate mass determination (<± 4 ppm) at a resolution of up to RFWHM 20.000 for all types of molecular ion. Additionally, low- and high-energy CID-spectra were acquired for tryptophan.

Conclusions:
We showed that an in-depth comparison of the data obtained with devices from different vendors with distinct performance characteristics is possible when the presented target system is being used. The possibility to obtain accurate masses can be useful in identifying unknown compounds in C chondrite meteorite samples.

Novel Aspect:
The developed target system allows the use of only one target on multiple LDI/MALDI-devices. Small organic molecules can be measured with LDI and accurate mass can be obtained.
33 - INVESTIGATING THE POSSIBILITY OF FORMING PREBIOTIC MOLECULES BY GAS PHASE ION-MOLECULE REACTION. APPLICATION TO GLYCINE.

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Keywords: Ion-molecule reactions; prebiotic chemistry; chemical dynamics simulations; Infra-red multi photon dissociation (IRMPD)

Introduction: (Limit of 400 characters)
In last years, progresses in astrophysics have revealed the presence in the interstellar medium (ISM) and in comets of many organic molecules, like glycine [1], urea [2] and formamide [3]. Due to the extreme conditions in space, it is not clear how such prebiotic molecules can be synthetized. Gas-phase ion chemistry, combining simulations and experiments, can propose some synthetic routes.

Methods: (Limit of 400 characters)
Chemical dynamics simulations and quantum chemistry were used to theoretically investigate ion-molecule reactions [4]. Simulations were done at different collision energies. Experimentally, we have used a Paul ion trap where we have introduced the ions and the neutrals. IR laser was used to characterize the products [5] and to induce the reactivity of the stabilized complexes [6].

Results: (Limit 900 characters)
To form glycine, we have considered the reaction: NH3OH+ + CH3COOH. Bohme and co-workers reported that it produces protonated glycine in a flow-tube [7].
Chemical dynamics simulations, tuning collision energy, form an ion with m/z 76 and water. However, the ions formed do not have the structure of protonated glycine [8].
Experimentally, the same reaction done in the ion trap conditions does not form the ion m/z 76, but the ion m/z 94, corresponding to the complex made by NH3OH+ and CH3COOH. It was irradiated by IR and characterized comparing IRMPD and theoretical spectra.
When doing IRMPD on the m/z 94, one of the photo-fragment is the ion m/z 76, corresponding to protonated glycine, suggesting a possible role of IR radiation in the synthesis of such prebiotic molecule.
Preliminary calculations [9] and experiments on urea formation suggests that a similar picture can occur also in this case.

Conclusions (Limit of 400 characters)
We have shown that glycine can be formed by ion molecule collisions between protonated hydroxylamine and methanol. This reaction can occur if some translational energy is given or if the formed complex is irradiated by an IR laser.
The species observed in simulations have the same m/z of protonated glycine but different structures, suggesting that radio-astronomy should consider this possibility.

Novel Aspect: (Limit of 150 characters)
Simulations and experiments of ion-molecule reactions suggest new synthetic route to form prebiotic molecules under astrophysical conditions.

References

For information please contact: scientific@imsc2018.it
THE THRESHOLD DISPLACEMENT ENERGY OF BUCKMINSTERFULLERENE C60 AND FORMATION OF THE ENDOHEDRAL DEFECT FULLERENE HE@C59

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Keywords: fullerenes, collisions, knockout, endohedrals, supernovae

Introduction:
The observation of fullerenes in various astronomical environments, and the identification of C60+ as the carrier of several Diffuse Interstellar Bands, has lead to a surge in interest in the formation and destruction mechanisms of fullerenes under different astrophysical conditions. Non-statistical fragmentation of fullerenes and other large molecules following collisions with energetic H and He atoms in supernova shocks is considered a key mechanism [1].

Methods:
C60- ions are produced by electrospray, selected with a quadrupole mass filter, and accelerated to 1-15 keV. Collisions with He at center-of-mass energies of 20-80 eV mimic supernova processing. Negatively and positively charged product distributions are measured with an electrostatic energy analyzer. Classical molecular dynamics (MD) simulations enable detailed analysis of collisions.

Results:
As the electron affinity of C60 is much lower than any of its dissociation energies, collisions depositing enough energy to induce statistical unimolecular dissociation most likely lead to electron loss and thus do not contribute to the negative ion product spectrum. Non-statistical fragmentation ie single carbon knockout is observed directly by detecting C59-. We observe for the first time the endohedral defect fullerene complex He@C59-. Secondary decay products of these ions, C58- and He@C58-, are also observed. We measure the threshold center-of-mass energy for knocking out a single carbon atom from C60 in collisions with He. Combining this with our MD simulations, we determine a semi-empirical value for the (projectile-independent) threshold displacement energy, the minimum energy needed to remove a single carbon atom from the C60 cage [2]. Our value, 24.1(5) eV, is much higher than generally assumed previously for fullerenes (around 15 eV), and is similar to that determined for graphene [3] and Polycyclic Aromatic Hydrocarbons [4].

Conclusions
Non-statistical fragmentation of fullerenes and other large carbonaceous molecules is an important destruction mechanism in collisions like those taking place in supernova shocks. The products of these collisions are surprisingly robust, and may serve as reactive seeds for bottom-up growth of larger molecules in the Interstellar Medium. The observation of He@C59- is a testament to the complexity of fullerene reactions.

Novel Aspect:
We report the first observation of endohedral defect fullerene ions and the first measurement of the threshold displacement energy of a fullerene.

References
INTRODUCTION
Islet Amyloid Polypeptide (IAPP), a 37 residue hormone, has been identified as a primary agent in the etiology of Type 2 Diabetes and the driving force for pancreatic cell death. There has been a substantial amount of research on the mechanism of the cytotoxic process but it is still not well understood. It is known that assembly of IAPP is involved and the oligomer states not the fibrils are the toxic agents. Here we provide new evidence bearing on the molecular details of the IAPP assembly process.

METHODS
Ion Mobility Based Mass Spectrometry (IMS-MS) will be used to characterize the assembly of IAPP and a number of N-terminal mutants designed to shed light on the importance of the disulfide bond in this process. These results will be complimented with data from Atomic Force Microscopy (AFM) using the same solutions utilized in the IMS-MS studies.

RESULTS
There is a disulfide bond linking cysteine residues at positions 2 and 7 in wild type IAPP. The stability of this loop appears to limit the participation of the N-terminal regions in the amyloid assembly of IAPP. However recent studies have shown that the N-terminal region is capable of self-assembly. Further, wt IAPP has an inhibitory affect on insulin secretion in beta cells but truncating the N-terminus reduces this effect. Here we look at a series of modified IAPP molecules and compare their assembly with wt: These include reduced IAPP, IAPP(8-37, and cysteine side chain Carbamidomethylation (CAM). We find reduction of the disulfide bond results in significant acceleration of the aggregation process while the CAM mutant greatly inhibits aggregation. The truncated form behaves similar to wild type in formation of oligomers but accelerates fibril formation. The implication of these results will be discussed.

CONCLUSIONS
The N-terminus has been shown to have a dramatic effect on the assembly of IAPP contrary to expectations. The results presented here contribute to clarifying the assembly mechanism of IAPP which is crucial to developing therapeutic agents to ameliorate its cytotoxic effect in Type 2 Diabetes.

NOVEL ASPECT
New data on the importance of the N-terminal region of IAPP is presented and the complimentary nature of IMS-MS and AFM emphasized.
Keywords: Non-covalent Complexes; cyclodextrin; amino acids; ion mobility-mass spectrometry; molecular dynamics simulation

Introduction:
Cyclodextrins (CDs) have the hydrophilic surface and hydrophobic cavity and enable CDs to form non-covalent complexes with other molecules. Electrospray ionization-mass spectrometry (ESI-MS), and ion mobility-mass spectrometry (IMMS) are suitable technology for the gas phase conformational study. Herein, non-covalent complexes involving amino acids and α-/β-CD are investigated using IMMS combined with molecular dynamics simulations.

Methods:
The gas-phase complexation of non-covalent complexes involving α-/β-cyclodextrin (α-/β-CD) and amino acids (AA) (AA=Gly, Leu and Phe) are investigated by ion mobility-mass spectrometry in negative mode. The complex ions are further identified by tandem mass spectrometry. The complex gas-phase conformations are clarified by IMS in combination with molecular dynamics simulations.

Results:
The 1:1 [α-/β-CD+AA]- adducts are observed when equimolar α-/β-CD:Gly:Leu:Phe solutions are electrosprayed. The complex ions are further identified by tandem mass spectrometry, in which complex ions are fragmented into [CD]- via losing one neutral AA molecule. Their gas-phase stability order of [α-/β-CD+Gly]- < [α-/β-CD+Leu]- < [α-/β-CD+Phe]-, revealed by survival yield curves, can be explained by gas-phase basicity difference. The anion is kinetically more stable with much smaller gas-phase basicity difference of [CD]- and [AA]-. The complex gas-phase conformations are clarified by IMS in combination with molecular dynamics simulations. The nearly equal collision cross sections of [β-CD+Gly/Leu/Phe]- anions were obtained by ion mobility analysis, while various cross sections were observed for the [α-CD+Gly/Leu/Phe]- anions.

Conclusions
The structure of [α-CD+Leu/Phe]- which obtained by IMS represented the exclusion structure in which the hydrophobic side chain remains outside the hydrophobic cavity of α-CD, and inclusion structure was proposed for other complex anions with the AA molecule being trapped inside the hydrophobic cavity of CD. The conformations are consistent with the most stable configurations obtained by molecular dynamics simulations.

Novel Aspect:
The gas phase inclusion or exclusion structure of non-covalent complexes of α-/β-cyclodextrin and amino acids can be identified by IMS and molecular dynamics simulations.

References
Keywords: mechanical bond, ion mobility, catenanes, knots

Introduction
Mechanically interlocked molecular architectures are exclusively used to convert energy into directed molecular motion on molecular level. Catenanes [1-3] and other mechanically interlocked systems are often hard to unambiguously characterize; the NMR studies need isolation and purification of the MIM as well as control experiments. On the other hand, in mass spectrometry (MS) unspecific binding often cannot be ruled out.

Methods
We are proposing a fast, selective, and simple approach based on collision induced dissociation combined with ion mobility spectroscopy (CID/IMS) to unambiguously characterize the topology for catenanes and knots.

Results
CID is used to induce the cleavage of the covalent bonds and IMS to separate the formed species based on their size in gas phase. Therefore, applying CID on catenanes results on two species an open macrocycle and a closed macrocycle, which can be easily separated with IMS and detected with mass spectrometry. The method automatically involves a quantitative control experiment: the amount of formed open and closed macrocycle sum up to the amount of catenane that has under gone cleavage. Similarly, this technique helps distinguishing knots from simple macrocycles. We have tested CID/IMS based method to characterize several libraries with already well known architectures including imine-bond based catenanes in water, oligolactame based structures, metal templated catenanes, etc. Thereafter, we applied CID/IMS method to probe the libraries with previously unidentified topology. Both catenanes and knots were identified; however, also libraries without mechanically interlocked bonds could be identified as true negatives. For the unknown libraries, the structures were later separated and confirmed also with other techniques.

Conclusions
We demonstrate that CID/IMS based method is rapid, conclusive, straightforward, and can be applicable to various types of catenane, including both for symmetrical and unsymmetrical catenanes, mixtures, or even crude products. Additionally, in case of dynamic libraries it can also be used to effectively probe the kinetic and thermodynamic properties of the mechanical bond.

Novel Aspect
MS, combined with IMS and IMS/MS/MS can be effectively used to characterize the structure of interlocked supramolecular structures.

References
Exploring the Inhibition Mechanisms of Amyloid Protofibrils Using Fourier Transform Ion Cyclotron Resonance-Mass Spectrometry (FTICR-MS)

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Keywords: Amyloid protein, Inhibition mechanism, Deamidation, Non-covalent interaction, Aggregation

Introduction:
Human Islet Amyloid Polypeptide (hIAPP) is an amyloidogenic protein that aggregates rapidly in humans. [1-5] Classic structure-based design for therapeutics cannot apply to amyloid protein drug development as most amyloid proteins are inherently disordered.[6-7] Herein, Fourier Transform Ion Cyclotron Resonance (FTICR) MS was used to study the potential inhibitors, identify binding regions through top-down MS/MS, and correlate with fluorescence and transmission electron microscopy (TEM) measurements to understand target regions for aggregation inhibition mechanisms.

Methods:
Collisionally activated dissociation (CAD) MS/MS and electron capture dissociation (ECD) MS/MS in a 12T FTICR MS were used to show the interaction strengths and regions between hIAPP/ deamidated hIAPP and the potential inhibitors respectively.

The performance of the aggregation inhibitors on preventing hIAPP/ deamidated hIAPP aggregation was quantified by a fluorescence spectrometer and relative MS quantification to determine the amount of fibrils formed in the incubated solutions. The overall fibrils structures were observed by TEM.

Results: (900 characters only)
Insulin, 3-APS, BISA, EGCG are all reported inhibitors of hIAPP aggregation. Insulin was found to specifically bind to the critical aggregation region of hIAPP (Ser-29 to Asn-35) which inhibited the formation of the early oligomers and the mature fibrils. The hIAPP in the heterodimer complex was easily converted back to soluble hIAPP in an aqueous solution, thus, 100% recovery of the soluble hIAPP was observed in an incubated solution. On the contrary, EGCG bound non-specifically to hIAPP and the interaction pattern of EGCG was very similar to the potential drugs which were found to be ineffective at inhibiting hIAPP aggregation (3-APS and BISA). Instead, EGCG was found to aggregate rapidly with hIAPP in a non-fibrillary complex to inhibit the aggregation.

For deamidated hIAPP, CAD MS/MS results showed insulin had a weaker interaction with the deamidated hIAPP compared to the wild-type and mature amorphous aggregates were formed during the experiment.

Conclusions: (400 characters only)
The results demonstrated there are two potential inhibition mechanisms adopted by the potential inhibitors to prevent hIAPP aggregation. Insulin directly inhibits hIAPP fibrilization by specific interaction with target sequence regions, and EGCG rapidly redirects hIAPP into non-toxic amorphous aggregates which prevents the amyloid fibrils formation. Only EGCG inhibitors with redirecting the protein into non-toxic amorphous aggregates was found to be applicable in inhibiting the deamidated hIAPP aggregation.

Novel Aspect: (150 characters only)
Understanding underlying mechanisms that prevent amyloid aggregation are the key to amyloid drug development.
References
DIFFERENT MEANS OF SOLUBILIZATION OF MEMBRANE PROTEINS FOR NATIVE MS

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Keywords: membrane proteins, nanodiscs, SMALPs, native MS, LILBID

Introduction:
Membrane proteins are of high interest, but still underrepresented in research due to the inherent difficulties for many investigation methods, arising from their hydrophobic nature. Depending on the feature of the membrane protein complex the researcher is interested in (protein stoichiometry, lipid affinity, annular lipids...), different means of solubilisation can enable MS analysis.

Methods:
LILBID (Laser Induced Liquid Bead Ion Desorption) is an ionization method which employs a droplet generator to produce analyte droplets of 30-50um diameter at a frequency of 10Hz [1]. These droplets are irradiated by a mid-IR laser leading to the explosive expansion of the droplet. The solvated ions are set free and are mass analyzed.

Results:
We investigated the usability of artificial membranes for the study of membrane protein complexes with LILBID-MS. Lipid bilayer mimics as nanodiscs and liposomes allow the analysis of the oligomerization state of a protein complex. Cell-free expressed membrane proteins can be solvated by lipid bilayer mimics without ever being in contact with detergents. This allows for example the study of lipid dependent oligomerization [2]. Even SMALPs, which allow to cut protein complexes out of living cells preserving their annular lipid belt, can be used to determine the constituting proteins of a complex, in dependence of growth conditions of the cells.

Conclusions:
LILBID-MS is well suited for the analysis of membrane proteins complexes solubilized by detergent or different lipid bilayer mimics, such as nanodiscs, liposomes or SMALPs. This allows analysis of the complexes’ constituting proteins, as well as their dependence on specific lipids or ligands.

Novel Aspect:
Analysis of membrane protein complexes directly out of lipid bilayer mimics

References
Introduction:
Mass spectrometry (MS) has the potential to monitor different modes of lipid binding to membrane protein complexes. Initial studies monitored the addition of lipids and deduced the kinetic and thermodynamic effects of lipid binding to proteins. Recently we have identified lipids already present, explicitly in plugs, annular rings or cavities. These lipids have higher residence times and consequently can be quantified and characterized by mass spectrometry.

Methods:
We have used native MS of membrane proteins liberated form micelles to study protein lipid interactions. Using an Orbitrap MS platform developed for membrane proteins (1)we show that it is possible to distinguish lipids, cofactors and drugs while in direct contact with the membrane protein target.

Results:
While most steps of lipid II transfer across the cytoplasmic membrane are understood, identifying the lipid II flippase has yielded conflicting results. We used native mass spectrometry to characterise lipid II binding to two candidate proteinsMurJ and FtsW. We showed that lipid II binding competes withcardiolipin to reduce lipid II binding to MurJ(2). We also showed how drug binding mediates phosphorylation in a class A GPCR (P2Y1R) (3)and that purification of three further class A GPCRs results in binding of specific lipids. These lipids in turn affect downstream coupling to certain types of G-proteins and consequently appear to enhance selective coupling primarily to class A GPCRs.

Conclusion:
The ability to detect lipid binding which haveboth structural and functional effects, for example on lipid flippase activity or G-protein coupling, is a powerful and important attribute of the study of membrane proteins by mass spectrometry.

Novel Aspect:
First mass spectra of folded GPCR complexes and demonstration of competition between lipidsaffecting function.

References:
819 - ARYL BIS-SULFONAMIDES BIND TO THE ACTIVE SITE OF A HOMOTRIMERIC ISOPRENOID BIOSYNTHESIS ENZYME ISPf AND EXTRACT THE ESSENTIAL DIVALENT METAL CATION COFACTOR

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Keywords: Native ESI-MS, oligomeric enzyme, binding mechanism, aryl bis-sulfonamides, IspF

Introduction:
Characterizing the mode of action of non-covalent inhibitors in multisubunit enzymes often presents a great challenge. Most of the conventional used techniques often fail because they do not reveal how the distribution of individual ligand-bound states changes with ligand concentration but instead describe an average property of the ensemble as a function of ligand concentration. Thus, mixed models comprising both competitive and noncompetitive binding are often used that proved very little insight into the mechanisms of ligand binding and recognition.

Methods:
Native electrospray ionization mass spectrometry (ESI-MS) has recently emerged as a powerful technique for direct, label-free analysis of intact protein complexes. Native ESI-MS has been increasingly recognized for its ability to directly probe such properties of intact macromolecular assemblies as the protein-ligand complex stoichiometry, binding affinities, and allosteric effects.

Results:
Here we apply native ESI-MS to comprehensively characterize an isoprenoid biosynthesis enzyme IspF from Arabidopsis thaliana, an example of homomeric protein complex with multiple binding sites for several types of ligands, including a metal cofactor and a synthetic inhibitor. Based on these combined with computational modeling, we propose a mechanism of AtIspF inhibition by aryl bis-sulfonamides that involves both the competition with the substrate for the ligand-binding pocket and the extraction of Zn2+ from the enzyme active site. This inhibition mode is therefore mixed competitive and non-competitive, the latter exerting a key inhibitory effect on the enzyme activity.

Conclusions
The results of our study deliver a profound insight into the mechanisms of AtIspF action and inhibition, open new perspectives for designing inhibitors of this important drug target, and demonstrate the applicability and value of the native ESI-MS approach for deep analysis of complex biomolecular binding equilibria.

Novel Aspect:
While standard biophysical techniques failed to reveal the mode of action of aryl-sulfonamide-based inhibitors of AtIspF, direct native ESI-MS titrations of the protein with the ligands and ligand competition assays allowed us to accurately capture the solution-phase protein-ligand binding equilibria in full complexity and detail.

For information please contact: scientific@imsc2018.it
385 - COLLISION INDUCED DISSOCIATION OF MULTIPLY CHARGED PEPTIDE AGGREGATES

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Keywords: clusters, charge separation, non-covalent complexes, peptides, tandem mass spectrometry

Introduction:
Self-association and interactions of amino acids and peptides is in the focus of intensive research since a long time. In this work, clustering properties and gas-phase dissociation pathways of multiply charged peptide clusters were studied. We have analyzed the cluster formation of tripeptides as well as the collision induced dissociation of their clusters in an ion trap mass spectrometer.

Methods:
Mass spectrometric experiments were performed using two tyrosine-containing tripeptides: H-Tyr-Ala-Gly-OH and H-Tyr-Gly-Phe-OH, on a Bruker Esquire 3000+ ion trap mass spectrometer equipped with ESI source. Optimized ion source parameters were: nebulizer gas pressure: 80 psi, dry gas flow: 0.5 l/min, dry temperature: 250 °C, skimmer voltage: 40V, capillary exit voltage: 106 V.

Results:
The relatively high peptide concentrations were found to enhance the formation of peptide aggregates. Ion source parameters were optimized to achieve high signal intensity for larger peptide aggregates. We observed, that high nebulizer gas pressure and low dry gas flow rate facilitate the detection of large, multiply charged clusters. Both peptides exhibit a characteristic cluster distribution profile in ESI-MS. Beside the protonated monomer, dimer and trimer species, peptides produce larger clusters in the 1200-2400 m/z. Most intensive peaks correspond to 5 and 6 peptide units with one charge. MS/MS experiments revealed that multiply charged metaclusters of peptides appear at the same mass-to-charge ratios as peptide aggregates with lower charge states. Peptide clusters of interest were isolated in the ion trap and fragmented to identify their fragmentation pathways.

Conclusions
The dominant fission products for the tripeptide clusters were the losses of the proton-bound dimers, presumably due to the high gas-phase stability of this species. Charge separation by cluster fission was proved to be a major dissociation pathway of these peptide clusters. Beside the formation of singly charged ion population, triply charged species were preferred products was well.

Novel Aspect:
Characteristic cluster distribution was detected for tripeptides, together with typical charge separation-based fragmentation pathways.

Acknowledgement
Gitta Schlosser acknowledges the support of the MTA Premium Post-Doctorate Research Program of the Hungarian Academy of Sciences (HAS, MTA).
803 - NOVEL, BILATERAL FLUOROUS ALKOXIDE-BASED CLUSTERS DISCOVERED BY AMBIENT IONIZATION MASS SPECTROMETRY

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Keywords: fluorous flying salts, fluorous clusters, ambient ionization, non-covalent complexes, energy-dependent tandem mass spectrometry

Introduction:
Cluster formation is an important research topic in fundamental mass spectrometry. Formation of alkali metal halide clusters using electrospray ionization has long been known. These salts can form very large cluster structures such as Na124Cl1222+ [1]. Here we report the discovery of novel, bilateral fluorous alkoxide-based clusters by ESI, showing unique structural properties.

Methods:
Experiments were performed using a Bruker Esquire 3000+ ion trap MS with ESI ion source and a Bruker microQTOF with DART ion source. MM/QM computational studies were carried out with Schrödinger Maestro 11.1 and Gaussian16 software packages. We optimized the structures with OPLS2005 force field and B3LYP/Def2SVP (GD3BJ) and calculated single point energy with B3LYP/Def2TZVPP level of theory.

Results:
Nonafluoro-tert-butyl alkoxides, MOC(CF3)3 (M = Li, Na, K, Rb, Cs), were synthetized to investigate their clusters using mass spectrometry. Solutions prepared in dry acetonitrile were directly injected into the ESI ion source. The salts show complexes of outstanding stability and high intensities both in positive and negative ionization modes, such as [M4A3+; M5A6-; where A = (OC(CF3))-].

Computational chemistry methods, MM/QM modelling, were used to calculate the stable gas phase structures. Energy-dependent MS/MS fragmentation experiments were also performed to quantitatively describe affinity and stability parameters of clusters. Experimental data were in good correlation with calculated values.

Conclusions:
Formation of fluorous alkoxide clusters are described here for the first time. These special compounds, referred to “fluorous flying salts”, represents a new cluster type, in which the anion has fluorous-ionic bilateral properties, which prevents the limitless polymer cluster formation, and support the outstanding stability of defined structures.

Novel Aspect:
Bilateral fluorous alkoxide-based clusters discovered by ESI-MS give novel opportunity for the study of electron pair acceptor compounds by MS.

Acknowledgement:
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NONCOVALENT COMPLEXES IN NATURAL EXTRACTS

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Keywords: noncovalent complexes in natural extracts

Introduction: Chemical evolution includes capture, mutation and propagation of molecular information via covalent and noncovalent associations [1]. Considering the high complexity of natural substrates, for the presence of molecular species of different chemical nature, the formation of noncovalent complexes has to be expected. They could play a role in the activity of the extract, leading to structures of active compounds different to that of the synthetic analogous products.

Methods: The presence of noncovalent complexes in different natural extracts (green tea, Ceylon tea, green coffee, mate) has been investigated by precursor ion scan on ions generated by ESI and neutral loss scans. In order to exclude that the complexes so detected were artifact due to ESI conditions, LC MS measurement were performed, and reconstructed chromatograms based on accurate mass values confirmed the presence of noncovalent complexes in the natural extracts.

Results: Some investigations were performed to establish unequivocally the presence of catechin/caffeine complexes in green tea (GTE), Ceylon tea, green coffee, mate extracts. By ESI-MS experiments performed on GTE by means of precursor ion scan and neutral loss scan methods, the presence of caffeine:catechin complexes was proved [2]. The same approach showed that in the case of Ceylon tea the abundance of these complexes is strongly reduced, while for green coffee and mate complexes of caffeine with chlorogenic acid present in the extracts were undetectable. Further experiments were performed on GTE by LC/MS method operating in high resolution conditions. The reconstructed ion chromatograms of the exact mass ions corresponding to the caffeine/catechin complexes have been obtained, showing the presence of the complexes of caffeine with gallate-type catechins [2]. This approach was able to put in evidence the presence of the same complex with different structures, exhibiting different retention times, in agreement with the data of Ujihara and Hayashi [3].

Conclusions The complexes between caffeine and catechins already observed in solid state and in solution by NMR in ad hoc prepared solution are detectable also by mass spectrometry, once specific methods are employed. These complexes are detected in green tea and, in minor extent, in Ceylon tea. In mate and in green coffee the complexes possibly originating by interaction of caffeine with chlorogenic acid are undetectable, reasonably due to the low affinity of the two molecular species.

Novel Aspect: For the first time the presence of caffeine/catechin complexes in green tea and in Ceylon tea extracts has been proved by mass spectrometric experiments.

References
Introduction:
Characterization of the overall topology and inter-subunit contacts of non-covalent protein complexes, and their assembly/disassembly and unfolding pathways, is critical because protein complexes regulate key biological processes, including those important in understanding and controlling disease. Native mass spectrometry is an approach that provides critical structural information at high throughput on low sample amounts.

Methods:
The power of native MS increases when coupled to ion mobility (IM-MS), a technique that measures rotationally averaged collisional cross sections and thus information on conformational changes, or to high resolution mass spectrometry (HRMS). This presentation illustrates CID,SID/IM, and SID HRMS for characterization of topology, intersubunit connectivity, and other structural features of multimeric nucleoprotein complexes.

Results:
Native MS is being used in our lab for characterization of soluble, membrane, and nucleo protein complexes. Native MS results for multiple systems will be presented in this talk, with a primary focus on RNA:protein complexes. The first nucleoprotein system under investigation (with Venkat Gopalan of OSU) is RNase P, an endonuclease that catalyzes 5’ maturation of t-RNAs. While we’ve been able to bind four RNase P proteins to the RNA or a fifth protein to kink turns in the RNA, we are still optimizing conditions to simultaneously bind all five proteins. A second system under investigation (with Karin Musier-Forsyth of OSU) involves the retroviral Gag protein involved in HIV-1 genome packaging and viral assembly, a system for which we’ve measured 3:1 Gag:Psi complexes. A third nucleoprotein project (with Sarah Woodson of Johns Hopkins) involves the hexameric RNA chaperone Hfq bound to RNA and recruiting messenger RNA, a system for which surfaced-induced dissociation of the hexameric ring is altered by the presence and absence of the bound RNA.

Conclusions:
Native MS coupled to HCD, SID, ion mobility, or high resolution mass spectrometry, along with complementary tools such as covalent labeling MS, provides information on conditions needed for optimal formation of the protein:RNA complexes (ionic strength, Mg2+ concentration, protein:RNA ratios) and on the protein-RNA connectivity and structure. Experiments are underway to determine the best ways to calibrate the CCSs of the RNAs and RNA:protein complexes.

Novel Aspect:
Native MS and SID of nucleoprotein complexes provides structural information unavailable or difficult to obtain by other structural biology tools.
1403 - STERIC STRUCTURE AND STABILITY OF THE GAS-PHASE COMPLEXES OF MACROCYCLIC RECEPTORS WITH ANIONS

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Keywords: ion mobility mass spectrometry, macrocycles, anions, noncovalent complex

Introduction: One of the most intensively developed topics of supramolecular chemistry is design, synthesis and studies of neutral molecular receptors capable of strong and selective binding of anions because of their crucial role in many chemical and biochemical processes. The ion mobility mass spectrometry (IM-MS) supported by theoretical calculations and CID methods are evaluated as fast screening methods in the development of new receptors.

Methods: IM-MS measurements were performed on Synapt G2-S HDMS. Collision-induced dissociation (CID) measurements were performed on the triple quadrupole 4000 Q Trap (MD SCIEX). Starting models of the molecules and ions were generated using Spartan software and were further optimized with DFT Methods: The theoretical collision cross sections for model structures were calculated using Mobcal program.

Results: The complexes of macrocyclic receptors with selected anions are stable enough in the gas-phase to survive the conditions of the IM-MS experiments. The experimentally measured collisional cross sections were used for establishing the steric structures of the studied ions by comparing them with the cross section values obtained from molecular modelling Methods: The type of calibrant ions significantly affects the accordance between theoretical and experimental values of collision cross sections. The effects of the deformation of macrocyclic receptors upon complexation with anions are recognized in IM-MS experiments.

Conclusions
The results confirm the usefulness of the IM-MS technique for studying the structures of complexes of the macrocyclic receptors with anions. They also prove that the IM-MS method is very sensitive to even small differences in the structures of the studied complex ions. The IM-MS and CID techniques are potentially very efficient and cost-effective methods to study supramolecular recognition of anions.

Novel Aspect: IM-MS and CID techniques evaluated as fast screening methods in the development of new receptors.
Keywords: molybdenum sulfide; catalysis; hydrogen evolution; action spectroscopy; dissociation

Introduction:
Molybdenum sulfide-based catalysts have the potential to replace platinum as a hydrogen evolution reaction (HER) catalyst in electrochemical water splitting [1]. A better understanding of the reaction mechanisms contributes to the targeted optimization of the catalysts. In this contribution, individual reaction steps are investigated quantitatively employing precisely defined gas-phase models, like Mo$_3$S$_{13}$(2-) and Mo$_2$S$_{12}$(2-).

Methods:
Fourier transform ion cyclotron mass spectrometry (FT-ICR MS) is used, as it is an excellent tool to examine ion-molecule reactions in the gas phase [2], including catalytic cycles. By combining FT-ICR MS with IR and UV/VIS laser spectroscopy, the reactants, products and reactive intermediates can be further characterized.

Results:
IR-spectroscopy experiments with the protonated species HMo$_3$S$_{13}$- show a distinct H-S stretching vibration. Comparing this result with density functional theory (DFT) calculations suggests that the proton is bound to an edge disulfide unit of the cluster. In gas phase, the cluster Mo$_3$S$_{13}$(2-) unfolds from the highly symmetric Mo$_3$-ring structure, found in the solid state, to form irregular structures with a variety of mono- and disulfide ligands. Structural changes are also expected for molybdenum sulfide clusters deposited on electrodes. Investigation of the structural flexibility and chemical properties of the different sulfur moieties contributes to an understanding of the catalytic activity of molybdenum sulfide surfaces.

Conclusions:
Structural features evolve in the gas phase that elucidate the conformational flexibility and relative stability of reactive molybdenum sulfide species. Hydrogen is bound at sulfur centers, as revealed by the infrared signature of the S-H bond. Electronic excitation spectra indicate the availability of low-lying excited states for some species generated by mild collisional activation.

Novel Aspects:
Molybdenum sulfide clusters are synthesized with isotopically enriched molybdenum and characterized by mass spectrometry in combination with optical excitation.

References
We have implemented cryo ion trap FT-ICR instrumentation with temperature control to as low as 11 Kelvin. With two such instruments we investigate the magnetism and the adsorption kinetics of transition metal clusters and complexes. We have upgraded one of these instruments by a cryo RF trap for tandem cryo operation. Spectroscopy comes into play through application of Infrared Photon Dissociation (IR-PD) by optical parametric oscillator/amplifier (OPO/OPA) photon sources. This enables one and two colour investigations of metal organic complexes[1] and of transition metal cluster adsorbate complexes[2-3].

Stepwise cryo adsorption kinetics of N2 on Ni+n+ clusters reveal clearly discernible mono layer like adsorbate shells which interpret in the light of IR-PD spectra of the product complexes Nin(N2)m+. It emerges a characterization of the cluster surface morphology and its adsorbate induced reorganization[4-5]. The according data on Fe+n+ cluster N2 adsorption are subject of current interpretation. Case studies on various metallophthalocyanine complexes found interpretation in terms of adsorbate induced spin quenching[6], and recent findings of non classical blue shifts of N2 stretching modes of adsorbates to Fe3O(OAc)6+ complexes received interpretation in terms of orbital interactions[7].

The tandem cryo trap option comes to live by studying consecutive co-adsorptions of N2 and H2 to selected Ru8+ clusters – as compared to the reverse order co-adsorption. Our findings allowed to elucidate the mutual interdependencies amongst the adsorption kinetics, their activation and migration[8].

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Keywords: Copper anticancer drugs, internalization of copper in cancer cells, Cu(I) and Cu(II), model tripeptides, human Copper Transporter 1 (hCtr1)

Introduction: Human Copper Transporter 1 (hCtr1) is a 190 amino-acids trans-membrane protein mainly devoted to the recruitment of metal ions from the extra-cellular fluids [1]. The extracellular domain of hCtr1 contains histidine-rich and methionine-rich sequences likely responsible of metal ions recruitment. The terminal portion of the protein extruding from the cell membrane contains the characteristic Met-Asp-His peptide sequence.

Methods: The interaction of Cu(I), Ag(I) and Cu(II) ions comprised in appropriate precursors (i.e. [Cu(P)4]⁺, [Ag(P)4]⁺ and CuCl₂ (P = tertiary phosphine)) with selected model tripeptides XYHis (X = Gly, Met; Y = Gly, Asn, Asp) and some polypeptides relevant to hCtr1 has been investigated by electrospray ionization mass spectrometry, and the interaction products have been characterized by multiple collisional experiments, using an ion trap mass instrument.

Results: Model XYHis tripeptides starting from the designed GlyGlyHis derivative of the ATCUN family [2] are able to mine Cu(I) and Ag(I) from [M(I)(P)4]⁺ inorganic precursors through the formation of the [M(I)(XYHis)]⁺ adducts under ESI(+)MS conditions [3]. Also Cu(II) is wrapped by XYHis tripeptides via [Cu(II)(XYHis) - H]⁺ adducts. MSn of these metal-tripeptide complexes indicate that His is bound strongly to the metal in all cases. While CID pathways of [M(I)(XYHis)]⁺ ions proceeded through losses of neutral fragments, MSn fragmentation of [Cu(II)(XYHis) - H]⁺ ions instead took place mainly through intramolecular electron transfer reactions including the reduction of Cu(II) to Cu(I) and the formation of fragment radical cations. The interaction of [M(I)(P)4]⁺ precursors with polypeptide sequences representing the terminal extra-cellular portion of hCtr1 (MetAspHis, MetAspHisSerHisHis and MetAspHisSerHisHisMetGlyMetSer) confirms the ability of these peptides to mine and recruit M(I) ions from inorganic precursors.

Conclusions: Model tripeptides having a free-amino group at N-terminus and either a free carboxylic or an amidated group at the C-terminus of His in third position, and polypeptide sequences relevant to hCtr1 are able to mine Cu(I), Ag(I) and Cu(II) from inorganic precursors via formation of [M(tri/polypeptide)] adducts. This experimental evidence is significant to shed light in the cell internalization process of anticancer phosphino-Cu(I) drugs.

Novel Aspect: The observation that Cu(II) comprised in the [Cu(II)(XYHis) - H]⁺ ions undergoes redox reaction under ESI(+)-MSn conditions might be relevant in the claimed reduction processes of physiological Cu(II).
Introduction:
While infrared spectroscopy of ions is a well-established field, the potential of UV-VIS spectroscopy of ions is barely touched. The field was pioneered by Ben Freiser in the 1980s, using lamps and monochromators as light source.[1] Nowadays tunable optical parametric oscillators cover a wide range of wavelengths from UV to infrared which can be used for spectroscopy, e.g. the photodissociation of peptides.[2]

Methods:
Two FT-ICR-MS setups are used: A modified CMSX47 instrument equipped with a 4.7T magnet, APEX II data station and a laser vaporization ion source and a Bruker Apex Qe, equipped with a nanobay console, electrospray ion source and a 9.4T magnet. Light from tunable OPO (EKSPLA NT342B) is guided into the FT-ICR cell for studying spectroscopy and photochemistry. Experiments are supported by theoretical calculations.

Results:
FT-ICR-MS is, compared to time of flight techniques or quadrupole mass filters, inherently slow, with a duty cycle in the range of seconds. High-resolution spectroscopy over a wide wavelength range, which requires the recording of 103-104 mass spectra, is not the prime application of FT-MS instruments. However, FT-MS offers a combination of high mass resolution, long trapping times, flexible mass selection capabilities in a collision-free environment and simultaneous detection of all fragments with high fidelity of absolute intensities. These features gives FT-MS significant advantages over other techniques for specific problems.

Selected examples which illustrate these advantages include photochemical hydrogen formation in the Mg+(H2O)n, n = 1-5, system, or the spectroscopy of the atmospherically relevant CO3-(H2O)1,2 ion in combination with a nitrogen cooled FT-ICR cell.

Conclusions:
The results show that FT-ICR-MS is a powerful tool in combination with theory for investigation of the photochemistry of cluster systems.

Novel Aspect:
Interplay between single and two photon processes play an important role in photodissociation of hydrated positively charged magnesium clusters.

References
Introduction:
Polyoxometalates cover a broad spectrum of interesting properties related to the nature and number of incorporated metals, their size and shape, their redox behavior, magnetic and photochemical characteristics. Possible applications are manifold. Yet to harness a certain functionality, comprehensive insights into their structure and reactivity are crucial.

Methods:
We are presenting the investigation of structure and reactivity in solution and in the gas phase of different antimonato polyoxovanadates by electrospray ionization mass spectrometry (ESI-MS), isotope exchange experiments in solution, collision-induced dissociation tandem MS and ion mobility MS experiments conducted at a SYNAPT G2-Si (Waters, Manchester).

Results:
The first water-soluble antimonato polyoxovanadate cluster was demonstrated to be present in solution as two distinct species, which both possess an intact, closed cage-like \{V15Sb6\} structure: a water-encapsulating and a water-free cluster core. Its chemical behavior is greatly dependent on the encapsulated water molecule, since a transduction of inner-phase reactivity of the guest results in changes in the outer-phase reactivity of the cluster cage.[1] Moreover, the surrounding cationic metal-complexes and the ligand environment, as well as additives influence the reactivity of the \{V15Sb6\} cluster in solution.[2] In this regard, a \{Sb6V15\}→\{Sb8V14\} cluster transition was discovered by ESI-MS. This reaction enables in combination with a change in the ligand sphere the formation of an unusual, meta-stable \(\alpha_{1^*}\)\{-V14Sb8\} isomer which forms dimeric super structures. These were investigated in the solid state, in solution and in the gas phase.[3]

Conclusions:
ESI-MS and IMS could serve as a probe for the elucidation of solution and gas-phase reactivity of metal-oxo clusters, as well as the identification and structural analysis of new products. These methods can provide the rational guideline for the synthesis of new compounds and for the assembly of resulting complex supramolecular architectures.

Novel Aspect:
We present the first comprehensive study on the structure as well as solution and gas-phase reactivity of antimonato polyoxovanadate clusters.

References:
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Keywords: Oxygen atom, Ozone, Ketone, Aldehyde, Ab initio calculation

Introduction
Non-thermal plasma induced reforming of fuel–air mixtures can improve the thermal efficiency of internal combustion engines by achieving its super-lean-burn operation. To address the general mechanisms of non-thermal plasma induced gasoline reforming in air, we used iso-octane, n-heptane, toluene, trimethyl pentene and methyl cyclohexane, which are components of gasoline surrogate fuel, as the models.

Methods
A mixture of air with liquid hydrocarbons was reformed by non-thermal plasma in a flow reactor, generated by a dielectric barrier discharge (DBD), and then directly analyzed using ion attachment mass spectrometry (IAMS). The reactor temperature was kept at 393 K and the equivalence ratio of the pre-mixture was set to 0.5. The simulation of the fuel reforming was performed by DFT calculation with Gaussian 16 program.

Results
The IAMSmass spectra of the alkane/air mixture with DBD irradiations showed intense signals corresponding to ketone and aldehyde. The DBD irradiation of the alkane/air mixture produced oxygen atom, which abstract a hydrogen atom from alkane. As a result, alkanes were converted to alkylhydroperoxide through alkyl radicals. The ketone and aldehyde were generated by fragmentation of alkylhydroperoxide. As in the case of oxygen atom generation, DBD produced ozone, which selectively react with alkene to produce molozone. The trimethyl pentene undergoes the cleavage of double bond by DBD irradiation, leading to the ketones. The DBD irradiation to toluene/air mixture provided the signal corresponding to the benzaldehyde. A hydrogen atom in toluene was abstract by oxygen atom. The produced benzyl radical was immediately converted to benzyl hydroperoxide by the reaction with oxygen molecule and subsequent fragmentation would produce benzaldehyde. The proposed fuel reforming processes are supported by the ab initio DFT calculations.

Conclusions
DBD irradiation of the fuel/air mixture produced oxygen atom and ozone, which react with fuel molecules. Hydrocarbon was converted to ketone and aldehyde by DBD induced fuel reforming. The presence of products due to DBD plasmairradiation, such as ketone, aldehyde, and the intermediates e.g., alkyl hydroperoxide and molozone, would contribute to the combustion enhancement resulted in the thermal efficiency and stability of lean internal combustion engines.

Novel Aspect
DBD induced fuel reforming of hydrocarbon provide ketone and aldehyde, which would contribute to improve the performance of lean combustion in internal combustion engines.

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Introduction:
Distonic radical ions can be used to explore the chemistry of transient radicals in the gas phase. While this approach facilitates the experimental observation of elusive radicals, it also perturbs their native reactivity. Direct thermodynamic and spectroscopic measurements of the influence of charge on radical stability within distonic radical ions have been undertaken and highlight perturbations over significant intramolecular separations.

Methods:
Radical ions have been synthesized in the gas phase using regioselective approaches based on collision-induced dissociation and photodissociation on modified ion trap mass spectrometers. The kinetics of ion-molecule reactions have been measured by seeding low concentrations of reagent gas in the helium buffer gas supplied to the ion trap.

Results:
Pioneering research by Kenttämaa has demonstrated the power of the distonic ion approach but has also highlighted the Faustian bargain that is entered into with the introduction of the charged moiety to the radical [1]. That is, while the presence of the charge facilitates the experimental observation and characterization of elusive radicals it can also perturb their native energetics. In our laboratory a homologous series of distonic ions incorporating nitroxyl and peroxyl radicals have been regioselectively prepared as both positively and negatively charged ions. The influence of the charge polarity and the distance between the charge and radical sites were systematically explored using a combination of ion spectroscopy; thermochemical measurements (using the kinetic method); and ion-molecule reactions. Key findings from these investigations reveal that radical moieties are stabilised in distonic anions and destabilised in analogous cations, with respect to their corresponding neutrals. Significantly, these effects could be measured over intramolecular distances of up to 10 Å.

Conclusions:
The thermodynamic impact of charged groups of radical stability provides the critical insight required to extrapolate distonic ion behaviours to their neutral archetypes. Moreover, the long range nature of charge-radical effects suggests that these phenomena are likely playing a significant role in the dissociation chemistries of the radical ion intermediates formed in contemporary ion dissociation technologies (e.g., ETD, ECD and RDD).

Novel Aspect:
Direct measurement of charge-radical interaction energetics in distonic ions

References:
ELUSIVE MONOFUNCTIONAL ADDUCTS OF CISPLATIN WITH NATURAL AMINOACIDS REVEALED BY IRMPD SPECTROSCOPY AND PHOTOFRAGMENTATION KINETICS

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Keywords: IRMPD kinetics, isomerism, conformer discrimination, cisplatin, aminoacids

Introduction:
Relevant intermediate species in the reaction path of cisplatin with aminoacids can be difficult to characterize due to the high reactivity of square planar complexes of PtII with nucleophiles. However, ESI-MS proved to be valuable in allowing to detect for the first time the primary complexes generated from direct substitution of one of the labile chloro ligands of cisplatin with aminoacids like His and Met, modeling cisplatin interaction with peptides and proteins.

Methods:
The complexes of interest were brought to the gas-phase using ESI and mass-analyzed using a Paul ion trap instrument (Bruker Esquire 6000) or a FT-ICR mass spectrometer (Bruker APEX-Qe and 7T Solarix). Vibrational features of the mass-selected ions were assayed using IRMPD spectroscopy in both XH (X = C, N, O) stretching and fingerprint regions using an OPO/OPA benchtop laser system and the free electron laser (FEL) at the Centre Laser Infrarouge d’Orsay, respectively.

Results:
The primary complexes of cisplatin with histidine and methionine, cis-[PtCl(NH3)2(L)]+ (L = His, Met), have been characterized using high-resolution MS and IRMPD spectroscopy. The IR spectrum of bare ions can be directly compared with calculated spectra, thus allowing the attribution of the assayed species to a particular conformer or a set of them. In the case of the cisplatin-histidine adduct, a complex mixture of isomers and conformers was hypothesized and finally resolved using IRMPD kinetics on conformer-specific vibrational modes.[1] Regarding the adduct with methionine, the comparison of the IRMPD spectrum with theoretical ones confirmed the preferred interaction of Pt with the thioether functionality of the aminoacid. However, when the complex of a structural isomer of the drug, transplatin, with Met was assayed, differences in the photofragmentation kinetics on a same, selected vibrational mode permitted to unveil the presence of a mixture of isomers presenting platinum coordination at either the amino or the thioether group.[2]

Conclusions
We show the ability of mass spectrometry to extract and isolate from solution species difficult to characterize using condensed-phase based techniques. Also, the coupling with IRMPD permitted to obtain spectroscopic information on the mass-isolated ions, proving the presence of complex isomeric mixtures. In this regard, the use of fixed-wavelength IRMPD kinetics permitted to discriminate and quantify isomers and conformers forming the gas-phase populations.

Novel Aspect:
Monofunctional adducts of cisplatin with His and Met have been characterized for the first time. IRMPD kinetics were used to discriminate and quantify isomers and conformers.

References
Characterization of a Nitrogen Dielectric Barrier Discharge Ionization

Keywords: ambient ionization techniques, nitrogen dielectric barrier discharge, fundamental, reactive species

Introduction:
In the last decade, ambient ionization coupled to mass spectrometry generated enormous attention due to the fast and sensitive analysis with nearly no sample preparation. [1] However, the development of new sources dominated over the understanding of the ionization mechanism of already existing methods: In this work, the active capillary plasma ionization source was characterized to get a better understanding of the behavior of the reactive species.

Methods:
The active capillary plasma ionization source contains a stainless steel capillary (ground electrode) which is separated by a glass capillary from a copper ring (sine-modulated high voltage). [2] Nitrogen or air was used as discharge gas. The plasma source is directly connected to the mass spectrometer. All optical measurements were performed at the cross section of the plasma source using either UV-VIS spectrometer in the range of 200-850 nm or a ns-gated iCCD camera.

Results:
The active capillary plasma ionization source based on nitrogen dielectric barrier discharge was characterized by optical emission spectroscopy. The two most abundant signals were the transitions of NO (γ system) and N2 (2nd positive system) and in low abundance the transition of N2+ (1st negative system) and O2+ (Schuhmann band) was obtained. It is known that N2+ is the reactive species for producing (H2O)H3O+ or SH+, which are mostly responsible for the protonation of the analytes (MH+). However, the lower abundance of N2+ was obtained in nitrogen plasma than in helium, which is due to the different ionization pathway. Nevertheless, the sensitivity of the ionization was similar for both discharges, since different geometries to the MS and sample introduction into the plasma was used. The nitrogen plasma ignites as irregular filaments between the inner electrode and the glass, which was confirmed by using an iCCD camera. Analytes can therefore travel through the plasma-free regions inside the capillary without fragmentation.

Conclusions
The properties of the filaments were studied by monitoring the transitions of NO (γ system) and N2 (2nd positive system) when applying different plasma parameters (voltage, frequency, humidity, etc.). Characterization of the plasma not only helps to improve the understanding of the ionization mechanism but also allows to optimize the performance of the plasma source when coupled with the mass spectrometer.

Novel Aspect:
This is the first report on spectroscopic characterization of the active capillary plasma ionization source which is used as a soft ionization source coupled to MS.

References
A targeted metabolomics approach for diagnosis of Inborn Errors of Metabolism

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Introduction:

Metabolome, the end product of the genome, can be studied through identification and quantification of small molecules. The global metabolome influences the individual phenotype through clinical and environmental interventions.(1, 2) Metabolomics has become an integral part of clinical research and allowed for another dimension of better understanding of disease pathophysiology. The clinical biochemistry laboratory routine workload (>95%) is based on small molecular identification, which can potentially be analyzed and discovered through metabolomics.(3, 4) However, multiple challenges in clinical metabolomics impact the entire workflow and data quality, thus the biological interpretation needs to be standardized for a reproducible outcome. The existing Mass spectrometry-based Newborn Screening assay has false discovery rate (FDR) that can be minimized once linearly integrated with other markers.

Methods:

Herein, we introduce the establishment of a comprehensive targeted metabolomics method for a panel of 225 clinically relevant metabolites using Liquid chromatography-tandem mass spectrometry (LC-MS/MS) standardized for clinical research. The sensitivity, reproducibility and molecular stability of each targeted metabolite (amino acids, organic acids, acylcarnitines, sugars, bile acids, neurotransmitters, polyamines, and hormones) were assessed under multiple experimental conditions. The metabolic tissue distribution was determined in various rat organs. Furthermore, the method was validated in dry blood spot (DBS), the standard sample type for newborn screening, collected from patients known to have various inborn errors of metabolism (IEMs).

Results:

The targeted LC-MSMS-based metabolomics method was developed for quantitation purpose, and the validity of this method was studied by following the United States Food and Drug Administration (USFDA) guideline.(5) This approach appears to be sensitive (LLOQ for 2-Ketobutyric acid is 1 nM) and robust (CV% ranges from 80-120%). The metabolic chemical stability and extraction recovery were evaluated in this approach using several biological matrices to control the method robustness in the routine analysis. The sensitivity of the method demonstrated differential and unique expression profiles in various rat tissues, where the overall metabolic expression varied, but was highest in the kidney tissue (35%) compared to the liver (25%), brain (17%), heart (15%) and muscle tissue (8%) for the same amount of tissue. Several optimizations and studies have been done on DBS samples such as relative
metabolic expression compared to whole blood and serum, the normal cutoff for each expressed metabolite in this panel, and the differential expression compared to patient samples. 56 patient samples with known diagnosis were analyzed using this platform for clinical validation. This method was capable to produce the same diagnosis as in the routine NBS platform using the same key markers, for instance, it gives the Phenylalanine/tyrosine ratio high in PKU patients compared to the others. Additionally, several other metabolites were found to be differentially expressed in these patients compared to control samples such as betaine, succinic acid, niacinamide, fructose 1, 6-bisphosphate. Several sugars (i.e. arabinose, ribulose) were overexpressed mainly in the disorders of mitochondrial metabolism (VLCAD and GA2 deficiency) compared to controls. In MMA (n=6) and PA (n=2) patients displayed distinct metabolomics profiles with minimal overlap including hyperglycinemia and related elevated glutamine levels. Interestingly, spermine appears to be markedly reduced in MMA compared to PA suggesting a preferential toxicity to the synthetic polyamine pathway in such patients.

Conclusions:

There is a strong clinical need to develop and validate quantitative methods capable of measuring the largest number of metabolites subject to wide and rapid dynamic changes present in a biological system. In the present study, we demonstrated the effect of sample matrix on the expression of profiles of a large set of clinically oriented metabolites which may help to guide the choice of the optimal sample type and matrix in which it is collected. We have also shown the capability of this assay to detect common inborn errors of metabolism. In addition to its role as a second tier assay, this targeted panel is also potentially useful as a screening tool for a large group of inborn errors of metabolism

Novel Aspect:

This panel has the ability to identify patients with IEMs and discover novel markers that minimize the assay FDR.

Reference list:

STERIOD BISCONJUGATES: UNEARTHING HIDDEN TREASURES OF STEROID METABOLISM

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Keywords: steroid bisconjugate, molecular di-anion, stable isotope label, MS fragmentation, ion loss,

Introduction:
Doubly or bisconjugated steroid metabolites have been known for decades and typically studied by indirect GC-MS.

Methods: Recently, the direct detection of steroid bis(sulfates) by LC-MS has been applied to doping in sport [1] and the prenatal diagnosis of inborn errors of steroid biosynthesis [2]. Access to labelled compounds for MS would increase our understanding of these fascinating compounds.

Methods:
General methods of chemical synthesis were developed to prepare stable isotope 18O-labelled sulfate esters and 13C-labelled glucuronides, including selectively labelled steroid bis(sulfate) and bisglucuronide compounds for use as MS probes or internal standards. The ionisation and fragmentation of these reference materials have been studied in detail on a triple quadrupole mass spectrometer.

Results:
Stable isotope 18O-labelled sulfate esters and 13C-labelled glucuronides were prepared from commercially available {18O4}-sulfuric acid (96 atom %) and {13C6}-D-glucose (>99 atom %). High levels of incorporation were observed with the conjugates suitable for use as MS probes or stable isotope labelled internal standards. Selectively labelled steroid bis(sulfate) and bisglucuronide compounds were used to study MS fragmentation pathways. Steroid bis(sulfate) compounds preferentially ionized as the doubly deprotonated molecule ([M-2H]2-) and this precursor typically underwent ion loss of the HSO4- ion (m/z 97) to afford fragment ions of higher m/z ([M-2H-HSO4]-). Other fragmentation processes including ion loss of •SO3- (m/z 80) and HSO3- (m/z 81), neutral loss of SO3 (40 Da) and ion molecule “roaming” were observed and correlated with analyte structure.

Conclusions:
Access to stable isotope labeled sulfate and glucuronide conjugates has unveiled the rich MS fragmentation behaviors of steroid bisconjugates. This knowledge paves the way for the development of new LC-MS approaches for the direct and untargeted detection and identification of these metabolites.

Novel Aspect:
Synthesis of stable isotope labelled sulfate and glucuronide conjugates, selectively labelled bisconjugates and their use in MS fragmentation studies.

References:
INVESTIGATING THE REACTION MECHANISM OF THE OXIDATION OF HEPTAFULVENES INTO TROPONES BY A MICROREACTOR COUPLED ONLINE TO MASS SPECTROMETRY AND DENSITY FUNCTIONAL THEORY CALCULATIONS

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Keywords: Reaction monitoring, density functional theory, microreactor, online, reaction mechanism

Introduction:
Understanding reaction mechanisms is crucial in studies of chemical reactions. When knowing the mechanism, reaction conditions can be designed to maximize the yield of desired product(s). Microreactors coupled online to mass spectrometry are ideal for experimental reaction mechanism studies[1],[2], while density functional theory calculations are well-established for theoretical studies.

Methods:
A 3D printed polypropylene microreactor with an integrated ESI needle[3] was interfaced with an ion trap mass spectrometer for online analysis of the oxidation of heptafulvenes to tropones. Density functional theory calculations were done with the software Gaussian 09, using the M06-2X functional and a 6-311++G(d,p) basis set to identify the most favored reaction pathway.

Results:
Online mass spectrometry allowed observation of the heptafulvene starting material, two key intermediates of the oxidation reaction and the tropone product as protonated molecules in positive mode ESI. MSn analysis of these species, as well as how their intensity change in relation to each other depending on the reaction time, support the proposed identifications. Based on the mass spectrometric results six competing reaction pathways were studied with density functional theory. The lowest energy reaction pathway, which thus is the most likely one, proceeds via initial formation of an epoxide, which is opened upon the addition of a second equivalent of the oxidizing species meta-chloroperoxybenzoic acid. The adduct then undergoes a Criegee-like rearrangement to produce a positively charged hemiketal, which upon deprotonation dissociates into acetone and the tropone.

Conclusions:
The most likely reaction mechanism for oxidizing heptafulvenes to tropones has been identified by experimental and theoretical investigations. These results are a good basis for optimizing reaction conditions to improve yields of the promising anti-cancer compound tropone[4]. Furthermore, these results highlight the usefulness of combining experiments and theory for studying reaction mechanisms.

Novel Aspect:
The synergy of combining results from a microreactor coupled to mass spectrometry and computational chemistry to find a plausible reaction mechanism.

References
HOW TO MODIFY THE ACIDITY OF CHARGED DROPLETS?

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Keywords: aerosols, electrospray, ionization efficiency, protonation, surface chemistry

Introduction:
The concept of acidity in confined spaces has been poorly understood, especially in media violating electroneutrality. Here [1] we present a study on the acidity of charged droplets to fill the gap in knowledge. We studied the effect of pH and different additives on the acidity of charged water and methanol droplets via protonation of nitrogen bases in electrospray. As a result, the work gives insight into how to modify the acidity of charged droplets.

Methods:
The protonation of 10 nitrogen bases was studied using ESI/MS. The extent of protonation (which is related to the acidity of charged droplets) was calculated using ionization efficiency values. To alter the acidity of charged droplets produced in ESI, 28 solvent compositions were used in water (pH_{H2O}=1.42–10.29) and 14 in methanol (pH_{MeOH}=3.63–12.24). The solvent composition was varied using 10 additives in different concentrations and combinations.

Results:
Generally, we observed that the ionization efficiencies of most analytes are pH dependent in specific pH range in both water and methanol. However, wrong-way-round ionization occurs in both solutions. Also, the extent of protonation decreases sharply in the pH_{H2O} range from 2 to 4 for all compounds, even though pK_{aH2O} values of these compounds range from 2.7 to 9.0. Close investigation of the pH range of 2 to 4 revealed that additive type affects the acidity of the droplets more than pH in aqueous solutions, enhancing the extent of protonation of the compounds when only acidic additives are present compared to droplets of the same pH containing also ammonium salts. Thus, we propose that in case of acidic additives, the excess charge on the droplet surface is a result of protonated acid and water molecules and, therefore, enhances ionization efficiency of compounds significantly. The effect of additives is less influential in case of methanol droplets, as the excess charge is situated in the interior of the droplets.

Conclusions:
The results show that the concept of acidity in solution phase cannot be transferred to charged droplets. We demonstrated that the acidity of charged water droplets depends on the additive; which means that the extent of protonation can effectively be modified by changing additive type even if the pH is kept constant. The opposite is true for charged methanol droplets where the extent of protonation can be adjusted with the conventional approach of changing the pH.

Novel Aspect:
While the protonation in charged methanol droplets depends on the solution-phase acidity, the protonation in charged water droplets can easily be modified with additives.

References:
Studies of a L-proline catalyzed inverse electron demand Diels-Alder reaction by ESI-MS

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Keywords:
Charge-tagging; electrospray ionization mass spectrometry; L-proline; organocatalysis; reaction mechanism

Introduction:
The synthetic scope of the L-proline catalyzed inverse electron demand Diels-Alder reaction between tetrazines and ketones has been published by Xie et al.[1] in 2008 alongside a postulated catalytic cycle. The postulated catalytic cycle includes three intermediates. First an enamine I derived from L-Proline and a ketone, which undergoes the Diels Alder reaction with the tetrazine forming II which loses nitrogen in a retro Diels-Alder reaction forming III. [1]

Methods:
Samples of the reaction solution were analyzed by ESI-MS and CID experiments. A charge-tagged substrate and a catalyst were utilized in different experiment setups as to enhance the ESI response factors of the respective intermediates.

Results:
While studying the reaction without implementing a charge tag into any of the participating species the third intermediate III could be detected and kinetic studies could be obtained. The charge tagged tetrazine was synthesized in hopes of detecting the second intermediate II as well, but to no avail; however again kinetic studies could be obtained. By using the charge tagged L-proline derivative from our group[2] as a catalyst all three intermediates could be detected and characterized by CID experiments. Within the CID experiments it was possible to mimic the reaction steps of the catalytic cycle.

Conclusions:
It was possible to thoroughly verify the catalytic cycle for the L-proline catalyzed inverse electron demand Diels-Alder reaction between acetone and 3,6-di-2-pyridyl-1,2,4,5-tetrazine.

Novel Aspect:
The superior effectiveness of utilizing a charge-tagged catalyst in ESI-MS studies of catalytic reactions could be demonstrated.

References:
AMINO-ACIDS OXIDATION: A COMBINED STUDY OF CYSTEINE OXO-FORMS BY MASS SPECTROMETRY, IRMPD SPECTROSCOPY AND SIMULATIONS

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Keywords:
IRMPD spectroscopy; cysteine oxo-forms; collisional induced dissociation; ab initio molecular dynamics simulations; chemical dynamics simulations;

Introduction: (Limit of 400 characters)
Cysteine residues in sulfenic (-SOH), sulfinic (-SO2H) and sulfonic (-SO3H) forms are key intermediates in the redox-switch chemistry of proteins [1]. Although sulfenic acids have been identified in several biochemical frameworks, they are transient and not isolable in small molecular systems. Low-energy collision-induced dissociation of L-Cysteine sulfate affords L-cysteine sulfenic acid.

Methods: (Limit of 400 characters)
Chemical dynamics simulations [2] have been employed to shed light on fragmentation mechanisms of L-Cysteine sulfate. A combined approach based on infrared multiple photon dissociation spectroscopy, calculations of IR frequencies and finite temperature ab initio molecular dynamics simulations has been employed to characterize the gas-phase structures of deprotonated cysteine oxo-forms.

Results: (Limit 900 characters)
IRMPD spectra of cysteine oxo-forms have been recorded in the highly informative mid-IR spectral range employing a Paul ion-trap mass spectrometer coupled to the IR beamline of the tunable free-electron laser at the CLIO facility in Orsay [3]. These ions show different structural motifs owing to the preferential binding of the proton to either the carboxylate or the sulfur-containing group. Due to the decreasing basicity of the sulfenic, sulfinic and sulfonic terminals, the proton is bound to SO- in [cysSO]-, migrates on the carboxylate in [cysSO3]-, while it turns out to be shared in [cysSO2]-. Evidence is gathered that a mixture of close-lying low-energy conformers is sampled for each cysteine oxo-form in a Paul ion-trap at room temperature [4]. An exotic roaming mechanism has been evidenced in the formation of L-cysteine sulfenic acid with other competing pathways.

Conclusions (Limit of 400 characters)
The spectroscopic signatures of the oxidative modifications enable the assignment of the deprotonation site and conformational landscape of the sampled [CysSOx] (x= 1,2,3) oxo-forms and may provide diagnostic probes to reveal critical intermediates in protein redox regulation.

Novel Aspect: (Limit of 150 characters)
The type of mass spectrometry instrument employed for collisional activation of L-Cysteine sulfate resulted to be “pathway” selective.


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311 - LOSS OF WATER FROM POLYGLYCINES AND THEIR [BN]+ IONS; IMIDAZOLONES AND OXAZOLONES

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Keywords: Loss of water; imidazolone; oxazolone; [bn]+ ions; interconversion between isomers

Introduction:
Loss of water in the fragmentation of protonated tetruglycine is mainly from the peptide backbone, resulting in [b4]+ ions with imidazolone structures. These [b4]+ ions have identical CID spectra indicating isomerization prior to dissociation. Here, the fragmentations of larger protonated polyglycines [Glyn + H]+ and their [bn]+ ions (n=5–10) are examined. Isomerization of the [bn]+ ions and their fragmentation mechanisms are studied.

Methods:
All experiments were carried out using an Orbitrap Elite Hybrid Ion Trap-Orbitrap mass spectrometer. Isotopically labeled peptides were synthesized and used to determine the structures of the fragment ions. DFT calculations at B3LYP/6-311++G(d,p) level provided reaction energetics and insight into the dissociation mechanisms.

Results:
18O-labeling experiment shows that loss of water in the fragmentation of protonated pentaglycines are predominantly from the first and second amide groups. Unlike the [b4]+ ion derived from tetruglycines, the [b5]+ ions show similar CID spectra but with different relative abundances. This suggests that the [b5]+ ions undergo fragmentations more easily than isomerize. This conclusion is supported by DFT calculations that give the energy barrier for the isomerization to be higher than that to fragmentation. Hexaglycinoloses water from the first three amide groups. The [b6]+ ions derived from the water loss from the first and second amide oxygens shows similar fragmentation patterns to the corresponding [b5]+ ions. However, further water loss from the peptide backbone is found from the [b6]+ ion that was formed when water was lost initially from the third amide group.

Conclusions
The [b4]+ ions from tetruglycine possess imidazolone structures and undergo isomerization prior to fragmentation. However, the larger [bn]+ ions have different fragmentation patterns indicating that the barriers to isomerization is high. These [bn]+ ions still feature imidazolone structures, and there is no evidence for oxazolone structures.

Novel Aspect:
Loss of water from protonated polyglycines are mainly from the backbone. With the increase of the peptide chain length, multiple water loss is observed. (164/150 characters)
Introduction
Arsenic-containing chemical warfare agents were used in World War I and World War II. Large part of the Axis stockpiles were destroyed after the World War II by burying in soil and dumping in sea. The environmental effect of these actions are still seen around the world. Mass spectrometry is widely used to analyze these chemicals and their degradation products. Arsenic atom induces a large number of rearrangement reactions.

Methods
The fragmentation pathways of selected organoarsenic chemicals were studies using gas chromatography-electron ionization/high resolution mass spectrometry (GC–EI/HRMS) and liquid chromatography-electrospray ionization/high resolution mass spectrometry (LC–ESI/HRMS). The latter instrument can produce also MSn/HRMS data using quadrupole, ion trap and Orbitrap analyzers. The spectra were carefully analyzed to identify fragmentation pathways.

Results
Some characteristic fragmentation pathways were identified based on the mass spectra. Arsenic atom induce rearrangement reactions, which lead formation of large number of cyclic fragmentation products. The chemicals discussed in this paper will include intact warfare agents, which are typically chlorine-containing chemicals, their degradation and oxidation products as well as some alkylthiol derivatives required for GC-based analyses.

For most of the chemicals there are several fragments, where neutral arsenic species are lost from the ionized molecule. One of the most exotic rearrangements is the formation of a phenyl cation from tris(2-chlorovinyl)arsine (“Lewisite I”). It is worth noting that in LC–ESI/MS, diphenylarsenic chemicals are producing highly characteristic radical cations, e.g. C12H10+•, C12H8+• and C6H5As+•. The current paper will present the suggested fragmentation pathways and highlight common ions formed from phenyl and diphenylarsenic chemicals.

Conclusions
Organoarsenic chemicals are an interesting group of chemicals, which fragment in unconventional ways to produce characteristic fragments. These fragment ions can be identified and then used for search of novel chemicals possibly forming in the environment. Several novel chemicals have been tentatively identified using these fragments in sediment samples taken in CHEMSEA, MODUM and DAIMON projects from dump sites in the Baltic Sea.

Novel Aspect
Novel chemicals have been detected in environmental samples. Their spectra have been analyzed using data obtained from other phenyl and diphenyl arsenic chemicals.
Kinetics of a Click Reaction in Charged Microdroplets with Time Resolved Reactive Laser Ablation Electrospray Ionization Mass Spectrometry

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Keywords: cycloaddition • kinetics • laser ablation electrospray ionization • reactive intermediates • time resolved mass spectrometry

Introduction:
Time resolved mass spectrometry (TRMS) provides insights into fundamental processes in (bio)molecular reactions, such as the direct detection of short-lived intermediates and the acquisition of kinetic data. Laser ablation based injection coupled to electrospray ionization (LAESI) can be used as a novel TRMS method to perform reactions in charged microdroplets, e.g., to accelerate synthesis or to study the chemistry of life.

Methods:
A Protea Biosciences LAESI DP-1000 system was electronically connected to a Waters Synapt G2-S time of flight mass spectrometer (MS). The distance between LAESI ion source and MS inlet was variable between 1 and 100 cm. Transport of ions from the ionization source to the MS inlet was assisted by a nebulizer gas via a grounded metal tube. Temporal resolution was obtained by the difference in time between the pulsed laser pulse (injection) and MS detection of product.

Results:
We demonstrate a reactive LAESI-TRMS method with the study of a cycloaddition reaction between dipyridyl-tetrazine and an amine derivative of bicyclononyne (BCN-amine) in charged microdroplets. In this reaction, a highly strained bicyclic reaction intermediate is formed before release of molecular nitrogen to obtain the final reaction product. For the first time, this short-lived intermediate was detected by a direct physical method. In addition, the ratio intermediate to product could be regulated by controlling the temperature of the MS source (thermal energy to overcome the low, intermediate to product, activation barrier). Kinetic data was obtained with the use of temporal resolution, which was demonstrated in the 1 to 500 ms time range. Interestingly, no drop was detected in product absolute intensity over a distance up to 100 cm (0.5s). Finally, reactive LAESI mass spectrometry imaging (MSI) experiments were conducted on an image containing dipyridyl-tetrazine solution.

Conclusions:
We have developed a novel method for the determination of reaction kinetics and the analysis of short-lived intermediates in charged microdroplets. We detected the reaction intermediate of a cycloaddition reaction and were able to obtain the pseudo-first order rate constant. Additionally, we demonstrated our methodology in a reactive LAESI-MSI experiment.

Novel Aspects:
Coupling of laser ablation injection with ESI-MS to study reactions in microdroplets with temporal resolution, and demonstrating reactive LAESI-MS(I) for the first time.
ACETONE AS PHOTO-INITIATOR FOR DISULFIDE REDUCTION – APPLICATION TO STRUCTURAL ANALYSIS OF DISULFIDE RICH PEPTIDES AND PROTEINS

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None, Tsinghua University/Chemistry, Beijing (1) - None, Purdue University/Chemistry, West Lafayette (2)

Keywords: Photo-Initiator, Radical Reaction, Disulfide Peptide, Tandem Mass Spectrometry, Proteomics

Introduction:
Pinpointing disulfide linkage pattern is necessary for characterizing proteins containing disulfide bonds. Often carefully controlled enzymatic digestion and multi-step partial disulfide reduction are needed before MS analysis to achieve confident assignment. Herein, we describe a new method based on coupling radical initiated disulfide bond cleavage with tandem mass spectrometry for rapid and detailed structural analysis of disulfide-rich peptides.

Methods:
A flow microreactor consisted of a 254 nm low-pressure mercury lamp (BHK, Inc., Ontario, CA) in parallel to UV-transparent fused silica capillary (100 μm i.d., 375 μm o.d) at 0.5 cm distance. The peptide/protein solution was infused to the reactor by a syringe pump and subjected to photochemical reactions before online electrospray ionization (ESI)-MS analysis. All data were collected on QTRAP 4500 and X500R QTOF mass spectrometers (SCIEX, Concord, ON, Canada).

Results:
Acetone (1% volume ratio) was used as photo-initiator (PI) to induce subsequent radical reactions. Upon 254 nm UV irradiation, acetone underwent Norrish type I cleavage, producing acetyl radical and methyl radical [1]. The presence of alkyl alcohol as co-solvent in the peptide solution converted the primary radicals into hydroxyalkyl radicals, which subsequently cleaved disulfide bond via substitution [2]. Disulfide reduction was the dominant reaction channel when using isopropyl alcohol, which completed in less than 5 s with >95% yield. More importantly, partial reduction of disulfide bonds could be achieved simply by shortening the reaction time. For structural characterization of disulfide-rich peptides/proteins, sequence information and disulfide bond connecting pattern could be obtained from coupling complete or partial disulfide reduction with MS/MS. The analytical utility of this method was demonstrated with peptides/proteins containing 2 to 4 disulfide bonds.

Conclusions:
Disulfides are highly reactive toward radical attack; however, uncontrolled radical reactions often lead to disulfide bond scrambling, which is undesirable for disulfide linkage pattern determination. In this work we demonstrated that controlled disulfide cleavage via hydroxyalkyl radical could be achieved. Furthermore, this reaction can be directly coupled with ESI-MS/MS analysis, allowing characterization of disulfide rich peptides.

Novel Aspect:
Photochemical reduction of disulfide bond in peptides and proteins and its online coupling with ESI-MS/MS for disulfide linkage pattern determination.

References:
232 - IDENTIFICATION AND INTERCONVERSION OF ISOMERIC 4,5-FUNCTIONALIZED 1,2,3-THIADIAZOLES AND 1,2,3-TRIAZoles IN CONDITIONS OF ELECTROSPRAY IONIZATION

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Keywords: HRMS, electrospray ionization, 1,2,3-thiadiazoles, 1,2,3-triazoles, isomer identification, Orbitrap

Introduction:
Investigation of 1,2,3-triazoles and 1,2,3-thiadiazoles has a great biological potential. However the problem of isomerisation of 4,5-functionalized 1,2,3-thiadiazoles and 1,2,3-triazoles in the presence of an acid or a base at room temperature makes it difficult to identify each isomer. The present study deals with ESI-MS application in positive and negative modes to identify each isomer by CID and HCD mass spectra and reproduce the rearrangement processes.

Methods:
All experiments were performed with Orbitrap Elite mass-spectrometer (Thermo Fisher Scientific, USA) with electrospray ionization source. The acetonitrile solution of each compound was introduced through syringe pump directly into the ion source at 5 μl/min. High resolution mass spectra were acquired both in positive and negative modes. MS/MS experiments were carried out using both CID and HCD fragmentation techniques at different energies.

Results:
The monomolecular transformations under (±)ESI-MS conditions of 4,5-functionalized 1,2,3-thiadiazoles and 1,2,3-triazoles in the gas phase were investigated varying the amino- and sulfanyl moieties. Fragmentation pattern of each isomer was carefully studied by scanning CID and HCD energies in positive and negative modes. It was demonstrated that basic fragmentation process in case of protonated 1,2,3-thiadiazoles involves the loss of N2 molecule. All other fragment ions in MS2 spectra originate from the primary [MH-N2]+ ion. Unlike 1,2,3-thiadiazoles, MH+ ions of 1,2,3-triazoles demonstrate a richer fragmentation pathway without losing N2 molecule. The main fragmentation process of MH+ ion involves the loss of sulfinylaryl/sulfinylalkyl or sulfonylaryl/sulfonylalkyl radical. Appearance of [MH-N2]+ ion in the spectra of 1,2,3-triazoles was proved to form due to the gas phase rearrangement during the ESI-MS analysis. The connection of all precursor and product ions was established in MS3 experiments.

Conclusions
Analysis of (±)ESI-MS/MS mass spectra of the isomeric 4,5-functionalized 1,2,3-triazoles and 1,2,3-thiadiazoles with HRMS allowed identifying each isomer. Moreover, only 1,2,3-triazoles undergo transformation into the corresponding 1,2,3-thiadiazoles under (±)ESI-MS conditions, thus mimicking the reaction in the condensed phase. Alkyl substituent next to the sulfone group in both isomers leads to new fragmentation pathways.

Novel Aspect:
1,2,3-Triazoles and 1,2,3-thiadiazoles have been differentiated using (±)ESI-MS/MS method. 1,2,3-Triazoles were shown to rearrange during the (+)ESI-MS analysis.
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Keywords: histone tail, ion mobility mass spectrometry, theoretical structural calculation

Introduction:
Nucleosome core particle (NCP) is the basic structural unit of nucleosomes and composed of four histone proteins and ca. 150 bp DNA strand. The behavior of histone tails in nucleosomes with a variety of lengths of DNA was investigated using nanoESI-MS and nanoESI-IM-MS. The MS data were evaluated by comparing the structures generated by computational calculation in solution and gas phases.

Methods:
Positive-mode nanoESI-MS and nanoESI-IM-MS data were obtained using a Waters Synapt G2 HDMS mass spectrometer. Nucleosomes were prepared with different lengths of DNA strands, 147, 250, or 342 bp DNA. Samples for MSexperiments were prepared with ammonium acetate solutions. Possible structures of NCP were investigated by theoretical calculation.

Results:
We found that the charge-state distributions of the nucleosomes were classified into two groups, narrow and wide, which was supported by IM-MS. By systematic analysis with ESI-MS in the positive-ion mode, it was suggested that the range of charge state of nucleosomes is related to the length of free DNA region. When the DNA strand has a region that is not involved in the NCP core structural formation, charge-state distributions of nucleosomes are narrow. In contrast, when the extra region of DNA does not exist, the charge-state distribution is widely spread. This suggests that histone tail regions contribute to generate structural diversity of nucleosomes in the absence of free DNA region. This was also supported by theoretical structural calculation of NCP.

Conclusions:
When eukaryotic chromatin is viewed under an electron microscope, NCPs are linked via histone-free DNA strands. Although further study is required, our findings that the free DNA regions affects the histone tails’ behavior may provide biological insight into the regulation mechanism of eukaryotic transcription initiation.

Novel Aspect:
It was suggested that flexibility of histone tail regions is affected by the existence of free DNA regions. [1]

References:
1. Saikusa k. et al., submitted.
Introduction:
Mass spectrometry (MS) coupled with electrospray ionisation (ESI) has been extensively applied to identify proteins and elucidate stoichiometry of protein complexes. Since desolvated species are affected by solvent conditions such as pH, buffer strength and concentration, ESI-MS is an appropriate method by which to consider the range of conformational states that proteins may occupy including natively folded, disordered, denatured and amyloid.

Methods:
Rotationally averaged collision cross sections of the ionised forms of proteins, provided by the combination of mass spectrometry and ion mobility (IM-MS), are measured and used to explore conformational landscapes in the absence of solvent. Measurements are made at a range of temperatures, on two model proteins as well as on permutants of the highly disordered protein p27.

Results: We present a novel method of monitoring the effect of charge location on structures, on unfolding transitions and the stability of transient intermediates using a variable temperature ion mobility mass spectrometer (VT IM-MS)[1]capable of measurements from 150-500K. We show with VT IM-MS, that for both ubiquitin and fully oxidised lysozyme, transient intermediate structures can be detected upon activation followed by trapping and ion mobility separation at cryogenic temperatures. We show that for permutants of p27 the location of charged groups has a dramatic influence on the structures adopted in the gas phase, and how this compares with solution phase measurements and predictions. We have obtained data providing direct evidence that restructuring of the compact states of ubiquitin and lysozyme proceeds through extremely diverse intermediate forms. In addition, our results indicate that intermediates are initially highly elongated and subsequently converge to compacted structures.

Conclusions
The processes of elongation and subsequent compaction indicates quasi-reversible folding transitions. The influence of charge location on protein structure is strong with implications for gas phase measurements. The presented novel data obtained on well studied “model” systems highlights the potential of utilizing this methodology in studying protein folding in the gas phase.

Novel Aspect:
VT-IM-MS on model proteins; consideration of the effects of sequence on stability.

References

Hydrogen-deuterium exchange (HDx) associated with mass spectrometry (MS) is emerging as a powerful tool to provide conformational information about membrane proteins [1]. Unfortunately, as for X-ray and NMR, HDx performed on reconstituted in vitro systems might not reflect the in vivo environment. We took advantage of the outer-membrane vesicles (OMVs) naturally released by E. coli to carry out conformational and dynamic analysis of native OmpF through HDx-MS.

Methods: A new protocol compatible with HDx analysis that avoids hindrance from the lipid contents was setup. Briefly OMVs purified from E. coli ΔompA were precipitated with TCA and washed with ice-cold acetone. In order to minimize the back exchange, all sample preparation steps have been shortened as much as possible. Each step required less than 5 s and was followed by centrifugation for 4 min at 4 °C.

Results: The extent of deuterium incorporation was in good agreement with the X-ray diffraction data of OmpF [2] as the buried β-barrels incorporated a low amount of deuterium, whereas the internal loop L3 and the external loops incorporated a higher amount of deuterium. Moreover, the kinetics of incorporation clearly highlights that peptides segregate well in two distinct groups based exclusively on a trimeric organization of OmpF in the membrane: peptides presenting fast kinetics of labeling are facing the complex surrounding environment, whereas those presenting slow kinetics are located in the buried core of the trimer.

Conclusions: The work presented here clearly demonstrates that HDx-MS is a versatile technique that can provide information on solvent accessibility and spatial arrangement of major bacterial outer-membrane proteins. This study was possible by taking advantage of the OMVs and by developing a protocol that allows the removal of most of the lipid content while limiting the back exchange.

Novel Aspect:
Gaining information on accessibility and dynamics of antigens in native conditions will be of pivotal importance for the future rational design of efficient vaccines.

References
Kefala G.; Ahn C.; Krupa M.; Esquivies L.; Maslennikov I.; Kwiatkowski W.; Choe S. Protein Sci. 2010, 19(5), 1117-25
HDX-MS REVEALS CONFORMATIONAL CHANGES OF IL-23 IN COMPLEX WITH IL-23R/IL-12RB1 AND PHAGE DISPLAY PEPTIDES

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Keywords: Interleukin IL-23, HDX-MS, computational approaches, binding interactions, phage display peptides

Introduction:
Interleukin IL-23 is a pro-inflammatory cytokine that contributes to the pathogenesis of autoimmune inflammatory diseases. The inhibition of the activity of IL-23 has promising therapeutic effects for psoriasis, psoriatic arthritis, inflammatory bowel disease, rheumatoid arthritis and multiple sclerosis.[1] IL-23 is a heterodimer composed of the cytokine subunits p19 and p40 that bind to the receptor complex IL-23R and IL-12Rβ1 respectively. The discovery of monoclonal antibodies designed to target IL-23 (either p19 and/or p40) have shown significant efficacy in human studies for some of these diseases. Here we present the application of HDX-MS [2] and computational approaches to interrogate the binding interactions of IL-23 with its receptors and the binding epitope of new IL-23 inhibitors such as phage display peptides [3].

Methods:
The exchange reaction was performed with a Tecan liquid handler modified to allow for freezing of samples into Millitubes arrayed in a 96 well plate format. Thus, samples were prepared triplicate of the following time course; Dmin, 10s, 30s, 90s, 270s, 810s, 2430s, 7290s and Dmax. The injection and sample thawing was performed in a robotic module equipped with an universal robotics UR5 arm designed to present samples in series to a LEAP CTC-PAL system in a refrigerated cabinet. Samples were thawed and injected into the injection port following digestion. The resulting proteolytic peptide mixtures were desalted and separated on a C18 analytical column and detected by mass spectrometry. HDX-MS data treatments were performed with HDX-Workbench software. [4-5]

Results:
Computational studies permitted the generation of a canonical model of the IL-23 receptor complex. To validate such model and to gain deeper insights of the interactions (dynamic view of conformational changes) between a protein and its corresponding protein partner in solution, HDX-MS experiments were performed. Thus, the proteins were exposed to D2O buffer for different periods of time to measure the deuterium incorporation in IL-23, IL-23/IL-23R, IL-23/IL-12Rβ1 and IL-23/IL-23R/IL12Rβ1 complexes. The HDX analysis of the Apo protein revealed that both subunits (p19 and p40) were dynamic in solution. Helices A and C and the A-B, B-C and C-D loops exhibited the highest deuterium uptake while helices B and D were identified as the less flexible regions (solvent protected) in this subunit. While some parts of domains D1-D3 in p40 exhibited faster and higher deuterium uptake, other regions in these domains showed less deuterium exchange, consequently suggesting more rigid structures. The HDX data of the IL-23/IL-23R complex indicated protection across helices A-D which allowed confirmation of the contact interactions of IL-23p19 when mixed with IL-23R. In a similar way, differential HDX analysis of IL-23 in the presence and absence of IL-12Rβ1 revealed protection from exchange in mainly some parts of domains D1 and D3 of IL-23p40. The interactions of the cytokine with its partner receptors were confirmed with the HDX results in the full trimeric complex IL-23/IL-23R/IL-12Rβ1.

Our results revealed that HDX-MS was also able to identify the binding epitope of phage display peptides previously identified as inhibitors of IL-23. The characterization of the interactions of these chemotypes by HDX-MS will also be presented.

Conclusions
In conclusion, our HDX-MS and computational analyses revealed valuable information about the interactions of IL-23 and its cognate receptors IL-23R and IL-12Rβ1. These studies provide new insights into the quaternary structure of this protein-protein interaction in solution and allowed us to discover novel anti-IL-23 peptides from phage display approach. The binding activity of these peptides was confirmed by biophysical techniques meanwhile the binding epitope against IL-23 was measured by HDX-MS.
To our knowledge, this is the first structural evidence of the interactions of the full trimeric complex IL-23/IL-23R/IL-12Rβ1. In addition, we confirmed by HDX-MS the characterization of the binding mode of macrocyclic small molecule to IL-23 for the first time. Indeed, this new chemotype can be used as foundation for discovery and characterization of small molecules targeting this cytokine and its receptors.

Novel Aspect:
HDX-MS and computational approaches to probe the binding interactions of IL-23 with its receptors and phage display peptides.

References
Introduction
The interpretation of ion mobility spectrometry-mass spectrometry (IMS-MS) data from large biomolecules relies on molecular dynamics (MD) simulations to model their putative structures. With the goal of enabling millisecond simulations approaching typical IMS-MS timescales, we explored possible coarse-grained (CG) schemes to replace overly expensive all-atom frameworks, while still meeting the level of detail necessary for proper structural elucidation.

Methods
Selected nucleic acid structures were modeled by using 2-, 5-, and 7-bead representations, and then submitted to MD simulations in GROMACS by using the Martini force-field. The collisional cross section (CCS) algorithms contained in MOBCAL were reparametrized according to a previously reported procedure to accommodate the pseudoatoms included in our CG frameworks, thus enabling the seamless calculation of theoretical CCS values directly from CG models [1].

Results
This report evaluated the merits of employing CG models to capture the global dynamics of large biomolecules, which involve the conformational rearrangement of entire domains completed in the ms range typical of IMS-MS determinations. The advantages of this approach were demonstrated by performing test MD simulations that provided a 0.009 s/ns wall time for a CG model versus 680 s/ns for the corresponding all-atom version, equivalent to 0.00025 hr versus 18 hr of computing time per 100 ns of simulation. Reparametrizing the MOBCAL algorithms provided the means for obtaining a full-fledge CG workflow to enable model building, MD simulations, and CCS calculations with no need for all-atom representations. This approach allowed us to test the possibility of employing the reparametrization process to correct for gas-phase effects on biomolecular structure, which are not adequately addressed in the leading force-fields, such as the apparent compaction of structures forced to establish self-solvating interactions in a solvent-free environment.

Conclusions
For large biomolecules, the resolution and timescale of IMS-MS analysis thwart the dissection of local dynamics involving side-chain/nucleobase displacement occurring in the ns timescale, whereas they enable the recognition of global dynamics in the ms range, which may produce distinguishable conformers. Therefore, CG approaches provide an excellent solution for completing MD simulations in the proper IMS-MS scale to enable their experimental observation.

Novel Aspect
Direct application of coarse-graining computational approaches to support the analysis of the structure/dynamics of large biomolecules by IMS-MS.

References
PROBING THE STRUCTURES OF NATIVE AND PATHOLOGICAL A-SYNUCLEIN SPECIES BY HYDROGEN/DEUTERIUM EXCHANGE MASS SPECTROMETRY

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Keywords: intrinsically disordered protein, alpha-synuclein, in-cell, H/D exchange, protein structural dynamics

Introduction:
It is well established that purified monomeric α-synuclein is an intrinsically disordered protein, but its structure in the cellular environment has been a matter of intense scientific controversy [1-3] fueled by reports that α-synuclein adopts a stable folded tetramer in cells, but only when they are intact.[4-7] This controversy is mainly due to the inherent difficulties associated with probing protein structures inside the cell.

Methods:
We here present a novel approach to probe the intracellular structure of endogenous proteins and we address the question whether the cellular structure of endogenous α-synuclein is folded or disordered. Our approach is based on in-cell hydrogen/deuterium exchange where α-synuclein is labeled in its cellular environment with D2O followed by immunoaffinity purification at acidic conditions (in-cell-HDX-IPAC) where the isotopic exchange is quenched.

Results:
Recombinant 15N-labeled monomeric α-synuclein was added to human erythrocytes prior to isotopic exchange as an intrinsically disordered extracellular reference protein. This allows for sensitive detection of protection of intracellular endogenous α-synuclein caused by any putative folded stable structure. To ensure that the equilibration of D2O across the plasma membrane of erythrocytes occurs sufficiently rapid, we determined the deuterium uptake of intracellular and extracellular hemoglobin and it was found to be identical. To enable in-cell HDX-MS analysis of endogenous α-synuclein, an effective enrichment of the protein at quench conditions is required. For this purpose, we developed a novel immunoaffinity approach (in-cell-HDX-IPAC) that maintains strong capture efficiency at acidic conditions (pH 3.5) to limit the amount of back exchange. The enriched α-synuclein was eluted at pH 2.0. Our results show that the deuterium uptake of intracellular and extracellular α-synuclein is identical.

Conclusions
The identical deuterium uptake of intracellular and extracellular α-synuclein suggests that the vast majority of endogenous α-synuclein in erythrocytes is intrinsically disordered. Furthermore, we have also shown that our in-cell-HDX-IPAC approach is able to detect the protection pattern of the presence of nonnative α-synuclein oligomers.[8,9] In addition, we have investigated the stability and dynamics of nonnative α-synuclein oligomers.

Novel Aspect:
In-cell H/D exchange followed by immunoaffinity purification at acidic conditions (in-cell-HDX-IPAC) is utilized to probe the intracellular structure of α-synuclein.

References


Mapping Conformational Dynamics to Binding Modes in TEM-1 \(\beta\)-Lactamase Using Millisecond Scale ESI-MS

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ATTN: Division of Mass Spectrometry Awards Committee

I am a third year graduate (Ph.D.) student in the Chemistry lab of Dr. Derek Wilson at York University. I am writing to you to request your monetary assistance to facilitate my travel to Florence, Italy to present my work at the International Mass Spectrometry Conference. To date in my graduate studies, I have submitted a paper to the Journal of the American Chemical Society (2018), published in Photochemical and Photobiological Sciences (2017) and coauthored a JoVE report (2017). In May 2017, I presented a poster in Sweden for the International Conference on Hydrogen Deuterium Exchange (IC HDX) for which I received the award for best mini presentation. More recently, last August I presented my work at the Trent (Lake Couchiching) Conference on Mass Spectrometry on a national travel award. Below, I provide an expanded abstract of my research Mapping Conformational Dynamics to Binding Modes in TEM-1 \(\beta\)-lactamase Using Millisecond Scale ESI-MS that I hope will interest you and lead you to granting my request for a travel award to attend the 2018 IMSC this August.

Keywords: catalysis, antimicrobial resistance, enzymes, HDX, \(\beta\)-lactamase

Introduction:
Current studies contribute to the work on antibiotic resistance by studying the enzymatic digestion of antibiotic drug families and mapping drug potency based on dynamic differences in drug binding modes. The induced fit model of enzyme function highlights the critical role of dynamics in substrate binding. However, there has been little evidence to support the notion that individual dynamic modes are required for catalytic processes after initial binding.

Methods:
Tandem mass spectrometry (MS/MS) is utilized to identify peptides after proteolytic digest within a chip. Without MS/MS, column chromatography would be used to identify the eluted peptides, leaving the experiment vulnerable to back exchange and unreliable dynamic information. Experiments are performed on a Synapt G2 Time-of-Flight instrument with Ion Mobility Separation (IMS). Microfluidic devices are constructed in house using a VersaLaser and machine drill.

Results:
By enabling deuterium uptake to be monitored on a millisecond timescale, we greatly enhance the manner that catalytic activity can be observed under native conditions(1). We use HDX-MS to identify dynamic modes linked to catalytic processes in the antibiotic resistant enzyme TEM-1 \(\beta\)-lactamase(2-4). Using a very active substrate (ampicillin), a poorly decylated substrate (cephalexin) and an off-pathway inhibitor (clavulanate), we isolate dynamics that are linked to 3 processes: substrate binding; lactam ring hydrolysis; and, deacylation. These separations translate to leads for dynamics-targeted antibiotic development, such as at the allosteric binding site at the C-terminal helix, H11 (5,6) and the link between known mutational sites and increased conformational dynamics upon drug binding(7-9). We propose that an inner \(\beta\)-strand, residues 245-257, is directly responsible for increased resistance to cephalosporin drugs(10) and should be conserved through a co-inhibitor to increase the potency of current on the market drugs.
Conclusions:
Our results identify dynamic modes that are uniquely linked to specific processes in the catalytic (or inhibitory) mechanism. In each case, these links could be rationalized in terms of what was known about the role of particular residues or structural elements within the identified region, supporting the reliability of the approach as a means to isolate catalytically important dynamics and to reveal new submolecular targets for drug design.

Novel Aspect:
Microfluidics HDX on the millisecond timescale is unlike traditional HDX that utilizes chromatography and operates on a minute to hours timescale.

Thank you very much for your consideration of my request for your financial support to present my research and to attend the conference to further my own development as a scientist.

Sincerely,
Ruth Knox

References
Introduction: 400/400
Structural stability plays an integral role in regulating protein function and activity. Stability and protein-protein binding interactions can be regulated by post-translational modifications that affect subunit binding affinities of protein complexes. Here, we use ion mobility spectrometry-mass spectrometry (IMS-MS) to explore how oxidative modifications affect subunit binding affinity and conformational stability of the hemoglobin tetramer complex.

Methods: 397/400
IMS-MS experiments were performed using a Waters SYNAPT G2 mass spectrometer. Hemoglobin solution was dialyzed against 150 mM NH4OAc (pH 7.4), and diluted to 15 µM [tetramer] for analysis. Protein solution temperature was controlled using a custom-fabricated heated nanoelectrospray device, in which the temperature of the analyte solution and nESI emitter can be heated. Collision cross sections (CCS) were estimated using the protocol described by Ruotolo et al.[1]

Results: 900/900
Unfolding and dissociation of the native hemoglobin tetramer is induced by incrementally increasing the solution temperature in the nESI source. Shifts in the average charge state, and CCS of the $\alpha_2\beta_2$ tetramer arise with increasing solution temperature, leading to dissociation into $\alpha\beta$ dimers, as well as populations of both holo and apo monomers. Stabilities of each oligomeric state and conformer are determined based on their midpoint dissociation temperature (Td). The $\alpha_2\beta_2$ holotetramer has a Td = 58 °C, while the $\alpha\beta$ holodimer has a Td = 62 °C, indicating the dimer is more stable at elevated temperatures relative to the tetramer. A subpopulation of $\alpha\beta$ dimers exists where one of the monomer units is in the apo state ($\alpha_h\beta_a$). A significant portion of this subpopulation shows evidence of an oxidation PTM at $\beta$Cys93. $\beta$Cys93 has been identified as a “hotspot” for oxidation, as well as having implications on $\beta$-subunit heme loss due to its proximity to the heme binding pocket.[2] The oxidized species has a midpoint Td = 57 °C, while the unmodified species has a Td = 61 °C.

Conclusions 393/400
The Td of the oxidized $\alpha_h\beta_a$ dimer species is 5 °C lower than the holodimer, indicating that $\beta$Cys93 oxidation has a considerable destabilizing effect on the $\alpha/\beta$ subunit interface, even more so than the loss of the heme group. In addition to decreasing the subunit binding affinity, the oxidized species favor structures with more compact CCS at increased temperature, suggesting a structural collapse or compaction may be involved indestabilizing the dimeric units.

Novel Aspect: 120/150
Thermal stability analysis by IMS-MS reveals conformational destabilization induced by oxidative stress in hemoglobin tetramer complex.

References
Introduction

Micropollutant emissions from industrial wastewater are poorly understood. Challenges arise from missing substance information due to confidentiality of production data. Moreover, short-term emissions from industrial batch processes are unlikely captured by conventional grab sampling. Hence, we developed a strategy for comprehensive identification of potential industrial peak emissions from treated wastewater to surface water.

Methods

Daily effluent samples (24-h composite) were collected for three months at two wastewater treatment plants (WWTPs) receiving different proportions of industrial wastewater. The samples were then analyzed by large volume direct injection LC-HRMS (orbitrap technology) in positive and negative ESI mode. Data processing followed a non-target approach using the freely available enviMass workflow [1] to extract time profiles of all detected signals from full scan spectra.

Results

After data cleaning and cross-sample componentization (isotopologue and adduct grouping), more than 20,000 and 60,000 time profiles of a priori unknown compounds were detected at the two WWTPs. More profiles were detected at the WWTP treating larger amounts of industrial wastewater. Based on observed time profile characteristics of compounds with known origin we developed a workflow to differentiate between continuous domestic emission and potential industrial peak emission profiles. Of the considered descriptors, profile intensity spread was the most conclusive, i.e. values for peak emissions were twice as high as for domestic emissions. The percentage of profiles finally classified as potential industrial peak emissions differed substantially between the two investigated WWTPs, being 3-fold higher for the plant receiving more industrial wastewater. For several of the observed peak emissions the underlying compound was identified and the emission was further assigned to a pharmaceutical production site in the catchment of the WWTP.

Conclusions

Our strategy of source attribution based on time profile characteristics proved to correctly identify industrial peak emissions and accurately predicted a higher share of peak emission profiles for the WWTP treating larger volumes of industrial wastewater. This approach is currently being applied to a national field study to estimate the contribution of industrial discharges on the overall micropollutant contamination of Swiss surface waters via WWTPs.

Novel Aspect

This study presents a non-target approach for systematic inspection of entire HRMS full scan time series datasets to identify potential industrial peak emissions.

References

Transformation products (TPs) are formed in the water cycle through biological and technological processes. Despite their potentially altered toxicity compared to parent compounds, TPs formed by drinking water treatment are not routinely monitored and remain elusive. This is mainly due to the technical challenges in analyzing the often unknown, low concentration compounds. Their analysis requires non-target HRMS/MS methods and novel data analysis approaches.

Methods:
Here, we performed lab scale experiments to monitor TP formation of the three organic micropollutants carbamazepine, clofibric acid and metolachlor during rapid sand filtration and ozonation, two readily applied biotic and abiotic drinking water treatments, respectively. TP identification from non-target data was facilitated through prediction of potential TPs based on literature and models, halogenated and/or isotopically labeled parent compounds.

Results:
The experimental results showed that the degradation of parent compounds did not per se lead to mineralization of the compound, but rather to an abundance of TPs, in often low concentrations. Some of these TPs were bigger and less polar than their parent compounds, which was somewhat unexpected. The identification of peaks representing TPs was straightforward and semi-automatic with the developed workflow, based on statistical testing and peak area filters. The suspect screening based on TP suspect lists manually curated from literature mining and prediction tools was efficient for TPs in the lists. However, the majority of TPs identified did not match suspect list entries. Furthermore, the structural identification of these features, as well as of isobaric suspects remained labor and time intensive.

Conclusions
The majority of TPs remained structurally unidentified, and for the majority of identified TPs toxicological risk assessment was missing. Follow-up work should target and hopefully alleviate these issues. Finally, the developed workflow can be applied to pilot-scale experiments to allow TP monitoring in actual drinking water production.

Novel Aspect:
develop and test an efficient workflow to monitor TP formation and identify drinking water treatment specific TPs on a lab-scale
For information please contact: scientific@ims2018.it
Introduction:
The oil spills occurred at the end of the Gulf War is one of the serious environmental problem in history. Although 30 years have gone after the gulf war, the world is still concerned about the environmental impact of spilled oil. It is important to characterize and understand the chemical compositions of oil spills for effective cleaning of the oil spill area. In this study, extracts from contaminated soil in oil spill area were analyzed using 2DGC and FT-ICR MS.

Methods:
Two soil samples were collected each between 15~20cm below the surface (top soil) and about 1m below the surface (bottom soil). The soil samples were extracted using sonication in hexane and acetone. Soil extracts were filtered and dried. The extract and oil samples were further diluted with solvent for FT-ICR MS. For 2DGC analysis, Rtx-5MS (30m x 0.25mm, 0.25μm) is used as the first non-polar column and Rxi-17sil-MS (1.3m x 0.15mm, 0.15μm) as the second polar column.

Results:
Class distributions obtained by (+) APPI, ESI and LDI MS did not show significant difference between the soil extracts and non-degraded crude oils. On the other hand, (-) ESI showed a noticeable difference. Increased abundance of O2 class was observed from the top soil extract. Relative abundance of O2 class compounds with DBE 2 or 3 were most abundant in the top soil extracts. And the A/Cratio(acyclic/∑(2–4 ring cyclic) O2 species) of topsoil extract was the lowest.[1] In addition, when the soil samples were extracted with water, a significant number of polar components were detected only in the topsoil extract. About 300 peaks were observed from 2DGC MS data obtained from soil extracts and oil. Volatile alkane peaks were disappeared and only aromatic compounds were observed from soil extracts.

Conclusions:
Comprehensive analyses of soil extracts and oil were done by use of 2D GC and FT-ICR MS. The most of the light compounds in the soil were evaporated because of hot weather in Kuwait. And the top soil extract showed significant abundance of oxidized peaks. However, it was surprising to find that extensive oxidation has not occurred in the bottom soil. This study shows that chemical compositions are different in contaminated soils located at different depth from the surface.

Novel Aspect:
This is the first study to analyze contaminated soils from spilled oil during the Gulf War using 2DGC and Ultra-High resolution MS.

References
OCCURRENCE OF ORGANOPHOSPHATE FLAME RETARDANTS IN SWIMMING POOLS AND THEIR ENDOCRINE DISRUPTING POTENTIAL

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Keywords: Organophosphate flame retardants, Endocrine disrupting activity, In vitro bioassays

Introduction:
The use of organophosphate flame retardants (OPFRs) has been increasing because of the restriction and phase out of brominated flame retardants. Environmental contamination of OPFRs is of growing concern since they have been reported to show various toxic effects, such as carcinogenicity, neurotoxicity, and endocrine disrupting activity [1-4]. The aim of this study was to investigate the occurrence of OPFRs in swimming pools and their potential health effects.

Methods:
Swimming pool water samples were separated into dissolved phase (W) and suspended solids phase (SS) by filtration. W and SS samples were extracted by solid phase extraction and Soxhlet extraction, respectively. Concentrations of selected OPFRs were quantified using an isotope-dilution liquid chromatography-tandem mass spectrometry (LC-MS/MS) method. Endocrine disrupting activities of OPFRs and pool samples were investigated using in vitro bioassays [5].

Results:
LC-MS/MS results revealed that 7 and 6 OPFRs selected for analysis were detected in W and SS samples, respectively, and the detection frequencies ranged from 65 to 100%. Tris(1-chloro-2-propyl) phosphate was the dominant OPFR in W samples, which was the major OPFR found in Australian public swimming pools as well [6]. Tri-n-butyl phosphate was the dominant OPFR in SS samples, and its concentrations were about 3 orders higher than other OPFRs. Bioassay results showed that selected OPFRs exhibited antagonist activities for androgen receptor (AR), glucocorticoid receptor (GR), and progesterone receptor (PR). AR and GR antagonist activities were also frequently found in W samples collected from southern Taiwan. By contrast, no AR antagonist activities were found in SS samples, and PR agonist/antagonist activities were not detected in W or SS samples as well.

Conclusions:
The present study demonstrated that OPFRs were ubiquitously present in W and SS samples collected from Taiwanese swimming pools. Also, OPFRs exhibited AR, GR, and PR antagonist activities, suggesting potential health risks of OPFR exposure from swimming pool water. Further investigation will be undertaken to evaluate the contribution of OPFRs to the receptor antagonist activities detected in pool samples.

Novel Aspect:
Trace concentrations of OPFRs in Taiwanese swimming pools were successfully analyzed for the first time by using the isotope-dilution LC-MS/MS method.

References:
Keywords: Ultra-High Pressure Liquid Chromatography Quadrupole Time of Flight (UHPLC/MS-QTOF), di-2-ethylhexyl phthalate (DEHP), bisphenol A (BPA), Gas Chromatography-Mass Spectrometry (GCMS)

Introduction

BPA and DEHP are endocrine disruptors (ED) widely used as plasticizers and associated with the development of endocrine and metabolic diseases. In the LIFE PERSUADED project, we developed UHPLC/MS-QTOF and GCMS methods to monitor exposition to BPA and DEHP (by the evaluation of metabolites MEHP, MEHHP, MEOHP) in 900 healthy pairs mother-child living in rural and urban areas from North to South Italy. Questionnaires on lifestyle and nutrition were recorded [1-3].

Methods

Urine samples (500 µl) were treated with enzymatic deconjugation to hydrolyze the glucuronide and separate free phthalates and BPA. Compounds with 13C standard were purified with C18SPE. DEHP metabolites were quantified by UHPLC/MS-QTOF (ZORBAX SB Phenyl 2.1x100mm 1.8 µm); the sample was then derivatized with BSTFA 1% TMS and acetonitrile and BPA was quantified by GCMS in selective ion monitoring (J&WDB-5MS30m, 0.25mm, 0.25µm) and normalized by creatinine.

Results

We were able to quantify DEHP metabolites in all urine samples. Preliminary data in 350 children (4-14 years, males, M and females, F) and mothers (Mo) showed that the range were: MEHP 1.3-187.5, 1.49-117.26 and 1.9-51.8; MEHHP 0.74-1502.68, 0.0052-953.75 and 2.69-165.3; MEOHP 0.82-303.82, 1.27-827.03 and 0.024-51.96 µg/g of creatinine (respectively in F, M vs Mo).

BPA analyses by UHPLC/MS-QTOF were affected by a matrix effect that was evident only in deconjugated samples. For this reason, after UHPLC/MS-QTOF analysis samples were dried and derivatized with BSTFA to be analyzed in GCMS. Total BPA range was 0.69-1036.55 µg/g of creatinine (F) and 0.951-1146.786 µg/g of creatinine (M) in children and 0.84-43.78 µg/g of creatinine in their mothers.

Internal exposure was different according to living area, age (young children showed higher values), with gender differences. Data evaluation of questionnaires and food diaries is in progress and will be integrated with ED exposure data to provide the determinants to be used in risk assessment.

Conclusions

Although not persistent, BPA and DEHP metabolites have been detected in all enrolled children, with different exposure was different according to living area, age (lower range), with gender differences.

Novel Aspect

We set up a method to quantify DEHP metabolites by UHPLC/MS-QTOF and BPA concentration by GCMS starting from 500 µl of urine.
References


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Keywords: Time-of-flight mass spectrometry; duloxetine; transformation products, in silico toxicity prediction.

Introduction
This study aimed to elucidate the transformation products (TPs) of duloxetine (DUL), a serotonin–norepinephrine reuptake inhibitor, under controlled experimental conditions, to simulate processes that can occur in the environment and WWTP: photodegradation under UV irradiation and chlorination. Moreover, the ecotoxicity of the transformation products was predicted based on in silico tools [1].

Methods
The experiments were carried out on spiked distilled water. The chlorination consisted of adding sodium hypochlorite in the solution and for the photodegradation an UV irradiation source was used (450 W medium-pressure mercury-vapor lamp). The time profiles of formation/degradation of the transformation products were analyzed. Samples were directly injected in the LC-QTOF/MS system in full-scan and MRM mode.

Results
The parent compound completely degraded in the photodegradation experiments after 30 minutes of UV irradiation whereas the chlorination process accounted for about 90% of its removal. On the whole, eleven TPs were formed, nine from the contact with UV light and two from the chlorine reactions. These TPs were identified and their structures elucidated by analysis of their fragmentation patterns. Their formation has been attributed to, namely, oxidation, hydrolysis and dehydrogenation reactions. All TPs formed were totally degraded in both experiments: photodegradation in 4 hours and chlorination in 1 day. In comparison with DUL all TPs presented low ecotoxicity with the endpoints LC50 F. minnow, D. magna and T. pyriformis from ECOSAR tool, and the quantitative structure–activity relationship (QSAR-TEST) analysis for mutagenicity resulted in five TPs with Ames-positive prediction.

Conclusions
Our experiments showed that duloxetine reacts with chlorine and under UV irradiation, producing transformation products, the structures of which were elucidated by analysis of their fragmentation patterns obtained using a LC-QTOF/MS equipment. These experiments also showed that the TPs and the parent compound degraded in both processes. Moreover, the in silico analysis enabled to conclude that some TPs presented mutagenic potential.

Novel Aspect
Duloxetine is frequently detected in effluents, but little is known about its TPs. This study enables to throw some light on the behavior of this compound in the environment.

References

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Introduction:
Triple quadrupole mass spectrometry is well suited for targeted quantitative analysis of large numbers of compounds including pesticides, which can belong to a wide range of chemical classes and span a wide range of volatilities. Different pesticides tend to respond to a variety of ionization and separation techniques, such as LC-ESI, LC-APCI, GC-EI, etc. It is advantageous to support analysis of as many of these compounds as possible on one hardware platform.

Methods:
A test suite of pesticides spanning different chemical classes and volatilities was selected for analysis using a single hardware platform consisting of a QSight 300 triple quadrupole mass spectrometer fitted with a versatile dual ion source capable of analyzing LC and GC amenable compounds using a variety of ambient ionization techniques. Methods were developed to demonstrate highly sensitive analysis of diverse pesticides using a common hardware platform.

Results:
Using a highly sensitive QSight 300 triple quadrupole mass spectrometer equipped with a versatile dual ion source it was possible to efficiently and quantitatively analyze both LC and GC amenable pesticides, including difficult to ionize compounds such as chlordane or pentachloronitrobenzene. Multiple strategies are possible. One approach is to use HPLC to separate all compounds. The output of the LC was connected by T or switching valve to both probes on the dual ion source which was fitted with one LC-ESI and one LC-APCI probe. Traditionally difficult to ionize compounds, often analyzed by GC, can respond to APCI. Fast mode switching between the ESI and APCI probes was used to analyze both LC and GC amenable compounds using a single method, and within a single injection. The dual probe ion source can also be coupled to two separation devices, e.g. two LC’s, or one LC, one GC, allowing for additional versatility.

Conclusions
A QSight 300 triple quadrupole mass spectrometer equipped with a versatile dual probe source was used to demonstrate efficient, highly sensitive, quantitative analysis of both LC and GC amenable pesticides using a single hardware platform.

Novel Aspect:
Simultaneous analysis of both LC and GC amenable pesticides using a single hardware platform.

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Keywords: resonance enhanced multiphoton ionization, membrane introduction, mass spectrometry, (poly)aromatic hydrocarbons, marine environment

Introduction:
Fast and sensitive analysis of (poly)aromatic hydrocarbons (PAH) is of high importance due to their impact on human health and the environment. However, very low concentrations of PAHs in marine environments complicate their detection and monitoring. A promising approach, regarding to direct determination of PAHs in marine systems, is resonance-enhanced multiphoton ionization (REMPI) coupled to membrane introduction mass spectrometry (MIMS).

Methods:
In MIMS analytes are extracted from the water phase into the vacuum of the MS by using semipermeable membranes. No laborious enrichment techniques are needed. With REMPI ions are formed by the subsequent absorption of two photons via an excited molecular state. The unique ionization scheme of REMPI provides selective and sensitive detection of PAHs. For the analysis of water samples, a REMPI-MIMS system with external inlets for sheet or hollow fiber membranes was built.

Results:
In this proof of principle study we present measurements of selected small (poly)aromatic species in different simulated (tap water, artificial sea water) and real world water samples. Both inlet designs show promising results with respect to the fast and sensitive determination of small PAHs. For a hollow fiber membrane setup, the trap-and-release technique can be utilized for direct determination of small (poly)aromatic species in water samples down to ppb range in less than 5 minutes. Here, the pervaporating compounds are trapped inside of the membrane tube for a certain time and are released by fast heating of the membrane as a sharp peak into the MS. By using a sheet membrane assembly fast response times and low limits of detection were obtained for toluene, p-xylene and naphthalene. Hereby, the membrane is heated indirectly by tempering the inflowing water. With increasing water temperature improvement of response time and signal heights can be achieved. For both inlet designs limits of detection can be improved by using high repetition lasers.

Conclusion:
REMPI-MIMS is a powerful technique for the direct measurements of PAHs in aquatic environments. With this early-stage system concentrations down to the mid-ppb range for selected small (poly)aromatic compounds are easily accessible in minutes without any sample preparation.

Novel Aspect:
Hyphenating external inlets with REMPI-MS is utilized for the first time. Additionally, we present the first measurements of real world water samples with REMPI-MIMS.
MULTICLASS ANALYSIS OF ANTIBIOTICS IN SEDIMENTS BY LC-Q-ORBITRAP HRMS. APPLICATION TO THE NERA RIVER.

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Keywords: veterinary drugs, sediments, liquid chromatography-high resolution mass spectrometry, Nera River

Introduction: (Limit of 400 characters)
Environmental research showed that pharmaceuticals from wastewater treatment plants, industries and hospitals can contaminate waters, sediments, aquatic flora and fauna [1]. Aquaculture plants also play an important role in this contamination. This study focused on the analysis of 58 antibiotics in sediments of Nera river (Umbria, Italy), an area in which several trout farms are present.

Methods: (Limit of 400 characters)
Sample preparation protocol consisted in three consecutive extraction steps followed by a tandem SPE clean-up (Oasis HLB + Strata-X-C). The LC-Q-Orbitrap conditions were the same described in our previous work [2]. The validation study has been performed at 1 and 10 µg/kg dry weight (dw). Samples from Nera River were collected monthly at three sites from March to December 2017.

Results: (Limit 900 characters)
The optimized sample treatment involved three extractions with mixtures at different pHs (7, 3 and 10, respectively) to take out the wide range of substances included in the method scope: 58 analytes belonging to ten different antibiotic classes widely used in veterinary practices. After validation, the sediments collected from Nera River were analyzed (30 samples). Two tetracyclines (oxytetracycline and tetracycline), three quinolones (ciprofloxacin, flumequine and oxolinic acid) and trimethoprim were measured. In particular, oxytetracycline (and its epimer) was detected in the 100% of the samples with concentrations from 2 to 159 µg/kg dw. The other drugs were always lower than 10 µg/kg dw and their detection frequencies ranging from 3% (tetracycline) to 73 % (ciprofloxacin).

Conclusions (Limit of 400 characters)
The results revealed the presence of drugs used in trout farming, but other antibiotics were from other sources (ciprofloxacin and oxolinic acid). Nera River collected at the same time [3]. On the other hand, it is well known that the sediment composition favors the adsorption of only certain drugs.

Novel Aspect: (Limit of 150 characters)
This is the first survey on the presence of antibiotics in the Nera River sediments.

References
Giusepponi D., Moretti S., Saluti G., Scoppetta F., Capuccella M., Galarini R., Book of Abstract - 8th Symposium on Recent Advances in Food Analysis, p. 405 - 7-11 November 2017 - Prague (CZ).
DETERMINATION OF 16 POLYCYCLIC AROMATIC HYDROCARBONS BY CORE-SHELL MOLECULARLY IMPRINTED POLYMERS PREPARED BY REVERSIBLE ADDITION FRAGMENTATION CHAIN TRANSFER POLYMERIZATION WITH APGC-MS/MS

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Keywords: Magnetic molecularly imprinted polymers; dispersive solid phase extraction; atmospheric pressure chemical ionization gas chromatography; reversible addition fragmentation chain transfer polymerization

Introduction:
Polycyclic aromatic hydrocarbons (PAHs) with carcinogenic and mutagenic properties are considered priority pollutants by regulatory agencies world-wide. Liquid–liquid extraction and solid phase extraction (SPE) are non-selective techniques influencing the sensitivity, accuracy and precision of MS analysis [1]. Molecularly imprinted polymers (MIPs) as selective sorbents can improve the efficiency, and reproducibility, and reduce matrix effects [2].

Methods:
MIP particles were prepared by surface polymerization on magnetic Fe3O4@SiO2 nanoparticles through reversible addition fragmentation chain transfer (RAFT) [3]. The RAFT-MIPs were used for dispersive SPE (dSPE) of 16 PAHs from aqueous matrices, then isolated by magnetic collection. The analytes were desorbed into organic solvent prior to quantitation with atmospheric pressure chemical ionization gas chromatography tandem mass spectrometry (APGC-MS/MS).

Results:
To obtain maximum performance by the RAFT-MIPs, the composition of polymeric coating as well as the parameters influencing extraction efficiency for enrichment of PAHs were assessed and optimized. The synthesized particles were also analyzed using XRD and IR measurements to confirm formation of selective binding sites through surface polymerization onto core-shell nanoparticles. Under optimized conditions (15 mg RAFT-MIPs, 40-mL sample volume, 1 min ultrasonic dispersion extraction time, 1 mL desorption solvent and 10 min desorption time), RAFT-MIPs were used for selective extraction of 16 PAHs from water samples. Good recognition properties (IF between 1.7 and 2.7) for extraction of PAHs in water samples were obtained without matrix manipulation, such as filtration or removal of interferents. The developed method with good detection limits (ng/L levels) and high precision (RSD<8%) provides a sensitive and reliable procedure for analysis of PAHs in any complex matrix.

Conclusions:
RAFT polymerization, as a controllable technique relying on living radical initiators, was used to create cavities with accessible and homogenous binding sites for the target molecules. The RAFT-MIPs yielded a rapid, selective and efficient procedure for extraction of PAHs from water samples. Indeed, application of a hydrophilic comonomer enhancing the water compatibility of magnetic MIP makes the sorbent suitable for the analysis in aqueous matrices.

Novel Aspect:
Water compatible magnetic RAFT-MIPs were produced for rapid, selective and simultaneous extraction of 16 PAHs with reduced interferences for instrumental analysis.

References
FATE AND BEHAVIOR OF OIL SANDS NAPHTHENIC ACIDS IN A PILOT-SCALE TREATMENT WETLAND AS CHARACTERIZED BY NEGATIVE-ION ELECTROSPRAY IONIZATION ORBITRAP MASS SPECTROMETRY.

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Keywords:
Double bond equivalent
Naphthenic acids
Constructed wetland
Kinetic analysis
Orbitrap-MS

Introduction:
Oil sand process-affected water (OSPW) is a complex mixture containing naphthenic acids (NAs) as the primary acid component [1, 2]. High-resolution Orbitrap mass spectrometer is well suited for the characterization of complex samples and is useful for monitoring the changes in distribution of OSPW-NAs during treatment. In this work, the effectiveness of constructed wetland treatment (CWT) in the transformation of naphthenic acid fraction components (NAFCs) was evaluated in a non-aerated wetland design using high-resolution Orbitrap-MS.

Methods:
NAs transformation in non-aerated CWT was assessed using high-resolution Orbitrap-MS technique. The NAs from aqueous wetland outflow samples were extracted using WAX SPE method described previously [3]. Class distributions were established with acquired accurate mass data and Composer version 1.5.3 with mass accuracies of less than 2 ppm. In addition, kinetic and statistical analysis was performed with SigmaPlot software.

Results:
The distributions of NAs in the untreated and CWT were examined for selectivity based on the changes in carbon number numbers and double bond equivalents (DBE). The results demonstrated that the non-aerated CWT preferentially transformed O2-NAs with larger carbon numbers than small carbon number NAs. The oxidized NAFCs (O3-NAFCs and O4-NAFCs) were more recalcitrant towards transformation relative to O2-NAs in the wetland system. Furthermore, kinetic data indicated that rate of transformation of O2-NAs with larger carbon numbers was faster than small carbon number NA species and there was no direct correlation between the increased transformation rate and size of the DBE observed in the treatment system.

Conclusions
The non-aerated wetland treatment system was effective for treatment of OSPW-NAFCs and favors the transformation of higher carbon number over small carbon number species. This suggests that preferential removal of NA species may be related to design of the system. Work is ongoing to compare with other wetland designs such as aerated wetlands to determine whether these designs have impacts on their selective transformation.

Novel Aspect:
The present study reported herein is the first study to evaluate the feasibility of OSPW-NAFCs transformation in constructed wetland treatment (CWT) using high-resolution negative-ion electrospray Orbitrap-MS for detailed understanding of their fates and behavior in the CWT.

References:
Keywords: Photocatalysis; Titania; Visible light; Organic Pollutants; Electrospray.

Introduction:
Some metal oxides, such as titanium dioxide (TiO2), can be employed as photocatalysts because of their ability to promote oxidation of organic molecules when activated by UV-light [1] due to its wide band gap. In this work, we showed via ESI(-)-MS that thin mesoporous films of TiO2 nanoparticles can degrade hazardous environmental pollutants, as 2-naphtol [2], under visible light incidence.

Methods:
Three identical aqueous solutions of 2-naphtol (photosensitive substance) and ammonium hexafluorophosphate (internal standard) were prepared. To one of them, a glass plate was immersed (control), and into the others two plates of TiO2 films were dived. One of the tubes containing titania was kept in the dark, and the other two tubes were maintained under incidence of blue LED light.

Results:
To conduct a quantitative experiment, NH4PF6 was employed as an internal standard for the analysis. This compound was chosen because it easily ionizes in aqueous solution generating anions that are stable and inert under these experimental conditions.
The photodegradation rate was carried by periodically sampling a small aliquot of the solutions in contact with the glass plates every 60 minutes for 6 hours. The decay rate of 2-naphtol was controlled by the ratio of the intensities of the [2-naphtol-H]- to the PF6-over time.
It was observed that TiO2 films can easily promote photooxidation of 2-naphtol under incidence of visible light and in the presence of oxygen gas by showing an almost complete photodegradation after about 6 hours of reaction. The systems that were set up with TiO2 film in the dark and with the bare glass under blue light did not demonstrate any kind of degradation.

Conclusions:
TiO2 films can photodegrade organic pollutants, as 2-naphtol, in the absence of UV radiation and under incidence of visible light. A quantitative MS technique was developed and NH4PF6 was used as internal standard. This procedure worked well for evaluating photodegradation and allows the degradation products to be detected.

Novel Aspect:
The use of ammonium hexafluorophosphate as an internal standard and the determination that visible light can induce photocatalysis in TiO2 films.

References:
Study on the degradation mechanism of atrazine in sewage by UHPLC-MS/MS

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Keywords: degradation mechanism, atrazine removal, nanoparticles, electrochemical reaction, UHPLC-MS/MS

Introduction:

CuFe2O4 (CFO) magnetic nanoparticles, worked as both particle electrode and catalyst for persulfate (PS), are applied to the three-dimensional electrochemical processes for atrazine removal. In this study, the effects of several key parameters, such as the content of Na2SO4 electrolyte, CuFe2O4, persulfate, other inorganic anions, the initial pH and current density on atrazine removal were investigated. In addition, the degradation intermediates were determined by UHPLC-MS/MS. Based on the identified intermediates, the degradation pathway or mechanisms for atrazine in the CFO/PS system were confirmed. Finally, the possible electrochemical reaction mechanism of the CFO/PS system was proposed according to the comprehensive study.

Methods:

The degradation intermediates of atrazine were determined by coupling PerkinElmer UHPLC to a QSightTM 220 mass spectrometer. A C18 column was used for separation with gradient elution at a flow rate of 0.3 mL/min, with mobile phases consisted of acetonitrile (A) and pure water containing 0.1% formic acid (B). The mass spectra with electrospray ionization (ESI) source were recorded across the range of 50-300 m/z with the positive scan mode and MRM mode. The operation parameters were as follows: drying gas of 100, HSID temperature of 250 °C, nebulizer gas of 200, electrospray voltage of +5500V, and source temperature of 450 °C.

Results: (Limit 900 characters without spaces)

In this electrochemical reaction process, CFO magnetic nanoparticles have been successfully applied as a catalyst for the activation of persulfate, exhibiting excellent performance for atrazine removal in CFO/PS system after 35 min treatment. The optimal operating conditions (i.e., CFO dosage of 3.0 g/L, PS dosage of 4.0 mM, initial pH of 6.3 and treatment time of 35 min) were obtained by investigating the effects of key parameters on the atrazine removal. The high-efficiency of the CFO/PS for atrazine removal was confirmed through seven control experiments. It was found that CFO particles retained high activity after four excessive cycles, demonstrating the stability and recyclability of CFO in the studied system. The comparative study of the freshly prepared and reacted CFO also confirmed the stability of CFO. In this study, the sulfate radical was identified as the main reactive radical in the atrazine degradation process. In addition, the degradation mechanism of atrazine was ascertained according to the 12
degradation intermediates detected by UHPLC-MSMS. Finally, the possible electrochemical reaction mechanism of CFO/PS system was proposed according to the aforementioned analysis.

Conclusions (Limit of 400 characters without spaces)

In this study, CFO magnetic nanoparticles have been successfully applied as a catalyst for the activation of persulfate, exhibiting excellent performance for atrazine removal in CFO/PS system. The high-efficiency of the CFO/PS for atrazine removal was confirmed. It was found that CFO particles retained high activity after four excessive cycles, demonstrating the stability and recyclability of CFO in the studied system. The sulfate radical was identified as the main reactive radical in the atrazine degradation process and the degradation mechanism of atrazine was ascertained according to the 12 degradation intermediates detected by UHPLC-MSMS.

Novel Aspect: (Limit of 150 characters without spaces)

The degradation mechanism of atrazine was ascertained according to the 12 degradation intermediates determined by UHPLC-MSMS.

References
None
Keywords: suspect screening, high resolution mass spectrometry, pesticide metabolites, groundwater

Introduction:
Groundwater contributes to 80% of drinking water supply in Switzerland [1]. However, various contaminant sources such as pesticide application in agriculture pose risks to groundwater quality. Only for few pesticide metabolites reference standards are commercially available so that groundwater quality is partly unknown. Thus, high resolution mass spectrometry combined with a screening of exact masses of potential metabolites is a possibility to detect new contaminants.

Methods:
31 groundwater samples were enriched via water evaporation, and analyzed with liquid chromatography coupled with electrospray ionization to high resolution tandem mass spectrometry (LC-ESI-HRMS/MS). The HRMS data were searched for the exact masses of more than 1000 pesticide metabolites, most of them observed within the European pesticide registration process [2, 3], using Compound Discoverer (Thermo Scientific).

Results:
After background and target removal 8400 compounds were annotated as suspects in positive and negative ionization mode. Despite optimization of the parameter settings in Compound Discoverer using target compounds, a high number of compounds were grouped insufficiently across samples and noise peaks were not fully removed. Therefore, visual inspection was required. The remaining 590 compounds, which showed a signal/noise >10, were checked for plausibility based on isotope pattern, retention time and in silico fragmentation with MetFrag [4]. The pesticide producers provided reference standards for 28 metabolites originating from 16 pesticides. So far, 11 compounds have been confirmed; 4 out of the 11 compounds were already detected in a target screening study by Reemtsma et al. [5].

Conclusions:
Due to the high-quality suspect list, the optimized workflow and the HRMS data the suspect screening successfully identified several new pesticide metabolites which have not been measured before in groundwater. However, despite advances in the evaluation softwares during recent years, suspect screening is still related to a large manual effort, a combination of computational tools and expert knowledge.

Novel Aspect:
Groundwater samples were screened for >1000 pesticide metabolites. Water evaporation allowed to minimize losses of polar compounds during enrichment.

References:
COMPARISON OF ORGANIC MIXTURES FROM PARTICULATE MATTERS COLLECTED IN KOREA AND CHINA BY USING GCXGC/HIGH RESOLUTION MASS SPECTROMETRY

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Keywords: particulate matter, PM2.5, GCxGC/HRMS, PAHs, atmosphere air

Introduction:
In Korea and neighboring China, airborne particulate matter (PM2.5) is very serious environment problems, having primary organic pollutants directly released from emission sources and secondary organic pollutants generated from atmosphere chemical reaction. Thus, the complex organic compounds extracted from PM2.5 collected day after day during one month in each country were analyzed to compare their identifications, relative quantities and emission sources.

Methods:
Samples of PM2.5 were simultaneously collected day after day for 28 days (4-31 January 2018) in Gwangju and Beijing using a high volume air sampler. The 90 cm² filter was extracted with dichloromethane through sonication twice. The two days of extracts were combined, filtered and concentrated under N2 gas. Comprehensive two-dimensional gas chromatography/high resolution time-of-flight mass spectrometry (LECO GC-HRT) was utilized to analyze the organic extracts. [1]

Results:
Approximately, 460 compounds were separated on the polar and sequential nonpolar GC columns and identified based on the mass spectral data from NIST and Wiley libraries, and exact mass accuracy (<1 ppm) of molecular ion from high resolution data, including alkanes, carboxylic acids, hopanes, PAHs, substituted aromatics and steranes, so on. A variety of PAHs were identified in Beijing samples such as 11H-benzo[b]fluorine, benzo[ghi]fluoranthene, benzo[a]anthracene, acepyrene, pyrene and benzo[A]pyrene. Their concentrations in Beijing were more 49 to 3 times according to PAH compounds than those in Gwangju for 16/17 days PM2.5. On the contrary to this, natural resin such as dehydroabiatic acid was much more in Korea sample. The organic compound markers which can make a distinction between the emission sources of PM2.5 in Korea and China are present. Among them, naphthalic anhydride found in this analysis may become a candidate marker. The study about what this compound in PM2.5 is originated from is currently ongoing. [2]

Conclusions
The complex organic mixtures from PM2.5 were analyzed by using GCxGC/HRMS. The identified organic compounds were different qualitatively and quantitatively between Korea and China. The PM2.5 generated in China has been sometimes transferred into Korea by atmosphere air. Thus, there is anticipated that the issues between two countries related about the emission sources will be discussed, based on these objective results obtained for PM2.5 collected in Korea and China.

Novel Aspect:
The organic compounds extracted from PM2.5 collected in Korea and China were different qualitatively and quantitatively due to their different emission sources.

References
Introduction:
Spray product emissions may be inhaled and some of the chemical ingredients may be respiratory irritants which may cause development of asthma or have other health effects. The aim of this project was to compare experimental exposure of 3 biocidal spray products (#1, #2, #3) applied in an exposure chamber with computed exposure calculations in existing models. The results were used to validate the models to contribute to a better risk assessment of spray products.

Methods:
Three biocidal spray products with different active compounds and spray nozzles were selected. They were applied in a 20.3 m³ controlled exposure chamber in accordance with the description provided by the manufacturers. Airborne organic compounds were sampled on Tenax and XAD and surface deposited by wiping with swabs. Samples were analyzed by (TD)-GC-MS/MS and LC-(QTOF)MS. Mass and number concentrations and size distributions of particles were measured on-line.

Results:
#1 had permethrin, piperonylbutoxide, and pyrethrum extract as active compounds, which along with alkanes were found in the air after spraying. Like the particles these followed a 1st order decay, however, the removal rates were higher than the air exchange rate (AER) indicating they were associated with the depositing particles. The alkane decay followed the AER indicating they were in the gas-phase.

#2 contained λ-cyhalothrin as the active compounds but it was not found in the air samples. Large particles (>0.5 µm) were found in very low concentrations (<0.1 mg/m³), while smaller particles (<0.3 µm) stayed at background levels. λ-cyhalothrin was found in wipe samples from surfaces.

#3 had benzalkonium chlorides as the active compounds, which were measurable in air up to 36 min after spraying following a 1st order decay. The particle pattern was the same as for #2. The wipe samples showed that the benzalkonium chlorides were mainly on surfaces near the spray area.

Preliminary results showed discrepancy between models and experiments.

Conclusions
The sprayed aerosols had shorter residence time in air than explained by the AER. Concentrations of the active compounds and low vapor pressure organic compounds decayed faster than explained by AER since they were associated with the aerosols, while more volatile compounds followed the AER. Aerosols of benzalkonium chlorides could be measured in the air more than ½ hour after application. The active compounds deposited mainly at the floor near the spraying position.

Novel Aspect:
Modelling is used extensively in risk assessment; however there is a research gap on the compliance between modelling and actual exposure data using standardized conditions.
Introduction:
Highly polar organic compounds are of special interest for drinking water utilities, since these substances are likely to end up in drinking water. Currently, there is an analytical gap, a monitoring gap, and a lack of toxicity data for persistent and mobile organic compounds. We aimed to close these gaps by applying a target and non-target screening using hydrophilic interaction liquid chromatography (HILIC) coupled to high resolution mass spectrometry in a monitoring study, followed by a toxicological risk assessment.

Methods:
HILIC-MS target and non-target screening approach including a pre-treatment was developed. The method was validated for the determination of 38 highly polar compounds in surface water and drinking water. 29 samples from surface water, river bank filtrate, groundwater and drinking water in The Netherlands and Flanders were analysed. A toxicological risk assessment was performed for the observed highly polar compounds.

Results:
More than half of the 38 highly polar target compounds were present in surface-, ground and/or drinking water at levels between LOD and LOQ or above the LOQ. Traces of several highly polar compounds were observed in drinking water. One compound, dichloroacetic acid, was introduced during disinfection treatment. Non-target HILIC-MS screening detected 145 features: compounds with a unique combination of accurate mass and retention time. The identity of eleven compounds could be confirmed by reference standards, the remaining compounds are yet to be identified. Due to threshold settings for detection not all target compounds that were observed with target HILIC analysis were observed with non-target HILIC-MS screening. A pGLV could be derived for 8 chemicals. For the remaining compounds present in the evaluated samples, insufficient information was available to derive a provisional drinking water guideline value (pGLV).

Conclusions
A HILIC-MS non-target screening approach was developed and validated for the determination of highly polar compounds. The HILIC target analysis and non-target screening were proven useful for the detection of highly polar compounds in source waters and indrinking water. Further identification of chemicals observed and toxicological risk assessment are imperative to evaluate the relevance of highly polar chemicals in (sources of) drinking water.

Novel Aspect:
An novel non-target screening approach using HILIC was developed for highly polar compounds revealed the presence of known and unknown polar compounds in the watercycle.

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OCCURRENCE AND HUMAN EXPOSURE TO BROMINATED AND ORGANOPHOSPHATE FLAME RETARDANTS FROM FINNISH HOUSEHOLD DUST

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Keywords: brominated flame retardants, organophosphate flame retardants, household dust, human exposure, exposure model

Introduction:
Flame retardants (FRs) are chemicals that are used to decrease flammability of consumer products. They are incorporated as additives or reactive ingredients in materials including plastics and textiles. Brominated (BFRs) and organophosphate flame retardants (PFRs) are the two main classes of FRs that are extensively used wherein the latter are considered as potential replacements to conventional BFRs but also part of those exhibit potential health risk to consumers.

Methods:
BFRs and PFRs were measured in Finnish household dust (n=40) by GC-HRMS. Children and adults exposure assessment (ingestion, inhalation, dermal) was based on measured dust concentrations, and it was made according to models published by Little et al. [1]. Exposure assessment needed calculation of dust-air distribution of FRs, the validity of which was evaluated using measured dust and indoor air concentrations of FRs from Norway [2].

Results:
The certified dust SRM2585 was used as the quality control of the analytical method: recoveries were 91-121% for BFRs and 65-120% for PFRs. In case of BFRs, BDE-209 (410 ng/g) had the highest median concentration followed by DBDPE (Decabromodiphenyl ethane) (120 ng/g), and BEH-TEBP (Bis(2-ethylhexyl) tetrabromophthalate) (110 ng/g) in household dust. TBOEP (Tris(2-butoxyethyl) phosphate) (11000 ng/g) was the most abundant PFR followed by TCIPP (Tris(2-chloroisopropyl) phosphate) (1870 ng/g) and TPHP (Triphenyl phosphate) (770 ng/g). From modelled exposure assessment, it was determined that exposure via dust ingestion contributes most for BFRs and also for less-volatile PFRs for children who are also more susceptible to FRs exposure than adults. But dermal adsorption governs the exposure pathway for more-volatile PFRs. However, the model used for determining air concentrations underestimated some low volatile BFRs and over-estimated some volatile PFRs, that results in the respective under/over estimation of exposure.

Conclusions
Measured dust and calculated air concentrations of FRs in Finland were found to be similar with those measured in Norway. Total daily FR intakes were some orders of magnitude lower than the oral reference doses from literature. This study presents simple analytical and exposure assessment methods which enable effective screening of exposure. However, further studies are required to develop cost-effective methods to study the distribution and exposure of FRs.

Novel Aspect:
We present a simple analytical-calculation method to assess total exposure from indoor environment to FRs that is based solely on the measured FR concentrations in dust.

References
Novel drinking water disinfection by-products

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Keywords: disinfection by-products, HPLC-HRMS, tap water, organochlorines, organobromines

Introduction:
Drinking water chlorination is used over 100 years. Destroying microbes chlorination however leads to formation of toxic disinfection by-products (DBPs), appearing due to reaction of active chlorine with natural and anthropogenic compounds dissolved in water. The list of DBP, contains over 700 compounds. However they cover only 50% of organic halogens in drinking water. In the present study over 30 new halogenated organic compounds were identified by HPLC-HRMS.

Methods:
Water samples from the Severnaya Dvina river at the water intake of Arkhangelsk (Russia), after purification and coagulation, and after chlorination were collected in March-June 2017. Two liters of water at pH 3 were made pass through SPE Bond Elute PPL cartridges (Agilent) cartridges. High resolution QTOF mass spectrometer TripleTOF 5600, (AB Sciex) with ion source Duospray connected to the HPLC system LC-30 Nexera (Shimadzu) was used.

Results:
Non targeted screening of pollutants in the Arkhangelsk tap water with HPLC- HRMS allowed detecting over 50 organohalogens. Among phenols pentachlorophenol is usually considered as an independent priority pollutant. However it was not present in the samples before chlorination. MS/MS allowed referring the largest group to halohydrines and dihalogenated fatty amides, the products of conjugated electrophilic addition of halogens to unsaturated fatty amides. The proposed structures were confirmed by oleamide chlorination in water. These compounds are a brand new class of DBPs, while their toxicities require special study. Five oxygenated species were 3,5-dichloro-4-hydroxybenzoic, dimethylbromobenzoic, and dimethyldibromobenzoic acids, trichlorophenylacetate, and 2,4-trichloro-1,3,5-benzotrialdehyde. ChemSpider search and spectra interpretation of N-containing compounds allowed identifying 3,4-dichloro-2-methoxy-5-oxo-2,5-dihydro-1H-pyrrol-1-yl)-acetic acid and highly substituted halogenated pyridines.

Conclusions:
Several groups of new disinfection by-products were identified in the tap water of Arkhangelsk (Russia). Mass spectral conclusions were confirmed by independent synthesis with standard compounds. Chlorinated and brominated compounds are formed at the stage of disinfection of the Severnaya Dvina river water rich with humic matter. Unfortunately there is no any available information on the toxicities of the novel DBPs.

Novel Aspect:
Several groups of novel disinfection by-products were established with HPLC-HRMS in the drinking water in Arkhangelsk, Russia.
Introduction:
For ensuring nuclear safeguards, we report the analytical signal-detection performance of thermal ionization mass spectrometry (TIMS) with continuous heating for the measurement of isotopic ratios in samples containing ultra-trace amounts of uranium.

Methods:
The isotope ratio measurements were performed using a thermal ionization mass spectrometer (TRITON Plus, Thermo Fisher Scientific, Germany). As methods for detecting uranium signals, peak-jumping mode using a single detector and static mode using multiple detectors were examined with U100 (10% 235U-enriched) uranium standard samples in the femtogram-to-picogram range.

Results:
Uranium isotope ratios, $n(235U)/n(238U)$, were measured down to levels of 1 fg and 3 fg in static and peak-jumping modes, respectively, while $n(234U)/n(238U)$ and $n(236U)/n(238U)$ values were measured down levels of 100 fg in both modes. In addition, the dependency of the 238U signal intensity on sample quantity exhibited similar tendencies in both modes. The precisions of the isotope ratios obtained in the static mode over all sample ranges used in this study were overall slightly higher than those obtained in peak-jumping mode.

Conclusions:
These results indicate that the analytical performance by TIMS with continuous heating are almost independent of the detection method, i.e., peak-jumping mode or static mode, which is characteristic of isotope-ratio measurements using the TIMS method with continuous heating.

Novel Aspect:
The results suggest that similar analytical performance can be obtained in the two detection of TIMS with continuous heating.
Introduction: (Limit of 400 characters)
According to the Act on Protective Action Guideline Against Radiation in the Natural Environment in South Korea has been planned and conducted the field investigation to monitor the radioactivity concentration surrounding usage facilities of raw materials and residues and protect from radiation in the natural environment. Anyone who handles raw materials or residues contained uranium or thorium whose activity concentration exceeds 1Bq/g should be registered the material informations (radioactivity concentrations, total amount, etc.). The objective was to monitor of potential pollution materials in naturally environment and offer some useful information for policy decision. Then it was important to pretreat the samples in order to accurately analyze the concentrations. There are various digestion procedures: open vessel digestions, closed vessel digestions, microwave digestions, alkali fusion. The digestion of monazite is an important aspect of thorium and uranium elements analysis using by quadrupole inductively coupled plasma mass spectrometry (ICP-MS). This paper describes comparison of two digestion methods for thorium and uranium elements analysis in monazite.

Methods: (Limit of 400 characters)
In this study, the monazite was dissolved by the conventional acid digestion and electric fusion system. Prior to the pretreatment, it was using a XRD in order to analyze the components of the suspicious materials, detected matter whose radioactive concentration exceeds or is suspected to exceed limits determined by NSSC in monitors at harbors. After homogenous sample preparation through milling or mixing, the sample were dried in a temperature controlled oven up to 450 °C for about 5hrs. The samples were dissolved by fusion with lithium metaborate and acid digestion with HF, HNO3, and HClO4 to break refractory complex. The dissolved solutions were precipitated at pH 7 using Fe co-precipitation and dilution HNO3 for analysis by ICP-QMS. In addition, the samples were completely sealed in D6H4 bottle to secure the confinement of radon gases. It spends time for 1 month to attain the secular equilibrium between radium and radon isotopes in decay series. Thorium and uranium series that represented 228Ac and 214Bi gamma nuclides measured by HPGe and 232Th and 238U atomic analyzed by ICP-QMS.

Results: (Limit 900 characters)
The reference sample with certificate values (zircon by ALML) was analyzed for the different digestions. The alkali fusion method showed great thorium and uranium yield up to 98% and the closed vessel acid digestion method was similar thorium and uranium yield. The results of 232Th and 238U using HPGe and ICP-QMS showed a similar distribution. However, the monazite showed different values between fusion method and acid digestion. The higher concentration of the samples, the bigger the difference values.
In addition, the radioactivity of the 232Th and 238U indicator decreased in the monazite and showed a different concentration of thorium and uranium between ICP-QMS and HPGe.

Conclusions (Limit of 400 characters)
It is the most important to monitor environmental radiation, raw materials, residues and products for protect from radiation exposure. NSSC enforced “Act on Protective Action Guidelines Against Radiation in the Natural
Environment” since 2012. Thus, this study establishes the procedures of radiation measurement or radioactivity analysis about NORM samples. In particular, it was necessary to analyze the thorium and uranium concentrations of monazite using acid digestion and evaluate the assessment of radiation risk for the Korean population and protect the health of the workers involved and the environment.

Novel Aspect: (Limit of 150 characters)
Comparison between alkali fusion and acid digestion for thorium and uranium analysis in monazite or products suspected as contaminant materials in the field survey based on NORM regulation in South Korea

References


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137 - DETERMINATION OF GLOBAL DNA METHYLATION AND OXIDATION OF DNA AND LIPID BIOMARKERS IN METAL OXIDE NANOMATERIAL-HANDLING WORKERS BY LIQUID CHROMATOGRAPHY-TANDEM MASS SPECTROMETRY

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Determination of global DNA methylation and oxidation of DNA and Lipid biomarkers in metal oxide nanomaterial-handling workers by liquid chromatography-tandem mass spectrometry
Keywords: metal oxide nanomaterials; 8-hydroxydeoxyguanosine; biomarkers; LC-MS/MS

Introduction
The nanotechnology industry is a rapidly growing. The most commonly used nanomaterials (NMs) are, titanium dioxide (TiO2), silica (SiO2) and zinc oxide. To assess whether oxidative stress markers of nucleic acids (8-OHdG), DNA methylation levels in urine and white blood cell (WBC), and EBC 8-isoprostane were associated with nanomaterial exposure. Moreover, the relationship between oxidative damage biomarkers and global DNA methylation was investigated.

Methods
In the present study, urinary and white blood cell (WBC) 8-hydroxydeoxyguanosine (8-OHdG), and exhaled breath condensate (EBC) 8-isoprostane were measured as oxidative stress biomarkers. WBC global methylation was measured as an epigenetic alteration. To examine global methylation, oxidative DNA damage, and lipid peroxidation in workers with occupational exposure to metal oxide nanomaterials using LC-MS/MS.

Results: (Limit 900 characters)
Exposure to TiO2, SiO2, and indium tin oxide (ITO) resulted in significantly higher oxidative biomarkers such as urinary 8-OHdG and EBC 8-isoprostane. However, significantly higher WBC 8-OHdG and lower global methylation were only observed in ITO handling workers. Significant positive correlations were noted between WBC and urinary 8-OHdG (Spearman correlation r = 0.256, p = 0.003). Furthermore, a significant negative correlation was found between WBC 8-OHdG and global methylation (r = -0.272, p = 0.002). These results suggest that exposure to metal oxide NMs may lead to global methylation, DNA oxidative damage, and lipid peroxidation.

Conclusions
Global DNA methylation levels, and levels of oxidative stress markers 8-OHdG and 8-isoprostanate were assessed in metal oxide nanomaterial handling workers. The results show that exposure to metal oxide nanomaterials may lead to global hypomethylation, DNA oxidative damages, and lipid peroxidation. Furthermore, we identified a negative correlation between WBC 8-OHdG and global methylation.

Novel Aspect
Exposure to metal oxide nanoparticles may lead to global methylation and DNA oxidative damage and lipid peroxidation.

References
Mass Spectrometry Study: When and How Green Solvents Ionic Liquids Turn into Toxic Compounds

Ping Jiang, Ian Vander Meulen and Xing-Fang Li

Introduction
Ionic liquids (ILs) are increasingly used in industrial processes as “green chemicals” because of unique properties of low volatility and customizability. ILs can be used to enable novel processes and/or replace conventional organic solvents in a wide variety of applications. Widespread use may increase the risk of accidental release of IL-containing industrial wastes into environmental waters. Most ILs are highly water soluble, and have estimated environmental half-lives of several days to a month. IL cations often consist of aromatic or alkyl quaternary amines that resemble previously confirmed N-nitrosamine (NAs) precursors. NAs are confirmed animal carcinogens and classified as probable human carcinogens. NAs are also potent, estimated to have negative health effects at ng/L concentrations. Therefore, this study is to investigate the formation of ILs of various structures to form nitrosamines under the conditions of water chloramination.

Methods
Laboratory reactions of ILs consisting alkyl and aromatic tertiary amines with chloramine will be performed. HPLC-MS and HPLC-MS/MS methods are developed and used to identify and quantify the nitrosamines produced in the reactions.

Preliminary Results
We have studied two ILs, 1-ethyl-1-methylpyrrolidinium bromide (EMPyr) and 1-ethyl-3-methylimidazolium bromide (EMIm), for their nitrosamine formation potential. Each IL species was reacted with pre-formed monochloramine under various conditions. After 24h, samples were extracted from water using dichloromethane. The extracts were analyzed for nitrosamines formed in the reactions. HPLC-high resolution MS analysis of the extracts identified the produced nitrosamines, while HPLC-MS/MS with multiple reaction monitoring mode quantified the concentrations of the NAs using a deuterated internal standard. Both EMIm and EMPyr can produce NAs: EMIm generating N-nitrosomethylethylamine, while EMPyr producing N-nitrosopyrrolidine. The formation yield of NAs is dependent on the structures of the ILs. EMPyr produced higher amount of NA than EMIm under all conditions evaluated. EMPyr can produce NA with a yield on the same order of magnitude as polydiallyldimethylammonium chloride, a confirmed nitrosodimethylamine precursor. We are investigating a variety of ILs for the formation potential of NAs. In addition, we will further investigate how to use UV/H2O2 to reduce the formation of nitrosamines from ILs. This study is the first report demonstrating the formation of NAs from ILs under water chloramination conditions. This study emphasizes the importance of prevention of environmental discharge of ILs to water bodies, and highlights a need for further evaluation of potential lifecycle impacts of ILs prior to their wide ranging applications.

Novelty: MS studies of Ionic liquids forming toxic nitrosamines under common water treatment conditions.
IODINE DISTRIBUTION AND BIOAVAILABILITY IN SOIL FROM FENYANG, SHANXI PROVINCE, CHINA

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Keywords: iodine, speciation, stability, distribution, soil

Introduction
Soil plays an important role in the environmental geochemistry circulation and transformation process of iodine. In this study, the primary interest was to understand the iodine species that were potentially mobilizable and bioavailable, rather than the total iodine species present in the soil. Investigation has been conducted on the status of iodine distribution in Fenyang, in order to elucidate the distribution characteristics of iodine in the environment.

Methods
Soil profile samples were mainly collected from farmland in Fenyang. The total iodine concentration was detected by inductively coupled plasma mass spectrometry (ICP-MS) using ammonium hydroxide digestion. The speciation analysis of iodine was determined by high performance liquid chromatography (HPLC) coupled with ICP-MS. Two methods that water extracting and phosphate extracting were used to investigate the bioavailability of iodine in soil samples.

Results
Good results were achieved using 10 % ammonium hydroxide (v/v) and 0.5 mM EDTA where the transformation between IO3- and I- was clearly minimized. Inorganic iodine was the main iodine species in the extraction solution. Iodine in soil exists as organic iodine has been reported elsewhere [1,2,3]. Our results indicated that the transformation of organically bound iodine to inorganic iodine must be occurred because the sums of inorganic iodine were agreed with the certified values of reference soils. But the transformation between IO3- and I- was not observed.

Water extraction and phosphate extraction were used to investigate the bioavailability of iodine in 6 soil samples. The results showed that I- was the main iodine species. It confirmed that iodine in soils was mainly transported into plants in the form of inorganic iodine. The iodine concentrations of all the soil profile samples ranged from 0.56~5.69 µg-I g-1. The maximum iodine concentration was at the depth of 0~20 cm. And the soil iodine content decreased with increasing sampling depth.

Conclusions
Efforts were made to preserve the oxidation of IO3- to I- by using EDTA. Although this method should be further tested for its application for a broad range of soil samples, the results obtained in this study are promising. The main species of iodine uptake from soil was iodide. The content of iodine in the surface soil was higher than that in the depth. It was not only related to iodine content in groundwater, but also related to geological structure and properties of soil.

Novel Aspect
The stability of inorganic iodine was achieved by adding EDTA into the extraction solution. The distribution and bioavailability of iodine in soil profile samples was discussed.

References
Introduction:
Nanoparticles are used in many areas of industry and everyday life and they are released in great quantities into the environment. Despite this, little is known about their (eco)toxicity and the risks they might pose. To be able to evaluate these risks and take appropriate precautions, it is necessary to understand their behavior and fate in the environment.

Methods:
The single particle ICP-MS was used to determine and characterize Ag nanoparticles in samples of river water taken at several places from Vltava and a few smaller streams in Prague and its neighborhood. NexION 350D (Perkin Elmer, Concord, Canada) spectrometer was used for all measurements. Samples were stabilized by addition of gelatin immediately after sampling and were analyzed the same day.

Results:
The developed method exhibited good characteristics and only minor matrix effects of the river water were observed: limit of detection (LOD) of particle size ca. 25 nm (depends on dissolved Ag concentration), LOD of particle number concentration 100 mL−1, 1.3% repeatability and 98% recovery. Small distortion of nanoparticle diameter was observed: mean diameter 92 nm was found for particles with diameter 96 ± 8 nm.
Ag nanoparticles were present in samples of water from Vltava river. The found Ag nanoparticles covered a wide range of diameters, ca. 30-120 nm. Their number concentrations were extremely low, ca. 300-2000 mL−1, however well above the LOD. The Ag nanoparticles number concentrations increased as the river flowed through the urban agglomeration.

Conclusions:
Single particle ICP-MS proved to be capable of determining Ag nanoparticles in surface waters. Ag nanoparticles with wide range of diameters were successfully determined in the Vltava river and an increase of number concentrations, likely due to human activity, was observed downstream of Prague. It should be evaluated whether these number concentrations can be harmful to the environment.

Novel Aspect:
To the best of our knowledge this is the first study of Ag nanoparticles in surface waters in the Czech Republic.

Acknowledgements:
Financial support from specific university research (MSMT No 21-SVV/2018) and Grant Agency of Czech Republic (project no. 17-00291S) is gratefully acknowledged.
Keywords: Antibiotics; river water; the Grand Canal; spatial distribution

Introduction
The Grand Canal passes through some industrial cities (e.g. Hangzhou, Suzhou, Wuxi, and Tianjin), and also vast rural areas. The aim of this work is to reveal the differences in contamination levels and composition profiles of antibiotics in waters of different sections of the Canal with comparisons of population density and economic scale in each section.

Methods
HLB column (500mg, 6cc, Waters, USA) was used as the solid phase extraction for extraction and enrichment of the target antibiotics. Analysis of antibiotics was performed using a high-performance liquid chromatograph tandem mass spectrometer. Separation of the analytes was performed by an Agilent HP1200 liquid chromatograph interfaced with API 4000 tandem quadrupole mass spectrometer operated in the electrospray ionization mode.

Results
Among the 20 target antibiotics, 14 antibiotics were detected. The predominant types of antibiotics were sulfonamides and macrolides. The spatial distribution pattern of antibiotics shows that the concentration levels at the sampling sites in the north end of the canal (average: 117.1 ng/L) and the south end sections (average: 96.5 ng/L) of the canal were obviously higher than those in the middle sections (average: 48.8 ng/L). At the north end of the canal there are two metropolitan cities Beijing and Tianjin, and at the south end of the canal there are cities with dense population and industries such as Suzhou and Hangzhou. Municipal sewage could be the main sources for sulfonamides and macrolides in these cities[1-2]. Most of the sampling sites in the middle part of the canal are in rural areas, especially in the northern Jiangsu Province, and Shandong and Hebei Provinces where animal husbandry and fish farming could be the main sources for sulfonamides and macrolides.

Conclusions
High concentrations of sulfadiazine, sulfamethoxazole and ampicillin were observed. Antibiotics showed large geographical variations from the North to the South, with high concentrations occurring at the sampling sites in the Yangtze River Delta area and Tianjin-Beijing urban and suburban areas, while low levels were generally found in the middle sections of the canal with vast agricultural areas, showing a V-shape geographic distribution along the river.

Novel Aspect
The impacts of urbanization and economic development on concentrations and compositions of antibiotics in waters of different sections of the Grand Canal are discussed.

References
Keywords: GC×GC-MS, environmental mass spectrometry, retention index, semivolitile compounds

Introduction: (Limit of 400 characters)
Gas chromatography-mass spectrometry (GC-MS) is one of the most precise, well developed, and reliable analytical tools for the analysis of small volatile and semi-volatile compounds. This method has been extensively improved and tested by enhancing the separation technique via comprehensive two-dimensional gas chromatography (GC×GC-MS). Application of GC×GC-MS is especially useful for the analysis of complex mixtures, which environmental objects usually are.

Methods: (Limit of 400 characters)
Various organic compounds as well as dichloromethane extracts of different water samples were analyzed using Pegasus® GC×GC HRT 4D instrument (LECO Corp., USA). Compounds from SV Internal Standard Mix (Restek, USA) were used as internal standards (IS) for retention index calculations (perdeuterated 1,4-dichlorobenzene and some PAH). Chromatographic separation was performed using Rxi-5MS and Rxi-17SilMS columns (Restek, USA) for both dimensions respectively.

Results: (Limit 900 characters)
Several examples revealing insufficiency of GC-MS and GC-HRMS methods in precise identification of some organic compounds put forward GC×GC-MS as one of the most powerful tools for non-target analysis. To improve the identification certainty a simple method for calculation of retention indices attributed to the secondary dimension column (2I) using single injection and PAH as internal standards commonly used in US EPA methods for the analysis of semi-volatile organic compounds was proposed. Using the linear combination of both retention time (tR) and retention temperature (TR): 2I = a(TR+ktR) + b, a series of 2I values for various organic compounds were calculated with good reproducibility under different separation conditions. a and b are proportional to enthalpy and entropy of interaction between analytes and stationary phases, k – non-linearity compensation coefficient. Time constituent of the 2I function by itself was found useful for identification of homologous series of some classes of compounds (alkanes, fatty acids).

Conclusions (Limit of 400 characters)
GC×GC-MS was demonstrated to be crucial for non-target analysis of complex environmental mixtures. A simple and reliable approach is proposed for the calculation of retention indices in GC×GC experiments. The proposed method uses the Lee indices based on PAH, that may be used as a reference under certain conditions, and doesn’t require any additional runs. The method is applicable for both target and non-target analyses for a wide range of compounds.

Novel Aspect: (Limit of 150 characters)
A simple and reliable approach is proposed for the calculation of retention indices in GC×GC analyses as one more analytical parameter to increase the identification certainty.
Introduction:
There are a very wide number of PFAS compounds that are actually present in environmental samples that are not part of the current EPA methods for PFAS analysis. The goal of this work was to use a QTOF mass spectrometer with Non Target Screening workflow to locate and identify unique PFAS compounds that were not known to be in the sample.

Methods:
Water samples were analyzed using a Sciex X500R QTOF system set up to acquire data in a Non Target Screening workflow. The instrument first performed a TOF scan from 100-1500. This scan was followed by a data dependent scan where ions above a set threshold were selected for fragmentation and acquisition of a full scan MS/MS spectrum. The resulting MS/MS spectra were searched against a high resolution library to identify potential PFAS of compounds present in the samples.

Results:
Perfluoroalkyl substances (PFASs) encompass a range of fully fluorinated alkyl compounds and are prevalent in Aqueous Film Fire Foam (AFFF). In addition, PFASs are ubiquitous as they are used in many household goods and have been found in various environmental and biological samples. Here, we demonstrate the use of QTOF technology to exploit the power of high resolution mass spectrometry and the use of product ion spectra to identify novel compounds in AFFF contaminated samples.

Novel Aspect:
Novel PFAS compounds are identified using high resolution mass spectrometry.
Characterization of organic compounds in aquatic sediment by ion profiles obtained using GC/MS system

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Keywords: GC/MS, river sediments, ion profiles

Introduction: The analysis of sediments is an efficient way of reconstructing the depositional history of organic compounds and environmental changes in aquatic environment [1-3]. The organic compounds are released to the environment through anthropogenic (industrial releases) and natural sources (aquatic organisms and terrestrial plants). Some compounds in sediments are widely used as a sensitive indicator for source identification. In respect to this, one of priority tasks is to determine the environmental distribution of organic compounds in the aquatic sediments. The present paper purpose is the characterization of main families of compounds by characteristic ion obtained by Ion Impact Mass Spectrometry using GC/MS system.

Methods: Sediment sample were prepared by Ultrasonic Solvent Extraction (USE). An aliquot (2g) of freeze-dried sediment was extracted successively with 10 ml CH2Cl2+methanol (2:1). The extract was fractioned on Al2O3 with following organic phases: n-hexane, n-hexane+CH2Cl2 (1:2), CH2Cl2+methanol (1:1) and methanol respectively. The final extracted was dried to 1ml in n-hexane. The analyses were performed using a GC/MS system with mass spectrometer operated in EI mode to 70 eV. The gas chromatograph was equipped with a capillary column HP-5MS (30x0.25mm) with 0.25 film thickness with programmed temperature from 90°C to 3150°C.

Results: The families of compounds are visualized by chromatograms on diagnostic ions. The main group of compounds is: 1) n-Alkanes which show compounds with the number of carbon atom ranging from 12 to 30. 2) Acyclic isoprenoids compounds with a large number of methyl branching points in their structure. This group of compounds include pristine (C19) and phytane (C20) often used as an indicator of depositional environments. 3) Pentacyclic triterpanes (Hopanes) a class of compounds that has a great impact on petroleum geochemistry, they being useful as source indicators. 4) An important group of compounds found in the sediment samples is the Lineal Alkylbenzenses (LABs), with number of carbon from 10 to 13. 5) An abundant class of compounds consists from Polyaromatic Hydrocarbons (PAHs) of environmental concern due to their carcinogenic proprieties. 8) Sulphonated Polyaromatic Hydrocarbons (SPAHS), other important group of pollutants used very often as biomarkers for the source information.

Conclusions: Are obtained characteristic profiles of organic compound families present in sediments. Their profile shapes is a function of their sources and reflect the natural, industrial and domestic activities at regional level. Novel aspect: An efficient method based on GC/MS technique is developed for detection of eight families of organic compounds in sediment. Every family is presented as characteristic ion profile having a large number of compounds.

References:
Study of pharmaceuticals removal rates in Wastewater Treatment Plant using GC/MS analytical technique

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Keywords: GC/MS, PPCPs, WWTP, isotopic dilution, removal rate,

Introduction: Many xenobiotics including Pharmaceuticals and Personal Care Products (PPCPs) are released into the environment from Wastewater Treatment Plants (WWTPs) [1]. The pharmaceuticals persist in the environment, remaining active and affecting aquatic life [2-4]. The PPCPs could act as persistent compounds, because of their continual discharge into aquatic sustaining a multigenerational exposure for the resident organisms [3]. In recent years, reliable methods have been established for analysis of these pollutants down to low ng/L levels in water environment, based on HPLC-MS and GC-MS. The purpose of this work was to quantify some pharmaceutical compounds in the aqueous phase at different stages of a conventional WWTP situated in Cluj-Napoca (Romania) with aim to determination of removal rate.

Methods: The compounds were extracted by SPE procedures and derivatised with N-methyl-N-trimethylsilylevfluoroacetamide (MSTFA) and than analyzed by Gas Chromatography-Electron Ionization Ion Trap Mass Spectrometry (GC/EI-ITMS), in full scan mode. For quantification isotopic dilution was applied.

Results: An effective analytical method for the simultaneous determination of pharmaceuticals from various therapeutic classes in aqueous samples has been developed. The optimized GC separation was obtained for 38 PPCPs. The complete mass spectra were registered for natural, derivatised and isotopic labeled compounds. Are detected also compounds originating from thermal decomposition of pharmaceutics during GC separation. Were analyzed sample collected from different places of WWTP leading to removal rate for different treatment units.

Conclusions: Were obtained: 1) GC/MS separation of 38 compounds from family PPCPs; 2) Mass spectra for natural, sylilated and isotopic labeled compounds; 3) Mechanism of thermal decomposition for cyclophosphamide and carbamazepine; 4) The removal rate for every compound in WWTP in the range 19.5-92 %, depending of chemical structure.

Novel aspect: a) Detection and quantification of PPCPs in an efficient analysis by SPE extraction, derivation by MSTFA and GC/MS analyses; b) Registration of mass spectra of the natural, sylilated and isotopic labeled for the same compounds; c) Determination of the removal rate for different units of WWTP.

References:
CITALOPRAM PHOTODEGRADATION: IDENTIFICATION OF TRANSFORMATION PRODUCTS BY MASS SPECTROMETRY

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Keywords: Transformation products; time-of-flight mass spectrometry; citalopram; wastewater samples.

Introduction
Citalopram (CIT), an antidepressant drug, is an emerging contaminant frequently detected in environmental matrices, but there are few data on its transformation products (TPs) formed in sewage treatment processes [1,2]. The objective in this study was to elucidate the formation in real matrices of CIT TPs by photodegradation under UV irradiation using LC-QTOF/MS for identification purposes.

Methods
Photodegradation experiments were conducted using a photoreactor with 100 mL of spiked distilled water (4 mg L⁻¹) cooled by water circulation. The UV irradiation source was a 450 W medium-pressure mercury-vapor lamp. The experiments consisted of 0.1 mL aliquots withdrawn at certain times. The QTOF Impact II mass spectrometer (Bruker Daltonics, German) was operated in the ESI positive mode in full scan and multiple reaction monitoring (MRM) mode.

Results
The photodegradation experiments almost completely removed CIT (C₂₀H₂₁FN₂O) in 4 hours and it was possible to identify 12 probable TPs with different kinetics of formation and degradation behavior. Analyses were based on accurate mass and on the fragmentation patterns observed in the MS/MS spectra and the mass errors were less than 5 ppm. Reactions of oxidation (neutral loss of CO), hydroxylation (neutral loss of H₂O), formation of amide (neutral loss of NHCO) and dehalogenation (neutral loss of HF) were observed. The most abundant TP, namely TP4 (C₂₀H₂₂N₂O₂), was formed by the dehalogenation of fluorine and hydroxylation on the furan ring. Desmethylcitalopram (TP2, C₁₉H₁₉N₂OF) and citalopram amide (TP11, C₂₀H₂₃N₂O₂F), human metabolites, were also detected in these experiments and showed that the compounds can be formed under UV-light. A pathway for the formation of TPs was proposed. Two TPs (TP2 and TP3, C₂₀H₂₁N₂O₂F) were detected in effluent samples, where the tertiary treatment is UV disinfection.

Conclusions
LC-QTOF/MS proved to be a useful tool for elucidating TPs identified formed under laboratory conditions. UV irradiation showed good efficiency in the removal of CIT and all TPs formed completely or partially degraded until the end of the experiment (4 hours), most of them having the maximum intensity between 1 and 2 hours. Analysis of wastewater samples showed that only TP2 and TP3 were detected, presumably because in sewage treatment plant complex interactions may occur.

Novel Aspect
The results obtained are relevant to elucidate the identity of TPs formed in photodegradation and also which ones may exhibit greater toxicity than the parent compound.

References
For information please contact: scientific@ims2018.it
Keywords: neonicotinoid, high-resolution mass spectrometry, neurotoxic effects, multiple reaction monitoring

Introduction:
The neonicotinoid pesticide imidacloprid (IMI) has been developed to act selectively as agonists of nicotinic acetylcholine receptors (nAChRs) in insects. However, recent evidences show that it is able to bind mammalian nAChRs, leading to toxic responses in cultured neurons [1]. To properly assess the risk in humans, we developed an integrated analytical strategy combined to the analysis of biological effects in a mouse model of prenatal exposure.

Methods:
The passage of blood-brain barrier (BBB) and placenta was verified analysing IMI concentrations in mouse dam brains and embryos by a multiple reaction monitoring (MRM) method. Different metabolites of IMI were identified in biological samples by high-resolution mass spectrometry (HRMS). Biological effects was evaluated in brains from newborn mice in utero-exposed to IMI by immunochemical techniques.

Results:
Results of MRM analysis showed quantifiable IMI levels in both plasma and brain (about 0.3% of the total administered amount of IMI) of treated dams, suggesting a passage of mammalian BBB. HRMS analysis allowed the identification of eight main metabolites, mainly due to phase I metabolic reactions in both plasma and brain, including desnitro-IMI that has a binding affinity to nAChRs similar to nicotine. Analysis of embryos showed detectable levels of IMI (0.03%) and its metabolites, suggesting a passage through placenta. The measured concentration of IMI in biological samples has been correlated with neurodevelopmental impairments in newborn mice (verified as expression of synaptic protein and marker of immature neurofilaments in brains) and immune system alterations (reduction of microglial cells in different brain areas).

Conclusions:
The newly developed analytical methods, including both MRM and HRMS, allowed an accurate quantitation of IMI in biological samples and the evaluation of relative distribution of its main metabolites. The results highlighted the passage of IMI and its main metabolites through the biological barriers (BBB and placenta) after oral exposure in a mouse model and neuro/immune system development impairments in newborn mice exposed in utero to IMI.

Novel Aspect:
Combining an accurate and sensitive analytical strategy to neurological investigations at human exposure levels, our approach paves the way for a proper risk assessment.

References:
Introduction
Dissolved organic matter (DOM) is a complex and ubiquitous organic mixture involved in a multitude of biogeochemical processes. In order to better understand its role in the global carbon cycle, the investigation on the most elusive DOM fraction is of critical importance. The goal of this study is the development of a non-targeted method for the analysis of the labile (biologically reactive) DOM, in order to monitor its depletion and transformation on short timescales.

Methods
Labile DOM, mainly characterized by hydrophilic compounds, was produced by cold water extraction of Norway maple leaves. This material was incubated with native bacteria over four weeks. Samples were pre-concentrated and analyzed by hydrophilic interaction chromatography (HILIC) coupled to high resolution mass spectrometry (Orbitrap –MS). Data analysis and interpretation were performed by R and Matlab statistical software.

Results
The analysis of leaf-derived DOM showed a consistent transformation over the incubation period. Compounds found in the leaves exudates, characterized by high oxygen-to-carbon and hydrogen-to-carbon ratios (typically identified as sugars) were gradually removed from the solutions. Preliminary data revealed that from the transformation and depletion of the labile DOM fraction, a molecular signature typically found in the aquatic environments emerged. This suggests that recalcitrant DOM can be derived from the fast degradation of the elusive labile DOM by rapid microbial activity. Contrarily, a large part of the organic material showed no reactivity in the tested time scale suggesting that many species are not easily accessible for bacterial degradation.

Conclusions
The preliminary data show promise for the development of mechanistic studies involving the investigation of labile DOM material. The method allows the monitoring of natural compounds in the complex mixture and might be applied to find biomarker useful in environmental metabolomics analysis. Such experiments can also be used to gain insight into the global carbon cycle.

Novel Aspect
Easy and flexible non-targeted method for determination of labile DOM compounds.
Screening of pharmaceutical and their metabolites in fish by HRMS using data-base support

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Keywords: Organic pollutants, HRMS, Data-base support, Fish

Introduction: (Limit of 400 characters)
Occurrence of pharmaceuticals in the aquatic environment has raised concerns about adverse effects on exposed wildlife. Little is currently known on exposure levels of drugs in fish [1], but studies already reported detection of pharmaceuticals in fish samples [1-4]. Pharmaceuticals could be much more concentrated in fish than in polluted living waters. Additionally, fish are known to possess a hepatic detoxification system which are likely capable of metabolizing pharmaceuticals taken up from polluted waters.

Methods: (Limit of 400 characters)
Riverine fish from were collected between 2014-2017 from different regions of Spain. Bile samples were analyzed after a protein precipitation and isolation by UPLC-HRMS. Muscle were treated with organic solvents and analyzed similarly. UPLC-HRMS system present great value in establishing the fragmentation pathways to identify pharmaceuticals and elucidate the structure of fish metabolites.

Results: (Limit 900 characters)
The HRMS data allowed screening for suspected pharmaceuticals and their metabolites and provided plausible chemical formulae. The comparison of MS/MS spectra of the parent compounds and their metabolites allowed to propose chemical structures for possible metabolites in fish bile. With this analytical methodology some metabolites, corresponding to different reactions that includes products of hydroxylation, glucuronide conjugates were identified, similar to the metabolites reported previously in our group in a similar study for carbamazepine. The suspect analysis of bile samples allowed the detection of several pharmaceuticals. Psycho-active drugs were one of the most commonly detected drugs. Their identities were proposed by matching their accurate MS and MS/MS data against different libraries.

Conclusions (Limit of 400 characters)
The UPLC-HRMS analysis of fish bile and muscle extracts allowed the detection of pharmaceuticals and metabolite. We are presently identifying more metabolites which they still require confirmation. Psycho-active drugs have been the most commonly detected drugs in fish. Thus, this analytical methodology highlights that UPLC–HRMS is a powerful tool for qualitative analysis, allowing the search for suspected metabolites, their identification and possible quantitation of target compounds.

Novel Aspect: (Limit of 150 characters)
we propose the evaluation of the metabolism of frequently detected drugs in fish, performing a rapid screening of bile and muscle extract by HR-MS for the presence of stable intermediates.

References

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679 - SUSPECTED SCREENING OF NATURAL TOXINS IN SURFACE WATER BY LIQUID CHROMATOGRAPHY HIGH RESOLUTION MASS SPECTROMETRY.

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Keywords: Q-exactive Orbitrap, natural toxins, surface water, non-target screening, HPLC-ESI/HRMS

Introduction:
Natural toxins are the prevalent contaminants in surface water reservoirs that may cause several health effects to humans [1]. Only microcystin-LR was regulated by the EU legislation [2]. High-resolution mass spectrometry (HRMS) is a powerful approach used for the non-targeted screening of natural toxins in surface water [3]. In this context, a HRMS non-targeted analysis was carried out using an in-silico approach for the identification of a wide group of biotoxins.

Methods:
A mix of natural toxins representative compounds has been used as standards. After solid phase extraction (SPE) enrichment and elution, compounds were separated by a C18 HPLC column and analyzed with a ESI-QExactive-MS. Data acquisition was based on full scan in positive ionization mode from 100 to 1500 Da with a resolution of 70000 FWHM. A homemade dataset was used as a library with Compound discoverer 2.1 software, in order to obtain a biotoxin tentative list.

Results:
A double layer solid phase for SPE enrichment has been optimized for the widest range of natural toxins. The method allows good recoveries ranging from 60 to 70% and LOD below 1 ug/L. To the authors knowledge’ no previous works reported an approach intended to retain such a large class of compounds. No cleanup was applied since loss of polar analytes was observed. The method was applied to perform a natural toxins suspect screening in water samples obtained from several rivers in Catalonia (NE Spain). The spectral data have been processed by Compound Discoverer 2.1 revealing the possible presence of several natural toxins in the “Riera de Rubí” river (Catalonia). Several suspected toxic compounds have been found, most of them being plant toxins and mycotoxins (but no cyanotoxins), with a level 3 of confirmation[4].

Conclusions:
A novel approach able to carry out a screening of suspected natural toxins in water samples has been developed and validated. To the authors knowledge’ this is the first method able to detect various groups of natural toxins simultaneously in one analysis.

Novel Aspect:
This is a novel method to analyze a large group of biotoxins. A home-made dataset coupled with an in-silico approach coupled to HRMS enhanced the screening performances.

References
Introduction

Organic gases and particles are important trace constituents of the Earth’s atmosphere. They affect climate and human health, and play an important role in atmospheric chemistry. Aircraft measurements of organics are analytically challenging because of the high sampling frequency requirement (> 1 Hz), low analyte concentrations in the remote atmosphere (ppt levels), and the chemical complexity of the sample.

Methods

Proton Transfer Reaction – Mass Spectrometry (PTR-MS) is an online chemical ionization mass spectrometry technique for detection of VOCs [1]. The recently developed CHARON ("CHemical analysis of aeRosolONline") inlet enables PTR-MS instruments to measure the organic composition of submicrometer particles [2]. Significant sensitivity improvements have been recently achieved by the inclusion of radio frequency ion lenses.

Results

Our PTR-MS instruments are nowadays routinely flown on NASA’s airborne science laboratories for measuring trace organics in the Earth’s atmosphere. During the three most recent deployments, we i) measured a set of organic trace gases (methanol, acetone, dimethyl sulfide) that are emitted from or taken up by the North Atlantic Ocean, ii) chased another research aircraft that was fueled with a biofuel blend to measure its emissions both on the ground (during taxing) and in the air, and iii) test flew the new CHARON inlet to characterize particulate organic matter emitted from wildfires and anthropogenic sources. We will show selected data examples to illustrate how the latest generation of PTR-MS instruments has been successfully used on NASA’s airborne platforms for characterizing the organic composition of gases and particles the Earth’s atmosphere.

Conclusions

A PTR-MS instruments has been successfully used for airborne measurements of selected organic trace gases (e.g., methanol, acetone, dimethyl sulfide) at high measurement frequency (1-10 Hz) and low detection limits (single-to-double-digit ppt at 1 second signal integration time). First test flights have been performed with the new CHARON particle inlet.

Novel Aspect

Airborne VOC measurements have been performed with unprecedented instrument sensitivity and detection limit. The CHARON particle inlet has been flown for the first time.

References

Environmental research continues to expand beyond traditional, regulated contaminants to emerging contaminants, such as sucralose and other artificial sweeteners, nanomaterials, perfluorinated compounds, pharmaceuticals, hormones, drinking water and swimming pool disinfection by-products (DBPs), 1,4-dioxane, sunscreens/UV filters, flame retardants, benzotriazoles, naphthenic acids, algal toxins, and new contaminants on the horizon: ionic liquids and microplastics. These are now frequently being found in water samples, including rivers, lakes, ground water, and drinking water. Moreover, understanding their fate and transport in the environment and in wastewater/drinking water treatment is vitally important, and as such, one of the major trends continues to be in identifying their transformation products. Because environmental samples are inherently complex mixtures with trace-level contaminants, mass spectrometry has been key for their identification and measurement. This presentation will provide an overview of the state of the science for emerging contaminants, their formation and transformation in the environment, and the modern tools used to measure them.
Introduction:
Dimethachlor, which belongs to the class of chloroacetanilide, is a selective herbicide. It has a variety of metabolites detected in soils, surface water or air[1] but they have not included in the maximum residue level of dimethachlor yet. This herbicide was not authorized in Spain, but it was used in several countries as France, and there are scarce studies focused on the degradation pathway of this herbicide.

Methods:
Extraction methods based on QuEChERS approach, using acetonitrile and magnesium sulfate and sodium chloride as well assolid phase extraction, using Oasis HLB cartridges have been developed in order to determinate dimethachlor and related metabolites in soil and water, respectively. Then, ultra high performance liquid chromatography (UHPLC) coupled to high-resolution mass spectrometry, applying Exactive-Orbitrap as analyzer, has been used.

Results:
In relation to the degradation studies of dimethachlor, it has been carried out in two types of soils, sandy loam and loam, at normal and double dose and in water under sunny and darkness conditions at normal and fivefold dose. It can be concluded that it degrades slowly in both matrices. For instance, when spiked soils were monitored, the initial concentration was 35 mg/kg and in seven days, the concentration is 31 mg/kg. In relation to metabolites, a targeted analysis determines that metabolites like dimethachlor CGA, dimethachlor SYN, dimethachlor SYN Na salt and dimethachlor oxalamic acid are detected 15 days after application in soils and after 30 days in water. On the other hand, untargeted analysis can allow us to discover other metabolites like CGA 39981, CGA 369873, CGA 72649, CGA 42443 and CGA 102935.

Conclusions
A new analytical method has been developed and validated for the determination of dimethachlor and related compounds by UHPLC-Orbitrap-MS. Due to the importance of the appearance of metabolites, UHPLC-Orbitrap-MS was used in targeted mode to monitor all the compounds generated in the process and untargeted mode to look for new compounds. Degradation of the parent compound occurred slowly in soils and water.

Novel Aspect:
Evaluation of the degradation of dimethachlor in soil and water applying UHPLC-Orbitrap-MS for the identification of metabolites by targeted and non-targeted approaches.

References
838 - PAVING THE WAY FOR THE ANALYSIS OF TRACE COMPOUNDS IN METALLURGICAL GASES BY PROTON-TRANSFER-REACTION TIME-OF-FLIGHT MASS SPECTROMETRY. IMPORTANCE OF BACKGROUND

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Keywords: Proton-transfer-reaction time-of-flight mass spectrometry (PTR-TOF-MS), Volatile organic compounds (VOCs), coke oven gas, blast furnace gas, catalyst poisoning

Introduction:
In the detailed trace analyses of industrial gases and catalytic processes the background level of carrier gases is of paramount importance. Wrong estimation may result in the underestimation of purification units [1]. Here, we focus on the analysis of the background of inert gases with different qualities at various humidity levels by proton transfer reaction (PTR) using PTR-QiTOF-MS.

Methods:
Measurements were performed with a Proton-Transfer-Reaction Quadrupole interface Time-Of-Flight Mass Spectrometer (PTR-QiTOF-MS) [2]. Prior to the measurements of the background, the measurement parameters for the substances of interest were optimized, which focuses on the minimization of the intensities of parasitic ions like N2H+, NO+, O2+, and fragmentation products. By means of a commercial gas calibrator, toluene was used for the optimization process.

Results:
With regard to the typical parasitic ions (N2H+, NO+, O2+, N3+), our results revealed that the most important effect was the change of humidity in the system. It was observed that some of these parasitic ions, such as N2+ and N2H+ showed a significant signal reduction in the presence of humidity, whereas ions like NH3H+ and NO+ remained unchanged. To date only the parasitic ions NO+, O2+ and water cluster H3O+(H2O)n have been considered in the literature as most dominant. In our study, the most dominant parasitic ion is N2H+ even after the optimization process. Our developed methodology is straightforward and allowed for assigning the origin of individual parasitic ions. Parasitic ions that are independent of the gas quality are exclusively produced in the ion source, whereas some parasitic ions which depend on humidity may be produced in the quadrupole interface. For most VOCs humidity showed an increase in sensitivity. For toluene no significant change was observed, which is in disagreement with previous results [3].

Conclusions
The characterization of VOCs in inert gases of different qualities with a PTR-QiTOF-MS and their humidity dependency in background measurements, were beneficial for the identification of instrument specific peaks. By changing the humidity it was possible to sense substances, which in dry conditions due to fragmentation were not measurable. Humidity is necessary in order to reduce the presence of parasitic ions generated in the quadrupole ion guide.
Novel Aspect:
A comprehensive determination of trace contaminants present in the background of typical inert gases by means of the prototype PTR-QiTOF-MS has not been reported before. A straightforward method to identify instrument specific substances is shown.

References

568 - NOVEL APPLICATION OF MOLECULARLY IMPRINTED POLYMERS WITH GAS CHROMATOGRAPHY-ATMOSPHERIC PRESSURE IONIZATION-MASS SPECTROMETRY FOR HIGH THROUGH-PUT DETERMINATION OF 16 PAHS

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Keywords: APGC-MS/MS, PAHs, Thin film extraction, water sample, Porous material

Introduction
Polycyclic aromatic hydrocarbons (PAHs) are common contaminants in marine and freshwaters. Identified as toxic, carcinogenic, and teratogenic, limits on the content of PAHs in water, air, and food are set by national and international regulatory agencies, e.g. the United States Environmental Agency (US-EPA). Traditional methods for extraction of PAHs from water are LLE and SPE [1,2] with excessive organic solvent use, and poor recoveries for certain species.

Methods
A thin film of molecularly imprinted polymer (MIP), designed to be selective for PAHs in water, was prepared on a glass substrate using a drop-casting method and UV-initiated radical polymerization. PAHs were extracted from 20 mL of aqueous sample into a MIP film over a fixed interval. Sorbed PAHs were extracted into a small quantity of solvent and analyzed using gas chromatography-atmospheric pressure ionization (APGC) coupled to a Xevo-TQ-S (Waters).

Results
APGC parameters can be optimized for soft ionization ideal for MS/MS measurements, e.g., dry source conditions were used here to produce molecular ions (rather than protonated molecules), to give the highest PAH signal-to-noise. Our PAH-specific MIP films are used much like solid phase microextraction and share common approaches to method optimization. For example, sample salinity, stirring rate and extraction time, desorption solvent, and desorption time, were optimized for the 16 PAHs. The best limits of detection (LODs) were achieved when sorption to the MIP reached equilibrium at ~3 h, but since there is little sample handling simultaneous extraction of many samples allows for high throughput. Since these MIP films are intended for single-use to eliminate any carry-over and cross contamination, inter-film reproducibility was evaluated and found to be <10%. The optimized MIP-APGC-MS/MS method was applied to real samples, e.g., produced water from oil and gas operations, to give fast, reproducible results at sub-ng/mL LOD.

Conclusions
A high throughput method is reported using porous thin MIP films with APGC-MS for detection of 16 PAHs from water. The methodology offers simplicity, reproducibility and speed, while minimizing consumption of organic solvents and waste generation. Results showed high extraction efficiency with good LOD which meet the maximum allowable concentrations set by the USEPA.

Novel Aspect
Single use MIP thin films designed for compatibility with APGC-MS/MS are developed as adsorption material for a high-throughput sample preparation for regulated PAHs in water.

References
369 - NEW ANALYSIS METHOD FOR ORGANOTIN COMPOUNDS BY TANDEM-LC/MS WITHOUT THE DERIVATIZATION STEP

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Keywords: Organotin compounds, LC/MS, Derivatization, Direct analysis

Introduction:

The analysis of organotin compounds, which are endocrine disrupter, is important in consumer products. The derivatization step with NaBEt4 for the analysis, however, the derivatization reactant NaBEt4 is hard to obtain because it’s highly expensive, flammable and only air delivery except Europe countries. In this study, we have developed a method for direct analysis method for organotin compounds by Tandem-LC/MS without the derivatization step.

Methods:

According to international environment regulatory trends, 8 kinds of organic tin compounds with one, two, or three alkyl substituents were analyzed. MBT, DBT, TBT, TPhT, MOT, DOT, TOT, and TCyT were obtained from Accustandard and Chiron. Analytical instrumentation was developed using a Tandem-LC / MS system consisting of Agilent Technologies' 1200 series HPLC and 6460 Triple Quad Mass Spectrometer.

Results:

Determination of optimum extraction solvent using Design of Experiment (DoE)

Different polarity of organotin compounds should be considered when determining the best extraction solvent for simultaneous extraction of various types of organotin compounds. For example, tri-alkylated organotin with a relatively large number of alkyl groups can be regarded as a nonpolar compound compared to moni, di-alkylated organotin, and non-polar compounds such as hexane may be more suitable as the extraction solvent. Therefore, in this study, Tropolone, a complexing agent, was added to methanol used as an extraction solvent in EN ISO 17353 and ISO / TS 16179, which is a conventional organic tin compound analysis method, and used as an extraction solvent.

Determination of Tandem-LC/MS analysis condition for direct method development

Tandem-LC/MS generates ion fragments from the ion source of injected analytes using the MRM mode and transfers only the precursor ion to the collision cell in the primary mass filter (Q1). It is a system that detects only the most characteristic ions by passing through secondary mass filter (Q2) by shipping. Direct analysis of organotin compounds using Tandem-LC/MS has developed in this study does not need a derivatization process through complicated pre-treatment steps.

Conclusions

A GC/MS analysis for organotin compounds in food and consumer products are popular method, but it needs a difficult derivatization step. The new method without derivatization by LC/MS is much simpler and safe and not time consuming. The direct method is using methanol solvent containing complexing agent, tropolone. The optimum
condition of extraction solvent was confirmed then the peak of each compound was obtained in chromatogram. Also international round test showed the validation of direct method by LC/MS.

Novel Aspect:

Direct analysis of organotin compounds using Tandem-LC/MS does not need complicated derivatization step. Therefore the extraction step is very short, much simpler and safer operators

References

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For information please contact: scientific@imsc2018.it
SELECTIVE AND RAPID QUANTIFICATION OF ATMOSPHERIC MONOTERPENES

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Keywords: SIFT-MS, SIFDT-MS, VOCs, TD, FastGC, quantification

Introduction:
The main objective is to develop a new analytical method for selective and rapid quantification of individual monoterpene isomers. This is especially relevant for atmospheric chemistry because different isomers exhibit highly variable reactivity with the OH• radical [1] and their role in formation of pollutants may be different for individual isomers. Thus, the precise measurements of their fluxes and concentrations will refine input data for numerical models.

Methods:
Soft chemical ionization technique of SIFT-MS [2] and its drift tube variant [3] have been used for absolute quantification of monoterpene isomers. Because SIFT-MS can quantify only the total concentration of monoterpene isomers, we employed more traditional separation method of fast gas chromatography, GC, [4] and thermal desorption, TD, and coupled them with Profile 3 instrument.

Results:
Separation by the fast GC was carried out on an electrically heated 5 m long non-polar MXT capillary column. Elution times of the isomeric compounds through short, rapidly heated column were characterised and optimised. Using the fast GC coupled to a Profile 3 SIFT-MS instrument we successfully separated eight of the most abundant plant monoterpene isomers (α-pinene, β-pinene, Camphene, Myrcene, 3-carene, R-limonene, α-terpinene and γ-terpinene). A combination of TD with SIFT-MS showed the ability for partial separation of isomers achieved by a slow temperature desorption profiles in contrast to the common approach of rapid desorption from TD tubes. The characterization of several sorbents was experimentally researched and tested. The optimal conditions for the TD approach such as the amount of sorbent, sampling flow rate, or sampling volume were defined.

Conclusions
The combination of fast GC or TD with SIFT-MS represents a promising and robust analytical method for selective and rapid quantification of monoterpene isomers. The advantage of fast GC is better separation in time of a minute, can be used in-situ, but does not allow the use of eddy covariance method. TD based method is very useful for long term monitoring of atmosphere and sample storage on TD tubes.

Novel Aspect:
Separation by TD profiles of the isomeric compounds trapped on sorbent was researched. Separation by fast GC using short capillary column was characterised and optimised.

References
CONTINUOUS-INTRODUCTION AEROSOL-TO-LIQUID SAMPLER AND ITS COUPLED USE WITH ICP-MS FOR ON-LINE SENSITIVE ANALYSIS OF ATMOSPHERIC AEROSOL

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Continuous-Introduction Aerosol-to-Liquid Sampler and its coupled use with ICP-MS for on-line Sensitive Analysis of Atmospheric Aerosol

Keywords: Continuous-Introduction Aerosol-to-Liquid Sampler; ICP-MS; on-line Analysis; Atmospheric Aerosol.

Introduction:
With the use of sampling pump[1], self-aspiration assembly[2] or aerosol introduction interface[3], ICP-MS is capable of on-line monitoring of atmospheric aerosol.However, parameters matching and conditions optimization are required prior to analysis. Moreover, concentration enrichment or matrix removal are necessary to overcome the matrix effect and sensitivity deterioration. Many types of aerosol-to-liquid samplers have been developed for efficient collection of aerosol into the liquid flow for subsequent analysis, i.e., ion chromatography[4], flow cytometry[5], GC-MS[6], LC-MS[7], ESI-MS[8], novel sensor[9,10].

In order to make full use of the advantages of the aerosol sampler and ICP-MS while avoid the possible interfere, a Continuous-Introduction Aerosol-to-Liquid Sampler (CIALS) was proposed and used in couple with ICP-MS for on-line, sensitive analysis of atmospheric aerosol. This paper presents a preliminary study conducted in the laboratory.

Theory and Methods:
Taking into account the arrangement of the three nozzles in the commercial BioSampler (SKC Inc.), a new collector matching the top part of BioSampler was proposed. One inlet for makeup liquid and one outlet for hydrosol are placed in the tangential but opposite position at the bottom of the collector. When CIALS is used in couple with ICP-MS, the makeup liquid flow rate (Q1) is controlled precisely by the peristaltic pump, the hydrosol flow rate (Q2) equals the nebulization flow rate of the ICP-MS, and the liquid loss rate (Q3) in the collector is dependent on the sampling gas flow rate mainly due to evaporation. The sampling mechanism of this sampler is identical to the BioSampler. Because the relative position of the nozzle and the collector wall is not changed, the size-dependent collection efficient should be very close to that of BioSampler[11].

By adjusting the speed of the peristaltic pump, the liquid mass in the collector could be kept nearly constant, which is crucial for efficient sampling of aerosol into the liquid. Assuming that individual droplet is fully dissolved as soon as it’s collected in the liquid, the sample concentration in the collector liquid would decay exponentially with time. The time constant equals the initial liquid mass (M0) divided by Q2.

Experimental and Results:
The above model was verified preliminarily through experiment in the laboratory. Firstly, ICP-MS (Model 8800, Agilent Inc.) was tuned by introducing 2%HNO3 through the sample tube and multi-elements standard solution (with concentration of around 1x10-9 g/g) through the “IS” tube simultaneously. The multi-elements standard solution was introduced throughout the experiment to calibrate the sensitivity of the total system. Secondly, the CIALS was connected to the nebulization system of ICP-MS through a PTFE tube. The liquid flow was continuously extracted for sequent nebulization and analysis. Meanwhile, the makeup 2% HNO3 was introduced to compensate the liquid mass variation in the collector. The inlet of CIALS was placed downstream of a HEPA filter to sample filtered air. The background of target elements in the total system were monitored by ICP-MS when the vacuum pump was running. Thirdly, 10 microliters of standard solution containing natural Ce, Tb and Ho (214.5x10-6 g/g) was transferred through pipette and dropped into the collector liquid when the vacuum pump was turned off. Finally, the signals of 205Tl (only from “IS” solution), 140Ce (from “IS” solution and sample), 159Tb and 165Ho (both only from sample) were monitored by ICP-MS.
The relative variation of $^{205}$Tl intensity is $\sim$7%, much higher than the typical value ($\sim$2%) when CIALS was not coupled with ICP-MS. Hence, intensity correction using internal standard is necessary. It's clearly observed that $^{140}$Ce signal is a sum of stable signal from IS solution and variable signal from sample liquid. The signal profiles of $^{159}$Tb and $^{165}$Ho after adding of individual droplet both satisfy exponential decay law very well. The fitted values (magnitude and time constant) of the two elements math well with each other, indicating that they synchronously disperse, diffuse, and transport in the system. By using signal integration method and internal standard correction, it is possible to obtain the total amount of element in the individual particle. The fitted value of the time constant is $\sim$1000 s, which roughly agrees with the value (814 s) predicted by the model. This suggests that the current system is able to monitor atmospheric aerosol with time resolution of $\sim$15 min.

Conclusions and Novel Aspect:

a Continuous-Introduction Aerosol-to-Liquid Sampler (CIALS) capable of on-line sampling, efficient collection of particle into liquid and continuous introduction to ICP-MS was proposed. A theoretical model for predicting the sample concentration in the collector liquid was derived. The CIALS was used in couple with ICP-MS and preliminary experiments was conducted in the laboratory. Results show that the total amount of element in individual soluble particles could be determined by using the current system. The combination use of CIALS and ICP-MS has a time resolution of $\sim$15 min for on-line monitoring of atmospheric aerosol.

References
612 - ACETONE DEGRADATION IN A PLASMA REACTOR STUDIED IN REAL-TIME BY PTR-MS

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Keywords: CIMS, volatile organic compound, FTICR, PTRMS, pollutant removal

Introduction:
Acetone is one of the major pollutants in the Earth’s atmosphere [1]. So that it is becoming more and more necessary to reduce acetone anthropogenic emission. The new non-thermal plasma technique for pollution control has received increasing attention [2]. While there has been recent progress in acetone removal, clear identification of the reaction products, eventually toxic, to be done.

Methods:
We used a photo-triggered low-temperature plasma discharge [3] to study the mechanisms of acetone decomposition at different concentrations in N2 and N2/O2 mixtures. Degradation by-products are detected in situ by a compact FTICR mass spectrometer BTrap [4], that uses chemical ionisation by proton transfer reaction from H3O+ [5] or by charge transfer from O2+. Identification and direct quantification of the molecules is conducted in real-time in the instrument.

Results:
Presence of oxygen in the gas mixture reduces the discharge efficiency, even for a small O2 amount, up to 5%. The decomposition of the molecule entails the formation of many by-products. For acetone diluted in N2, at least 20 different compounds were detected. The major detected by-products include nitrile compounds (hydrocyanic acid and acetonitrile), formaldehyde and acetaldehyde, alcohols (mainly methanol and ethanol). Some products, such as hydrogen cyanide, appear at the first discharge and increase rapidly. These products are then reprocessed but not completely. These results are compared with those of acetone decomposition in N2/O2, which leads to oxygenated compounds such as nitro-alkanes.

Conclusions
The large number of by-products leads to better understanding of the mechanisms involved in plasma degradation. Our work shows that the non-thermal plasma technique degrades efficiently acetone in N2 and N2/O2 gas mixtures. We plan to extend these analysis methods to others discharge conditions with or without associating catalysts.

Novel Aspect:
Coupling of a high resolution compact FTICR to a VOC degradation plasma reactor and real-time monitoring of by-products.

References:
180 - COMPREHENSIVE DEPTH PROFILING OF STATEN ISLAND SOIL BY GC AND PETROLEOMICS

Mary Thomas (1) - Emma Collinge (2) - Matthias Witt (3) - Christopher Vane (4) - Mark Barrow (2)

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Keywords: Contaminated soil, petroleomics, GC, FTICR MS

Introduction: (Limit of 400 characters)
Staten Island is located in the New York/New Jersey (NY/NJ) Estuary, a region with one of the highest population densities in the United States. Surface sediment concentrations showed the NY/NJ Estuary to be among the most chemically contaminated waterways in the United States. Analysis of the extent of environmental contamination by complex petroleum-related pollutants is of rising importance.

Methods: (Limit of 400 characters)
Sediment cores were collected in a marsh region of Staten Island, NY and freeze-dried. Samples taken at intervals to 1 m depth were spiked with PAH standard mixture and subjected to solid phase extraction (SPE) before bulk analysis by GC MS. Sediment samples from 5 selected depths underwent Soxhlet extraction prior to compositional analysis by direct infusion APPI-FTICR MS and GC-APCI-FTICR MS.

Results: (Limit 900 characters)
Saturate, aromatic and resins (SAR) GC MS analysis at each sampling depth showed a sharp increase in concentration at two depths that may correlate with the timing of major spills in the area, including the 1990 Exxon pipeline spill. Direct infusion APPI-FTICR MS measurements showed a corresponding spike in the relative contributions to spectral intensity from radical hydrocarbon as well as oxygen and sulfur-containing compound classes at these depths. However, background sampling depths were also found to have strong contributions from petroleum-related contaminants, indicating continuously poor water and soil quality in the NY/NJ Estuary resulting from regular oil spills in the region. Using GC-APCI-FTICR MS additional compound classes were observed, revealing the impact of leaks of industrial chemicals and agricultural effluent on the estuary system.

Conclusions (Limit of 400 characters)
Several trends in the compositional profiles obtained by direct infusion APPI-FTICR MS and GC-APCI-FTICR MS were found to correlate with the bulk analysis obtained by GC-MS, including relatively strong contributions from OxSy[H] compound classes. Samples originally obtained for background study were found to contain significant contamination from anthropogenic sources.

Novel Aspect: (Limit of 150 characters)
Comprehensive study of petroleum-contaminated soil by GC-MS, direct infusion APPI-FTICR MS and GC-APCI-FTICR MS.
Uptake of Dioctyl phthalate by Populus alba Villafranca

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**Uptake of Dioctyl phthalate by Populus alba Villafranca**

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**Keywords:** DOP, micro-pollutants, phthalates, poplar, xenobiotics

**Introduction:** Phthalates are micro-pollutants detected in aqueous systems and their removal through wastewater treatment plants results ineffective [1,2]. The accumulation and metabolism of organic pollutants (caffeine, erythromycin, sodium dodecyl sulfate) in poplar has been demonstrated [3,4,5,6] and in this study we evaluated the poplar capability to uptake and translocate dioctyl phthalate inside the plant.

**Methods:** Plants of Populus alba Villafranca clone, growing in a hydroponics condition, were treated for 21 days with 0, 40 and 400 µg L-1 of dioctyl phthalate (d4-DOP). The health status of the plants was monitored through the plant growth, relative chlorophyll content and fluorescence analyses. The d4-DOP concentrations in roots, stem and leaves were evaluated through LC-MS/MS analysis.

**Results:** Treatments did not affect significantly poplar health at none concentrations tested: plants maintained a good photosynthetic performance and no variation amongst relative chlorophyll contents were observed between control and treated d4-DOP plants. After 21 of treatments, 400 µg L-1 d4-DOP induced an increase in root dry biomass. On the contrary, leaves number and leaf dry weight were reduced. Poplar revealed the ability to uptake the d4-DOP with the highest accumulation observed in roots. Indeed, the d4-DOP concentration in roots reached 7.1±3.29 µg g-1 FW and 3.4±2.28, after 1 day and 21 days of treatments respectively. In leaves d4-DOP concentration values were lower than 0.02 µg g-1 FW.

**Conclusions:** P. alba Villafranca showed a good tolerance to d4-DOP at both concentrations tested and the accumulation of d4-DOP occurred mainly in roots after one day of treatment.

**Novel Aspect:** For the first time tree removal of DOP from aqueous solutions was explored. Our findings can contribute to the development of green technologies for the remediation of phthalates.

**References**


IDENTIFICATION OF DEGRADATION PRODUCTS OF SELECTED PHARMACEUTICALS IN SOILS BY HIGH RESOLUTION MASS SPECTROMETRY

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Keywords: mass spectrometry, pharmaceuticals, degradation products, soils

Introduction
Pharmaceuticals are frequently detected in different matrices, including soils, in the last decade[1, 2]. Different degradation/transformation products can be formed by interaction of soil bacteria with pharmaceuticals [3]. Only limited information on this topic is available, we aimed to study the fate of the selected pharmaceuticals and their possible metabolites in the different soils.

Methods
Fortified soils samples were incubated under controlled conditions, extracted and analysed with LC-ESI-full scan HRMS (QExactive, Thermo Fisher Scientific) at a resolution of 70,000 FWHM. Compound Discoverer 2.0 software was used for data processing. The high resolution product scan (HRPS) at 17 500 FWHM resolution was applied to list of suspect metabolites to obtain the MS2 fragmentation.

Results
The degradation of 6 pharmaceuticals (carbamazepine, citalopram, clindamycin, fexofenadine, irbesartan and sulfamethoxazole) was investigated in 4 different soil types. The parent compounds degraded in all of the soils. Dissipation curves for the parent compounds of interest were constructed to evaluate a degradation rate by means of slope values. We obtained list of suspect transformation products from two different workflows in CD software for each compounds. The list was further evaluated using HRPS data to confirm or disprove proposed compounds. Tentatively identified TPs were semiquantified with method described by Koba[4]. Time trends of TPs concentration could further help in both degradation mechanism and kinetics clarification.

Conclusions
Using sequence of full scan HRMS, advanced data processing, HRPS structure confirmation and back semi quantification from HRMS we were able to identify degradation products of selected pharmaceuticals and obtained important information on transformation of pharmaceuticals in soil.

Acknowledgements
This study was supported by the by the Grant Agency of Czech Republic (No. 17-8937S)

Novel Aspect
Identification and semi quantification of degradation products of pharmaceuticals in soils significantly increase information value of analysis.

References
Abstract: The Study of Sampling Pipeline Effect for Gases Partial Pressure Measure with Mass Spectrometer

Keywords: Mass spectrometer, Gases partial pressure measurement, Sampling pipeline, Stability time

Introduction: In some gases analysis or partial pressure measure with mass spectrometer, The gases are far from the mass spectrometer because mass spectrometer is not portable or the gases environment is toxic. The sampling pipeline connecting gases and mass spectrometer has to be used. But the sampling pipe how to affect measure results such as stable time, ingredient aberration, which are worthwhile to study.

Methods: Based on the study of unsteady-state gas flow with the effect of pipeline adsorption, the physical mechanism of gases at molecular regime in pipeline flowing into mass spectrometer chamber and pumped out by vacuum system has been studied. The gases transportation equation in sample pipeline and gases partial pressure distributing equation in mass spectrometer chamber are set up. The effect of sample pipeline for some gases partial pressure in air are calculated.

Result: The relation equation of sample pipeline gases pressure, the mass spectrometer chamber gases pressure, analysis time, pumping rate to the chamber, gas molecular mass, pipeline length has been got.

The pressures in sampling pipeline gas are a complex exponential equation with sampling time and sampling pipeline distance;

The gases balance pressures in mass spectrometer chamber are direct proportion to the gases pressure of the sampling place;

The sampling pipeline change the ration of gases ingredient. After sampling equilibrium, the ratio of partial pressure is inverse to the square root of the ratio of gas mass. The pressure in mass spectrometer chamber is inverse to the length of pipeline.

(4) The minimum pressure stable time in chamber is proportion to the square of pipeline length and inverse to gas molecule thermal motion speeds.

Novel aspects: The mass spectrometer gas pressure equation with sample pipeline length and sample time was deduced. The stable time and ingredient aberration of gases partial pressure measure with mass spectrometer can be calculated.

References: abbreviation
Direct Analysis of 17 Perfluorinated Compounds in Water at Low Parts-Per-Trillion Levels by LC-MS/MS Workflow

Sha Josh Ye (1) - Jingcun Wu (1) - Feng Qing (1) - Frank Kero (2) - Luca Piatti (3) - Stefan Edler (3) - Derek Mattern (3) - Marco Gori (4)

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Direct Analysis of 17 Perfluorinated Compounds in Water at Low Parts-Per-Trillion Levels by LC-MS/MS Workflow

Sha Josh Ye, Jingcun Wu, Marko Gori, Luca Piatti, Derek Mattern, Stefan Edler, Frank Kero, Feng Qin, 501 Rowntree Dairy Road, Unit 6, Woodbridge, ON, L4L 8H1, Canada

Keywords: Direct Analysis, LC-MS/MS, perfluorinated compounds

Introduction: (Limit of 400 characters)
Perfluorinated compounds (PFCs) are used as surfactants, fire-retardants, nonstick cookware coatings, and coatings for paper packaging for over a half century[1,2]. PFCs have recently received attention as emerging persistent organic pollutants [3-14]. Presently, a LC-MS/MS direct sample injection method was developed for analysis of PFCs in water samples. The results indicate that a good sensitivity, robustness were achieved with LOQ below the proposal by EU.

Methods: (Limit of 400 characters)
The PFCs native and internal standards were obtained from Wellington Laboratories (Guelph, Ontario). 100 µL of lake or tap water samples were first filtered then injected onto a PerkinElmer Brownlee SPP C18 (100x2.1mm, 2.7µm) column for separation at 0.5 mL/min with total run time of 15 minutes. The mobile phases were A) water and B) methanol, both containing 5mM ammonium formate. Detection was via a QSight triple quadrupole LC-MS/MS system.

Results: (Limit 900 characters)
The calibration standards were prepared with different levels of PFCs to determine instrument detection limits and set up calibration curves. Detection limits for most analytes were <= 1 ng/L except for PFBA, PFTA, PFHxDA, and PFODA which were <= 5 ng/L. The calibration curves for all analytes were linear over three orders of magnitude with a R^2 value of > 0.995. Identification and confirmation of the analytes in investigated samples was achieved by comparing their respective retention times and peak area ratios between the quantifier and qualifier MRM transitions with those from the standards. Ten (10) samples were screened for PFOA and PFOS with the developed method. Five of them were detected with PFOA at a concentration of larger than 2 ng/L and six of the samples were detected with PFOS at a concentration of larger than 5 ng/L.

Future work would include further development of shorter LC separation time, robustness evaluation, and the analysis of other brands of bottle water and water from rivers or lakes using the workflow developed in this study.

Conclusions (Limit of 400 characters)
The fast and simple LC-MS/MS method for 17 perfluorinated compounds analysis in water samples was developed by coupling a UHPLC system to a Qsight 220 triple quadrupole mass spectrometer. This method can be employed to the direct determination of PFCs in drinking and surface water samples, with LOQs well below the limits set by regulatory boards from many countries.
Novel Aspect: (Limit of 150 characters)
Direct analysis of perfluorinated compounds in water at low ppt levels with improved sensitivity and robustness by a simple LC-MS/MS workflow.

References

10. FACT SHEET PFOA & PFOS Drinking Water Health Advisories, US Environmental Protection Agency (2016).
1332 - DIRECT ANALYSIS OF PHENOLIC COMPOUNDS IN CORK WASTEWATER BY HPLC-QTOF

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Keywords: cork, phenolic compounds, HPLC, QTOF

1.Introduction:
Cork is the outer surface of the cork oak tree and it is a versatile material that can be used for a variety of products, mainly stoppers. Industrial preparation of cork requires the immersion of cork planks for approximately one hour in boiling water. Cork boiling wastewaters (CBWs) are waste streams with high loads of phenolic compounds (1). This work studies the direct analysis of CBWs with HPLC/QTOF in order to identify useful by-products for their recovery.

2.Methods:
The samples were analysed by HPLC on a reversed-phase column RP-18 (250 nm x 4 mm x 5 µm). and detection was carried out using a diode array detector. Other sub-samples were analysed by HPLC-QTOF in order to confirm the target compounds.

3.Results:
Several low-weight phenols were detected with HPLC-DAD by comparing UV-vis absorption maxima in mass spectral analysis and the retention times of standards. Mass spectra of these phenolic compounds were compared with the corresponding standards by QTOF. 18 target compounds were studied: gallic, protocatechuic, ellagic, p-hydroxyphenyl acetic, p-hydroxybenzoic, vanillic and syringic acids; esculetin, vanillin, syringaldehyde, ferulic, caffeic, p-coumaric and salicylic acids; coniferyl aldehyde, sinapaldehyde, eriodictyol and naringenin. From these phenolic compounds, significative concentrations of vanillin and gallic, ellagic, vanillic, protocatechuic, ferulic and syringic acids were detected, confirmed and quantified. Besides, some characteristic ellagitannins were also analyzed but they were not detected.

4.Conclusions:
The direct analysis of CBWS by HPLC/QTOF confirmed several low weight phenolic compounds highlighting the presence of vanillin in and gallic, ellagic, vanillic, protocatechuic, ferulic and syringic acids. This kind of compounds and their concentrations coincides in general terms with other studies (1). Regarding extractable compounds from cork such as ellagitannins didn’t appear, maybe due to the higher temperature of the boiling process.

5.Novel Aspect:
This work shows that the direct analysis of phenolic compounds in CBWs without pre-concentration achieved good results and made the tests simpler.

References:
1. Isabel Paula Marques, Luis Gil, Francesco La Cara, Biotechnology for Biofuels, 7, 67 (2014).
MOLECULAR COMPOSITION OF DISSOLVED ORGANIC MATTER IN THE TALDICE ICE CORE.

Roberta Zangrando (1) - Veronica Zanella (2) - Ornella Karroca (2) - Elena Barbaro (1) - Natalie M. Kehrwald (3) - Dario Battistel (2) - Andrea Gambaro (2) - Carlo Barbante (1)

Consiglio Nazionale delle Ricerche, Institute for the Dynamics of Environmental Processes, Mestre-Venezia (1) - Ca’ Foscari University of Venice, Department of Environmental Sciences, Informatics and Statistics, Mestre-Venezia (2) - U.S. Geological Survey, Geosciences and Environmental Change Science Center, Denver (3)

Keywords: Untargeted analysis, ice cores.

Introduction:
Deep polar ice cores are archives that collect information on paleoclimatic and paleoenvironmental conditions during glacial-interglacial swings. This information is then obtained by detailed analyses of the ice to obtain past temperature, greenhouse gas concentrations, and sea ice extent etc. The presence of trace chemicals in ice, reflect changes in their sources, transport, deposition, preservation.

Methods:
An untargeted analysis of dissolved organic matter (DOM) provides a general over view of molecular species in the ice samples giving information on their sources and processes. For this kind of analysis typically 100 - 900 mL are required, this is not possible when dealing with deep ice cores. Using a nano-UPLC-nano-ESI-HRMS technique we have detected the major molecular species using only 4 µL of melted ice.

Results:
This work allowed us to tentatively identify generally aliphatic species, that were attributable mainly to the presence of saturated and unsaturated fatty acids, hydroxyl fatty acids and their degradation products, along with species from the oxidation of isoprene and monoterpenes and other compounds more difficult to interpret. Interglacial had more species while their number decreased in glacial samples. The reduction in oxidation products of isoprenes and monoterpenes is probably due temperature changes that affected terrestrial vegetation and the sea ice extent. Passing from interglacial to glacial times, unsaturated FAs almost disappeared because they are exposed for longer to oxidative processes.

Conclusions
Until now untargeted analyses have been limited to surface snow and shallow ice cores covering a short time span. To the best of our knowledge these are the first indications of the behavior of fatty acids and their oxidation products and the oxidation products of isoprene and monoterpenes in a deep ice core.

Novel Aspect:
The innovative aspect of this work was the creation of a micro-volume analytical method that allowed the molecular characterization of DOM in a deep ice core.
Keywords: Rare earth elements (REEs); (Inductively coupled plasma mass spectrometry) ICP-MS; Graphene oxide (GO); Adsorption;

Introduction:
Rare earth elements (REEs) are major resources for modern society but have the risk of environmental pollution. With a high surface area, carbon nanomaterial shows the good adsorption character and has the bright future to become a novel REEs separation material [1]. Herein, we demonstrate the special adsorption behavior on REEs by graphene oxide. The mechanism is also investigated and has the chance of being applied in the comprehensive ecological improvement.

Methods:
GO nanosheets were synthesized from natural flaky or expanded graphites using the modified Hummer Methods:
The physicochemical properties of GO nanosheets were characterized by SEM, FTIR and Raman respectively. We tested the adsorption behavior of GO nanosheets on Rare earth elements with different concentrations. After centrifugation and filtration, the solution was transferred into ICP-MS to obtain residual content of REEs.

Results:
We obtained GO with 5 different meshes from natural flaky graphites and 3 giant GO with pre-expanding process. The SEM results exhibited that GO surface morphology was well nanoscale thin layer. FTIR spectra indicated that GO had similar O-containing functional groups as mentioned [2]. It was worth mentioning that GO of 500 and 200 meshes had higher carboxyl, aromatic, sharp C=O and –OH, where as the giant GO of 100 mesh had higher –OH. Raman spectroscopy showed that GO of 300 and 100 meshes had more defects/edges. Through the key ICP-MS detection, we found GO had superior adsorption ratio on REEs to other inorganic elements even including heavy metals. Then, we compared the adsorption behavior on 17 REEs with 5 different concentrations. When at proper concentration of REE, GO with 500, 200 and 100 meshes exhibited more excellent adsorption ratio. Surprisingly, neither a lower nor a higher concentration of REEs could display adsorption difference. The accordance of adsorption and characterization made us to illuminate the adsorption mechanism properly.

Conclusions
Comparing with other inorganic elements, we verified the superior adsorption on REEs by GO which was fabricated by modified Hummers Methods: The corresponding adsorption mechanism was illuminated that aromatic, carboxyl, sharp C=O and –OH contribute to the whole special adsorption behavior. The proper GO meshes should have to be selected to separate or enrich REEs in the future environmental pollution.

Novel Aspect:
GO has special adsorption behavior on REEs superior to other inorganic elements and the mechanism is also been illuminated.

References
Development of LC-MS/MS Analytical Protocol for Artemisinin and Its Derivative Compounds Quantification in the Artemisia Annua L. Extracts for Cosmetic Applications

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Keywords: Artemisia annua, artemisinin, supercritical extraction, LC-MS/MS

Introduction:
Artemisia annua L., a plant used as traditional Chinese herb medicines, is rich in sesquiterpene lactones that present a wide range of biological activities, exhibiting therapeutic potential as anti-inflammatory and antimicrobial. Here, an improved liquid chromatography-tandem mass spectrometry (LC-MS/MS) protocol was developed to quantify artemisinin and its derivative compounds in A. Annua extracts obtained by supercritical CO2 fractional extraction.

Methods:
A. annua extracts, including essential oil, fraction rich in artemisinin and a residual fraction were obtained using supercritical CO2 fractional extraction, under optimized temperature and pressure experimental conditions. Thin layer chromatography (TLC) and liquid chromatography-tandem mass spectrometry (LC-MS/MS) were used, respectively, for qualifying and quantifying, artemisinin and its derivative compounds in the extracts.

Results:
The developed LC-MS/MS method covers both the unambiguous identification and simultaneous quantification of artemisinin and its derivative compounds, while TLC protocol was effective for artemisinin semi-quantification during the optimization of the supercritical extraction process, as a rapid screening method. Using multiple reaction monitoring (MRM) method, artemisinin, deoxiartemisinin and dihydroartemisinin were detected as ammoniated adducts. For validation purposes, an A. annua extract, denominated E4, obtained by supercritical extraction, was used. Calibration curves were prepared in the concentration range of 50-500 ng/mL. Analytical parameters such as linearity, precision, accuracy, limit of detection (LD) and limit of quantitation (LQ) were established. The data indicated that the linearities resulting from the three analytes of interest showed correlation coefficient (r) ≥0.99. LD and LQ were 25 and 50 ng/mL, respectively. The relative inter and intraday standard deviation (RSD) was <5%.

Conclusions:
The developed analytical methodology can be used for quality control of A. annua extracts due to be able to quantify simultaneously artemisinin, deoxyartemisinin and dihydroartemisinin. The standardized extracts could be use in cosmetic formulations.

Novel Aspect:
Standardized and certified extracts from A. annua using LC-MS/MS protocol could be used more safely in new and effective natural cosmetic products.
Introduction
Bioinformatic approaches are shaping multiple scientific fields and such tools have particularly impacted natural products chemistry in the latest years. The ever improving capabilities of mass spectrometry analytical platforms now allow to acquire signals of quality for the hundreds to thousands analytes constituting complex biological matrices. At the moment, such metabolomics approaches are the most appropriate to characterize natural products extracts in a comprehensive way.

Methods
In our sense, two main challenges emerge when implementing and developing such approaches. On one side, and this defy is well identified within the community, important progresses are to be done regarding metabolite identification tools and strategies.[1] On the other hand, we propose that what we define as contextualization approaches will be crucial to fully grasp the complexity of natural products and establish the discipline of pharmacognosy in the digital era.[2]

Results, Conclusions and Novel Aspects
Here we will present some of these contextualization strategies developed in our lab. After explaining the role of central tools such as molecular networking and in-silico fragmentation approaches,[3] we will present the establishment of a bioactive natural products prioritization pipeline based on the establishment of multi-informative molecular maps.[4] We will discuss application example, evaluation methods and possible further developments.

References
METABOLOMIC UHPLC-QTOF METHOD TO ANALYZE TANNINS IN NATURAL COMPLEX PRODUCTS USING ALL-IONS FRAGMENTATION ACQUISITION

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Keywords: Tannins, Mass Spectrometry, All-Ions, Metabolomic

Introduction
Tannins are a class of compounds naturally occurring in some plants. They can be classified into condensed tannins (or proanthocyanidins, PAs) and hydrolysable tannins (HTs), further sub-classified in galloyltannins (GTs) and ellagitannins (ETs). Structurally, they are the most complex class of polyphenols as they are polymeric molecules. Here is described a metabolomic LC-HRMS method using All-Ions Fragmentation acquisition (AIF)[1] [2] to increase natural tannins identification.

Methods
Green tea leaves dried-extract (GTE) has been analyzed by means of UHPLC-qToF using a reverse phase chromatographic column, in high resolution(2GHz) and using AIF. In negative ion mode, AIF has been acquired at four different energies: 0, 20, 30, 40 eV. The EICs of fragment ions, from the high energy scan data, have been correlated to the precursor ion, from the low energy scan data. The EICs of each tannin subclass typical-fragments has been used to get tannins semi-quantitative data.

Results
Tannins are polymers characterized by certain degree of polydispersity, so that actually it is not possible to find on the market reference standards for all the possible molecular structures. In this context, the analysis in AIF shows its potential to study natural complex products containing tannins, as using this algorithm the fragment ion of UHPLC eluted compounds is correlated to its precursor. Combining the MS/MS information with retention time, high resolution accurate mass and product/precursor ion intensity ratios, it has been possible the identification and annotation of various tannins in GTE. After the creation of a specific tannins in-house database and the study of the tannins fragmentation behavior, the following fragments common to each tannin subclasses have been identified: ions at m/z 125.0236 and m/z 169.0140 for GTs, m/z 300.9980 for ETs, m/z 289.0719 and m/z 577.1358 for PAs. The annotated GTE tannins have been semi-quantified by external regression curves of Gallic acid (ions at m/z 169.0140) for GTs, Ellagic acid (ions at m/z 300.9980) for ETs and Procyanidin B1 (ions at m/z 577.1358) for PAs.

Conclusions
If reference standards are available, tannins can be analyzed by comparison with the corresponding compounds. Incase this it is not possible, metabolomic screening by AIF is an effective alternative! The approach presented here has allowed us to identify more than twenty GTE tannins, increasing the quality of the information on tannins, opening new perspectives in routine quality control oftannins as well in understanding their fate in biological studies.

Novel Aspect
A new metabolomic method for analyzing tannins in natural complex products while references standards are unavailable on the market is now available.

References
438 - NEW APPROACH FOR THE STRUCTURE ELUCIDATION OF (ACYL)POLYAMINES IN SPIDER VENOMS BY UHPLC-HR-ESI-MS/MS

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Keywords: acylpolyamine, spider venom, H/D exchange, database, in-silico fragmentation

Introduction: Acylpolyamines, often found in spider venom, are promising lead compounds to treat cancer or neurodegenerative diseases like Alzheimer’s disease, epilepsy or stroke. [1,2] Polyamines follow characteristic fragmentation rules; therefore MS/MS is ideal for their structure elucidation. [3] However, low available sample amounts, co-eluting isomers and diverse nomenclature strategies in literature make the structure elucidation demanding.

Methods: The fragmentation rules for linear polyamines were used to develop a new database with in-silico fragmentation for known and unknown acylpolyamines. The spider venom was analyzed by UHPLC-HR-ESI-MS and MS/MS on a QExactive. The method was optimized for small, basic molecules. In addition, the number of acidic protons was determined in an on-line H/D exchange experiment (HDX).

Results: The database of spider venom acylpolyamines provides fast access to the related literature and acquired MS/MS spectra. Therefore, already known acylpolyamines are identified easily and time can be used to focus on the structure elucidation of unknown compounds. Database entries for new acylpolyamines can easily be generated by assembling the structure from simple building blocks. The separation of co-eluting isomers could be improved. The HDX experiment clarified the presence of N-methylated amines and supports the structure elucidation in general. The optimized method allowed the identification of more than 90 polyamines from just one microgram lyophilized venom of the spider Agelenopsis aperta. More than half of the structures were not described before.

Conclusions: The improved analytical method in combination with the developed acylpolyamine database enables the routinely identification of linear polyamine amides even from sources of limited sample amounts like spider venoms. The in-silico fragmentation enables the identification of polyamine structures without the analysis of expensive reference material.

Novel Aspect: The database with in-silico fragmentation supports the annotation of known and the structure elucidation of new acylpolyamines based on HR-MS/MS and HDX.

References:
CHARACTERIZATION OF BETA AMINO ACID ESTERS IN ONONIS SPECIES

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Keywords: phytochemistry, beta amino acids, isoflavonoids, fragmentation, quantitative

Introduction:
The non-protein amino acids are widespread in the flora as important molecules against insect herbivores. Based on our previous results, Ononis species contain beta amino acids mainly in the form of isoflavonoid glucoside esters. The beta amino acid structural motif is common in CNS drugs. As Ononis species are used in traditional medicine, we aimed to characterize these structures as well as to define their quantity.

Methods:
For the structural identification HPLC-HR-MS/MS, authentic standards, isolation and NMR experiments were used. HPLC-MS was applied to screen for these molecules in various Leguminosae plants. Since the quantitation of the beta amino acid was performed for its free form, a prior hydrolysis method has been developed. To minimize the matrix effect during the quantitative HPLC-MS experiments, a further SPE method was applied for purification purposes.

Results:
Based on the MS/MS fragmentation of the investigated molecules, six isoflavonoid glucosides and two beta amino acids could be assumed, resulting in altogether 12 compounds. The structures of isoflavonoid moieties were identified as formononetin, pseudobaptigenin, medicarpin, maackiain, onogenin and sativanone because of their specific MS fragmentation pattern [1]. The HR-MS/MS results combined with NMR data and comparison with authentic standards proved that homopipecolic acid and homoproline esterify the isoflavonoid glucosides. The presence of the esters was investigated in various Leguminosae plants (O. spinosa and arvensis, Trifolium repens, Glycine max, Medicago sativa), however, those were only characteristic for Ononis species. The roots of M. sativa also contained trace quantities while none of the other plants contained these molecules at all. Homopipecolic acid content of Ononis species ranged from 1mg to 35 mg/100 g dried plant, while homoproline levels were between 0.1 mg and 16 mg/100g.

Conclusions:
The structures of 12 isoflavonoid beta amino acid esters were elucidated. Screening various Leguminosae plants, only Ononis species contained them in significant amount, thus, these molecules could serve as chemotaxonomic markers. Levels of the beta amino acids were also quantified in these plants. Comparing these values with the ones required to induce CNS effects [2], [3] one can conclude that the rational use of Ononis species as traditional remedy, is safe.

Novel Aspect:
This work presents the structure of 12 new isoflavonoid beta amino acid esters and firstly describes the quantity of cyclic beta amino acids in Ononis species.

References:

For information please contact: scientific@imsc2018.it
AN UNUSUAL TMS ARTIFACT IN EI MASS SPECTROMETRY - PERRHENIC ACID TMS

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Keywords: TMS derivatization artifacts, surface reaction

Introduction:
Trimethyl silyl derivatives (TMS) are one of the most common methods for extending the range of compounds for which GC/MS methods can be used, however as with most methods where reactions are involved there are often undesirable by-products [1]. Here we report an unusual artifact that appears to be caused by the mass spectrometer filament – specifically Perrhenic acid-TMS (Re(O3)-O-TMS) appears to be formed during the ionization process.

Methods:
Pure samples of compounds were analyzed both as the native and TMS derivatized compounds. The derivatization was carried out with BSTFA and the analysis was done with an Agilent 5977 MS with an Agilent 8939 GC. Chromatography used 15M Agilent VF-5MS column, 40:1 split ratio, 270C inlet temperature, program 50C start, ramp 15C/min to 300, ion source temperature 250K. All data were analyzed using AMDIS.

Results:
Several TMS spectra had low intensity ions at m/z 307,309. In some spectra these ions were above the expected MW and in some they were unreasonable losses from the molecular ion. The AMDIS deconvolution showed that these ions did not have the same time history as the main ions in the spectrum – they always appeared slightly delayed. However, the ions could not be arising from a distinct compound formed in the derivatization process since the ion maxima were always nearly coincident with the derivatized compound regardless of the elution time. Some the data files had separations in time between the 307/309 maxima and that of other ions was sufficient that AMDIS could extract a distinct spectrum. Comparing this spectrum with the NIST library gave a match factor of over 900 to Perrhenic acid-TMS (Re(O4)Si(CH3)3. Careful examination of the smaller peaks in the spectra when the Perrhenic acid spectrum could not be deconvolved showed that the other expected peaks (294/292, 279/277, and 263/261) were seen with the isotope ratios expected for Re.

Conclusions:
TMS derivatives reacted with the rhenium filament in the ion source to produce Perrhenic acid-TMS. The reaction product was typically slightly delayed in time from the derivative, but in some cases the temporal separation was not sufficient to allow for deconvolution of the pure derivative spectrum. Aging of the filament over a 2 month period removed the active sites on the filament and the perhenic acid TMS was no longer observed.

Novel Aspect:
Perrhenic acid-TMS from reaction of the rhenium filament and TMS derivatization is reported. The time history was used to determine the identity using AMDIS.

References:
Introduction
Nowadays the study of chemical compounds from natural sources is an established method to obtain new active substances. This strategy is used for the treatment of many different diseases and natural products show anti-inflammatory, antidiabetic, analgetic and anticancer activity.[1] We engage ourselves with the extraction, separation and characterization of various natural materials to contribute to the treatment and prevention of intractable diseases.

Methods
We use a two step method for the extraction which includes the use of ultrasound and microwave radiation. The polar extracts are then separated via HPLC to obtain fractions containing multiple substances and pure compounds. The complex mixtures are analyzed with LC/MS and GC/MS. Full scan experiments with two scan modes give us a good overview of the extract composition. For further information we apply target molecule scans and tandem mass spectrometry (MSn).

Results
Following our two step extraction method we were able to produce different plant extracts. We extracted various natural sources like sea buckthorn, chokeberry, cornelian cherry and Chinese medical plants using mainly ethanol and ultra pure water as extraction solvents. With the help of our LC/MS and GC/MS devices the identification of many compounds from different substance classes was possible. Especially vitamins, organic acids, isoflavones, anthocyanins and other flavonoids were often found. Different glycosilated forms of those structures were also present within the extracts. We managed to obtain different fractions of our plant extracts after method developing with our HPLC system. Both full extracts as well as defined substance mixtures have been applied in cell culture studies. Some of them showed promising results in reducing cellular injury.[2] Our ion trap MS device enabled us to perform some MSn experiments. That way we collected structural information on several unknown compounds within the extracts.

Conclusions
We were able to prepare plant extracts which then were separated into fractions containing defined substances mixtures. The cell biological studies showed promising results for some of the tested compounds and we will continue to further separate the mixtures. The question remains if the biological activity is a synergistic effect of multiple natural products or only one active substance. We also need to obtain the pure unknown compounds for a full characterization.

Novel Aspect
The natural materials we extract in the PePPP-project are applied in the study of hereditary liver and pancreas disease, namely Wilson’s disease, pancreatitis and Johanson-Blizzard syndrome.

References
Introduction:
Polyketides are a large class of biologically active and structurally diverse compounds. They are synthesized by polyketide synthases (PKSs), classified as type I, II and III enzymes. The Aryl-polyene ester (APE) system is a PKS type II-like system, where ten unbound proteins are involved, but the exact process of the biosynthesis of APEs is mostly unknown [1,2]. Here we report our current results of protein interactions in the synthesis of APE.

Methods:
To reconstitute the APE biosynthesis, all proteins that are involved in this process were purified from X. doucetiae. Components were mixed in vitro, and samples were desalted with Micro Spin Columns (Thermo scientific) into ammonium acetate or Tris buffer directly before MS analysis. ESI mass spectra of the enzymes were obtained in positive ion mode using a Synapt G2S (Waters) mass spectrometer.

Results:
The acyl carrier protein (ACP) is a small protein in PKS systems, which is responsible for carrying substrates cargo between active sites [3]. We see binding of ACP to different proteins showing protein-specific differences in binding strength. Acyl-acyl carrier protein synthetase (AasS) is a monomer and forms dimers in presence of ACP, it binds one ACP in monomeric and two ACPs in dimeric form. Ketosynthase 2 (KS2) can oligomerize up to a tetramer, but the dominant species are the monomer and dimer; it binds ACP in the monomeric and dimeric states. We can show the regulating effect of ACP on the oligomerization states of the proteins. The thioesterase (TE) forms tetramers and is able to bind up to four ACPs. The ketoreductase (KR) forms tetramers, where the dimer is the building block for the tetramer. Surprisingly we find that in this system the ketosynthase 1 (KS1) and the chain length factor (CLF) as well as the two dehydratases (DH1/DH2) build heterodimeric complexes, and only in their heterodimeric form, they can interact with ACP.

Conclusions:
Incubation of all the proteins of the PKS and MS analysis supports the hypothesis that the APE system is a PKS II-like system. ACP has a regulating effect on the oligomerization of the proteins. We could determine the oligomeric states and binding behavior of the proteins that are involved in the APE process. These results are the first step in reconstructing the process of the APE biosynthesis.

Novel Aspect:
Native MS analysis of protein interactions in PKS II system with separate proteins that act together in a multienzyme complex, which are used iteratively to form polyketides.

References:

Introduction:
Our study aims to exploit the Yellow-legged hornet venom (Vespa velutinannigrithorax) in the cosmetic. It is known that environmental factors could conduct morphological and behavior modification of insect to adapt to climatic changes. Our hypothesis is that seasonal variation can have a qualitative and quantitative impact on venom. In general, insect venoms contain a complex mixture of molecules, including proteins, peptides and low molecular weight compounds.

Methods:
V. velutina venoms from winter or summer hornets were separated into two fractions using 10 kDa filter (Sartorius). Protein profiles were achieved from >10 kDa fraction by gel electrophoresis, their density was measured by TLC Visualizer (CAMAG). In parallel, <10 kDa fractions were analyzed by two techniques on UHPLC-qToF (MS or MS/MS) and MALDI-TOF MS to obtain peptide profiles. Their differences of seasonal samples were accessed by statistical analysis (PCA, PLSDA).

Results:
The protein profiles obtained in gel present a mass distribution from 10-95 kDa, in which the most abundant bands were observed from 20-50 kDa. Density profiles achieved from TLC Visualizer at white light, however, do not reveal the significant difference between winter and summer samples in this mass range. Besides, a separation method by UHPLC-qToF (MS and MS/MS) was developed that allowed us to analyze peptides and low molecular weight compounds in V. velutina venom. The rich peptide profile was observed in each sample. In parallel, to develop an analytical method for investigating the peptide profile on MALDI-TOF, four matrices were screened with venom. The peptide profiles obtained from MALDI-TOF correspond to one from LC-MS and in addition, they provide more information in the high mass range. For global comparison, the statistical analysis results for each analytical method are in process.

Conclusions:
There is no significant discrimination between winter and summer protein profiles on gel electrophoresis. The methodology for analyzing the profile variation of venom samples issued from different periods was established. The PCA and PLSDA approaches are essential for evaluating the difference and the results are in process.

Novel Aspect:
The intraspecific comparison of venom compositions will be carried out to achieve a completed view of Yellow-legged hornet venom.
Introduction:
Our study aims to explore novel bioactive molecules from the Yellow-legged hornet venom for preventing or reducing skin aging in the cosmetic.
In this study two challenges are set: i) evaluate the antioxidant potency venom and then identify the bioactive structure responsible for it ii) establish and compare the peptidome expression pattern between the winter and summer venoms to assess if seasonal sampling impact qualitatively and quantitatively venom compounds.

Methods:
We develop a technical investigation platform that includes DPPH (for radical scavenging capacity), FRAP (for ferric reducing antioxidant power) chemical assays as well as ROS (reactive oxygen species) production in HaCaT keratinocyte cells in order to determine the antioxidant capacity of hornet venom. To identify the active molecules responsible for this remarkable property, the crude venom was fractionated by HPLC and then identified by UHPLC-ESI-qToF MS/MS.

Results:
Our results revealed a significant antioxidant activity of the venom in three activity assays. Nine fractions were obtained from HPLC and again evaluated. Only one fraction revealed an antioxidant activity. Although the fraction is rich, the identification of the compound responsible for the activity could be achieved and validated by an antioxidant test directly on Thin Layer Chromatography (TLC). From the analysis in mass spectrometry, we identified one major compound responsible for almost the whole antioxidant activity of the crude venom. In parallel, the venom peptidomes of winter and summer hornet workers were investigated with UHPLC-qToF-HRMS or MS/MS and their differences were evaluated by statistical analysis (PCA, PLSDA).

Conclusions:
We have successfully identified the compound responsible for antioxidant activity in Yellow-legged hornet venom and compared the profile variation of venom samples issued from different periods.

Novel Aspect:
The other cosmetic applications will be carried out to obtain a complete view of Yellow-legged hornet potency.
Introduction:
Alkaloids (alks) are widespread in nature. These nitrogen-containing organic constituents occur mainly in plants as secondary derivatives of the amino acid metabolism, or arising from amination of other substrates, which may be, for example, terpenes or steroids [1]. The biosynthesis often involves a further glycosylation of the alks (aglycones) as glycoalkaloids (glyalks) [2]. One of the best-known glyalks is the steroidal Solanine, a poisonous compound present in the Solanaceae plant family, but, to date, only a few studies on glyalks belonging to other chemical categories have been reported.

Methods:
The chromatographic separation was performed using a UHPLC equipped with an SPE on-line system and a byphenyl column. The binary mobile phase was composed of 0.1% formic acid (FA) with 5 mM ammonium acetate (AAc) and MeOH/ACN 95:5 v/v with 0.1% FA and 5 mM AAc. A Full MS/AIF/NL dd-MS2 experiment was performed in positive ion mode with the resolution set at 140,000 FWHM (m/z 200; 1.5 Hz) for full MS spectra, at 70,000 FWHM (3 Hz) for AIF and at 17,500 FWHM (12 Hz) for dd-MS2.

Results:
Neutral losses of pentose (m/z 132.0423), deoxyhexose (146.0579), and hexose (162.0528), and of all the combinations of up to four of these sugar units were considered. Full MS and AIF spectra were processed comparing the possible glyalks masses with the corresponding aglycone mass of about 100 alks already reported in literature as characteristic of the examined herbs [3,4]. If both the glycoside (in full MS) and the aglycone (in AIF spectra) peaks were present at the same retention time, a manual interpretation of spectra was performed. The compound identification was assessed considering the matching of glycosidic experimental dd-MS2 spectra with the fragmentation patterns of previously studied alks. The study allowed us to describe the profiles of glyalks belonging to the most important chemical classes (indole, piperidine, protoalkaloid, pyridine, pyrrolidine, pyrrolizidine, quinoline, steroidal, terpenoid, and tropane alks) in a selection of 21 plants.

Conclusions
This high resolution mass/neutral loss experiment permitted us to describe the presence of the glycosidic forms of a large selection of alks, permitting higher awareness of the possible risks and benefits relating to the consumption of popular plant-based medicines with new attention to both the free and bond alk forms.

Novel Aspect:
To our knowledge, this is the first high-resolution mass - neutral loss approach proposed for a broad identification of plant glyalks belonging to the main chemical classes.

References
Aniszewski T., Alkaloid Chemistry, Biological Significance, Application and Ecological role, 7-10 (2007).
627 - ANALYTICAL AUTHENTICATION OF OPHIOGOPON JAPONICUS SAMPLES: AN ESSENTIAL STEP TO SECURE ITS SUPPLY AND ITS USE FOR THE DEVELOPMENT OF A SAFE AND EFFECTIVE DERMO-COSMETIC INGREDIENT.

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Keywords: Ophiopogon japonicus, LC-MS/MS, Authentication, secure supply

Introduction:
Ophiopogon japonicus tubers are used in Traditional Chinese Medicine and are currently traded for the treatment of pathological skins. Their supply has to be traced and secured to ensure safety and efficacy of the resulting dermo-cosmetic ingredient. They share botanical and morphological characteristics with other plant species, particularly Liriopespicata. To avoid falsifications, analytical authentication of these plants tubers is thus an essential step.

Methods:
In this context, we performed a comparative analytical study of various samples of Ophiopogon japonicus tubers, from different supply regions. Phytochemical profiles obtained by ultra performance liquid chromatography coupled with tandem high resolution mass spectrometry (UPLC-MS/MS) were then directly compared with those of Ophiopogon japonicus and Liriopespicata specimens from referent collections.

Results:
Chromatograms obtained from referent samples revealed specific markers of Ophiopogon japonicus and Liriopespicata, in particular the range of homoisoflavonoids[1-5] and alkaloids. Identification of the species could be performed thanks to comparison of parent and fragment ions to components described in the literature. Based on these data, our results demonstrated that one of the samples contains alkaloids not present in Asparagaceae (i.e. the family of Ophiopogon japonicus and Liriopespicata), thus suggesting falsification of this sample. Two other samples display trace amounts of homoisoflavonoids, a variation probably due to the cultivation region which does not totally refute Ophiopogon japonicus. The other samples have the specific markers of authentic specimen of Ophiopogon japonicus.

Conclusions:
This analytical analysis of samples from different regions allowed us to authenticate samples of Ophiopogon japonicus comparatively to certified specimens. This approach will allow to secure future supplies of this starting raw material and to detect early some falsifications. This work is essential to guarantee safety and efficacy of the resulting dermo-cosmetic ingredient.

Novel Aspect:
This study shows that an analytical approach is complementary to botanic to authenticate a plant species, avoid falsifications and secure its supply.

References:

For information please contact: scientific@imsc2018.it
Keywords: short chain peptides, anti-aging, cosmetic formulations, LC-ESI-MS/MS analysis

Introduction: Skin aging is a natural event characterized by progressive structural and physiological changes in the skin. The main outcomes are the atrophy of the skin followed by loss of elasticity and a slowed metabolic activity [1]. Peptides like GHK, pal-KTTKS, and pal-GHK stimulate collagen renovation and shows anti-aging activity [2,3,4,5]. Thus, cosmetic producers interested in the introduction of peptides in cosmetic formulations is greatly increased in last years.

Methods: 4 modified peptides: 2,5-dihydroxybenzoic acid-PLG-OH (Db-PLG), 2,5-dihydroxybenzoic acid-LG-OH (Db-LG), 2,5-dihydroxybenzoic acid-PG-OH (Db-PG) and 2,5-dihydroxybenzoic acid-GL-OH (Db-GL) was quantified in cosmetic samples using LC-ESI-MS/MS method with positive ion mode. The method was validated using quality parameters like linearity, calibration, correlation of determination, detection limit, precision and, recovery.

Results: Retention times for peptides were following: 18.29 min for Db-PLG, 22.3 min for Db-LG, 22.56 min for Db-GL and 6.74 for Db-PG. The calibration curves were found to be linear with an average correlation of determination R2 to 0.9926 for Db-PLG, 0.9903 for Db-LG, 0.9986 for Db-PG and 0.996 for Db-GL.

Detection of peptides was performed with an AB SCIEX 3200 triple quadrupole system in the MRM and positive electrospray modes. MRM transitions for Db-PLG, Db-LG, Db-PG, and Db-GL were m/z 422/234, 325.1/250, 309.1/234 and 325.1/194.0, respectively.

Limits of quantification was established for a signal to noise ratio equal to 10. This corresponds to 40 ng for 2,5-dihydroxybenzoic acid-PLG-OH, 10 ng for 2,5-dihydroxybenzoic acid-LG-OH, 50 ng for 2,5-dihydroxybenzoic acid-PG-OH and 10 ng for 2,5-dihydroxybenzoic acid-GL-OH.

Recoveries were checked at three different concentrations and it was varied from 99.3% to 101.8%.

The method was applied to detect and quantitate tri- and di-peptides in different cosmetic formulations like skin, lotion, and gel.

Conclusions LC-ESI-MS/MS method was developed for the quantification of tri- and dipeptides with anti-aging properties: Db-PLG, Db-LG, Db-PG, and Db-GL. The method was validated with the International Conference on Harmonization of Technical Requirements for Registration of Pharmaceutical for Human Use guidance note represents validation procedure of analytical Methods: The method was applied to determine short chain peptides in cosmetic formulations.

Novel Aspect: newly synthesized short chain peptides in different cosmetic formulas were quantified by using LC-ESI-MS/MS method.

References
Introduction:
Many chickens died because their food used sorghum contaminated with Crotalaria retusa (Fabaceae) seedswith high concentration of pyrrolizidine alkaloids (PAs), highly toxic substances. We determined the PAs and their N-oxides (PANO), in Crotalaria genus plants growing in Colombia. Alkaloid distribution was determined in root, stem, leaves, flowers, and seeds of six Crotalaria species and in Utetheis onatrix eggs, larvae and moths, in relation to herbivory.

Methods:
U. ornatrix larvae consumed leaves of C. retusa, C. pallida, C. spectabilis, C. incana, C. maypurensis, and C. nitens maintained in an experimental garden. Solvent extraction, matrix solid-phase dispersion were used with plant material and insect parts. Pyrrolizidine alkaloids (PA) were determined both free and as their N-oxides. GC-MS analysis of PANO required their previous reduction with Zn. Extracts were analyzed on an ExactivePlus orbitrap LC-MS system.

Results:
High-resolution mass spectrometry afforded the exact molecular masses of both pyrrolizidine alkaloids and their N-oxides (PANO). Monocrotaline, creatonotine B, crispatine, integerrimine and their N-oxides were found both in plant material (0.01 – 21 mg/g) and insect specimens (0.01 – 1 mg/g). U. ornatrix eggs contained callimorphine N-oxide additionally. PA + PANO content in Crotalaria spp. plant parts varied according to the species, but was highest in seeds, followed by flowers, leaves, and roots. C. retusa showed the largest PA + PANO content among the species studied. U. ornatrix larvae preferred Crotalaria spp. unripe seeds (2 mg PA + PANO/g) and apical leaves (0.3 mg PA + PANO/g). They accumulate PA and PANO, which are transferred to moth and eggs. Alkaloid content was higher in eggs (6 mg/g) than in other U. ornatrix stages. PA/PANO ratios of 1200 were determined in larvae fed with C. retusa leaves. PA and PANO content in various Crotalaria spp. parts did not change significantly in response to herbivory by U. ornatrix or mechanical cutting.

Conclusions
PAs and PANO were simultaneously determined with LC-MS. MSPD permitted alkaloid isolation from small sample amounts. U. ornatrix and Crotalaria spp. exemplify co-evolutionary adaptation. Pyrrolizidine alkaloids produced by the plant as a defense measure are used by U. ornatrix as a protection from predators. Crotalaria spp. seeds (29 mg/g) and U. ornatrix eggs (5 mg/g) showed the largest alkaloid content, in which the N-oxide form was prevalent (PANO/PA ~ 1200).

Novel Aspect:
With good reproducibility, matrix solid-phase dispersion afforded plant and insect extracts with high alkaloid recovery.
1331 - GC-MS/MS DETERMINATION OF CIS AND TRANS-Δ9-THC ISOMERS IN CANNABIS SATIVA L

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Keywords:Cannabis sativa L., cis-Δ9-THC, hemp, GC-MS/MS.

Introduction:Hemp (Cannabis sativa L.) is a complex plant able to produce many chemical entities. Its biomedical relevance has been focused on Δ9-tetrahydrocannabinol (Δ9-THC). Anyway Δ9-THC exists in two geometrical isomers: cis and trans. The trans-Δ9-THC is the active compound responsible for the narcotic action of hemp. The existence and the distribution of the cis isomer in cannabis is still unclear. Aim of this study was the determination of the cis and trans-Δ9-THC in cultivar of Cannabis belonging to different chemotypes.

Methods: A gas chromatography–tandem mass spectrometry (GC-MS/MS) method was developed for the characterization and quantification of cis and trans isomers of Δ9-THC. Calibration curves, with internal standard (IS), were prepared from standard solutions of both molecules. Linearity, LOD and LOQ were evaluated. Aerial parts (leaves and inflorescences) of 31 hemp varieties were extracted in acetone. The dry extracts, added of IS, were analyzed by the developed method.

Results: The method developed resulted suitable for the determination of both Δ9-THC isomers. The calibration curves were linear in the range 5-200 μg/mL. LOD and LOQ were assessed respectively 0.3 and 1.0 μg/mL. The study demonstrated the presence of cis-Δ9-THC in all the varieties analyzed in a range of concentration of 0.003 mg/g and 1.41 mg/g. The trans-Δ9-THC isomer, always present in higher concentration of the cis isomer, was assessed in the range of concentration of 0.004 mg/g and 15.50 mg/g.

Conclusions: The cis-Δ9-THC was detected in all samples analyzed and its amount evaluated in comparison with the more known trans-Δ9-THC isomer. The cis/trans rate is always less than 1 and decrease with the increment of the total amount of Δ9-THC present in the plant.

Novel Aspect: For the first time the cis-Δ9-THC isomer was characterized and determined in different cultivar of Cannabis sativa L..

References
Introduction
MALDI-TOF MS is an emerging technique for microbial identification, characterization and typing that has been successfully used in the clinical field. Though, studies related to the characterization of remain scarce. In order to fill this gap, protein profiles of various environmental microorganisms have been acquired and classified. Moreover lipid profiles have been obtained and are expected to be complementary to protein profiles for rapid classification.

Methods
The lipids of each strain are extracted after maceration in an MTBE / MeOH mixture and then recovery of the organic phase and evaporation of the solvent. These lipid extracts are solubilized in a CHCl₃ / MeOH mixture (2/1) and then analyzed by MALDI TOF in positive mode (DHB matrix at 20 mg/mL in THF) and negative (9-AA matrix at 10 mg/mL in MeOH). Protein extraction: (CH₃CN / H₂O / HCOOH: 50/15/35) then MALDI-TOF analysis (α-CHCA matrix at 10 mg/mL).

Results
We were able to confirm that the spectral fingerprint of a protein extract is dependent on the species of environmental microorganism, whether for a bacterium or a fungus. For example, 16S sequencing of strains belonging to genera such as Bacillus is not sufficiently discriminating for identification at the species level. On the other hand, clusters within the same genus appear well defined when observing the spectral imprints of these same strains. Unlike protein extracts, lipid fingerprints do not seem to depend on the species but only on the genus in most cases. Only data on fungi of the genus Fusarium allow discrimination at the species level. The study related to lipid fingerprints has shown that strain classification can be performed even though genetic sequencing has not been performed or if the protein fingerprint is too specific to be related to a known species.

Conclusions
In our study we observed a perfect concordance of "clustering" between genetics and spectral fingerprints. We have therefore been able to provide a proof of concept concerning the possible identification of the different environmental microorganisms by comparison of protein and / or lipid fingerprints and to show the important potential of MALDI-TOF mass spectrometry for the identification of these environmental microorganisms.

Novel Aspect
New MALDI-TOF methodology for the identification of environmental strains (phytopathogens, mutualist microorganisms, endophytes, etc.)
Introduction:
Cyclopeptides, widely spread natural products, exhibit various biological activities. The recent reports indicate that the native chemical ligation allows the synthesis of cyclic peptides. However, this process has a complex mechanism and optimization of this synthetic method needs extensive analytical characterization of the reacting system including mass spectrometric methods.

Methods:
The precursors of cyclopeptides susceptible to transamidation reaction were synthesized according to the solid phase peptide synthesis procedure. The synthesis and cyclization progress were monitored in microscale by Triple Quadruple LC-MS, and FT-ICR MS and MS/MS. The structure of protected cysteamine derivatives applied as substrates in this synthesis were confirmed by NMR spectroscopy, FT-ICR MS and MS/MS all with ESI ion source.

Results:
The new cysteamine derivative protected with Mmt was synthesized according to the procedure developed by Barlos et al., purified by LC and analyzed by ESI-FT-ICR-MS with MS/MS and NMR. During MS experiment the fragmentation in source was observed and highly stable Mmt+ ion appeared. Model peptides as well as natural ones were synthesized on solid phase. Then the N,S-transfer of acyl group, followed by transthioesterification was performed, which allowed the transformation of peptide into the form of MESNa thioester. The interaction of thioester moiety with N-terminal Cys results in cyclization of a peptide. The intermediates of this reaction were monitored by Triple Quadruple HPLC-MS as well as FT-ICR MS and MS/MS. The application of proposed methods for the synthesis of biologically active peptides will be discussed in presented poster.

Conclusions:
The presented LC-MS techniques allows the monitoring of peptides metathesis including the formation of intermediates. The developed method will allow highly efficient cyclopeptides synthesis combined with their liberation from the solid support during SPPS. The combination of the native chemical ligation with N,S-acyl shift and transthioesterification will allow the synthesis of any desired cyclopeptide containing cysteine residue.

Novel Aspect:
The novel aspect is an application of the mass spectrometric methods for real-time monitoring and optimization of the complex, biologically important chemical reactions.

References:
566 - STRUCTURAL INVESTIGATION OF NATURAL ORGANIC MATTER USING DIRECT FRACTIONATION AND SELECTIVE ISOTOPIC LABELING COUPLED TO FTICR MS

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Keywords: FT ICR, natural organic matter, isotopic exchange, fractionation, structure

Introduction
FTICR MS is an exclusive method, which enables exploration of extremely complex natural organic matter (NOM). Routine analysis resolves thousands of CcHhOoNnSs molecular compositions in NOM sample [1]. However, FTICR MS is tolerant to structural isomers. Here we show determination of specific structural sites in individual NOM molecules and isomers in different samples using selective labeling reactions and direct fractionation in combination with FTICR MS.

Methods
NOM samples were sealed with NaOD or DCl for skeletal HDX [2]. Deuteromethylation was performed by CD3OD/SOCl2 reaction of NOM. All samples were desalted [3] and fractionated using SPE on ppl cartridges operated at different pH. HDX of mobile protons was performed by incubation in D2O. All samples were analysed using 7T FT MS Bruker Apex Ultra with harmonized cell (Bruker Daltonics). The FTICR MS data were processed using the lab-made “Transhumus” software.

Results
Catalytic HDX coupled to FTICR MS was applied to determine structural fragments of individual molecules of NOM and its synthetic analog. Application of HDX showed isomeric lignin-like components, which differ by aromatic ring substitution pattern. Combination of skeletal HDX provides the reliable identification of isomers in various NOM samples isolated even from similar sources. In case of models obtained by oxidative condensation of phenols, the exact structure of components was suggested.

Deuteromethylation enabled determination of COOH-groups carried by individual molecules in NOM. This allowed formapping compounds with close elemental compositions but different protolytic properties on conventional Van Krevelen diagram. The number of COOH-groups matched model structures suggested for major NOM components. Based on this result we performed direct fractionation of NOM on hydrophobic resin performed at different pH. We observe distinct shift of molecular composition from COOH-depleted molecules to polycarboxylic compounds.

Conclusions
We found that shared molecular compositions identified in different NOM samples correspond to structural isomers. By visualization of labeling results, we discovered relationships between compartments of NOM — oxidation, decarboxylation, condensation, etc, including transformation of CHOS compounds. Due to distinct separation of carboxylic acids according to molecular compositions, the deeper fractionation of hydrophobic components of NOM was performed.

Novel Aspect
The developed approaches enabled for the first time mapping of NOM components with respect to their structural pattern, and prediction of their chemical properties

References
E.03 ORGANIC AND INORGANIC MS: CHALLENGES & APPLICATIONS - MATERIALS & NANOMATERIALS

442 - DEVELOPMENT OF ISOLATION TECHNIQUES AND CAPILLARY ELECTROPHORESIS MASS SPECTROMETRY (CESI-MS) METHODS FOR THE CHARACTERISATION OF NANOMATERIAL PROTEIN CORonas

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Keywords: CE-MS, Protein-corona, nanoparticle, proteomics

Introduction:
Since the nanoparticle corona rose to eminence a decade ago, it has been investigated by a wide range of researchers trying to characterise and derive its significance [1]. To date, LC-MS is the instrument of choice for the analysis of the protein corona [1,2]. However, CESI-MS offers an exciting prospect to increase sample throughput and separate and detect highly polar and very large peptides which may be missed by conventional nLC-MS platforms [3].

Methods:
Silica nanoparticles were incubated for 1 hr at 37 °C in plasma. The on-particle digest particles were washed following immediately by digest. In the case of the off particle digest, NP-corona complexes were boiled in SDS buffer and run on an SDS-PAGE gel prior to in-gel digestion. A Sciex CESI 8000 equipped with a 90cm neutral capillary with 30 kV separation voltage and 2 PSI pressure was used for peptide separation, detection was with a Thermo QExactive HF mass spectrometer.

Results:
This investigation began by developing a sample preparation method to isolate the corona prior to CESI-MS analysis. Here the off-particle digest method enabled the detection of 1581 peptides which correlated to 134 protein groups. Initial on-particle digest methods were limited to 1270 peptides and 147 proteins due to peptides adsorbing to the NP surface to reform a corona. To ameliorate this, the addition of urea and/or rapigest to the digest solution were investigated as potential methods to keep the digested peptides solubilised. The addition of 1.5M urea with and without 0.1% rapigest improved both peptide and protein coverage. However, the addition of 0.1% Rapigest only enabled the detection of 2215 peptides and >200 protein groups. Furthermore, this method resulted in the least number of missed cleavages whereas any addition of urea increased the number of missed cleaves compared to the initial ammonium bicarbonate digest buffer solution.

Conclusions:
The finalized sample preparation and CESI-MS methods enabled a much higher throughput analysis than a conventional nLC-MS method, 50 minutes versus 140 minutes injection to injection. This was achieved by using an on-particle digest (<12hrs) instead of an off-particle in gel digest (1.5 days). In addition, this study introduced CESI-MS to the field of nanoparticle protein corona analysis.

Novel Aspect:
This is the first example of CESI-MS being applied to the nanoparticle protein corona and the first to comprehensively compare on and off particle corona isolation Methods:

References:

For information please contact: scientific@imsc2018.it
Introduction:
Inhalation of particles may cause airway inflammation induced by reactive oxygen species (ROS) generated by the particles. ROS are often measured by non-specific methods e.g. using 2’,7’-dichlorodihydrofluorescein (DCFH2) and comprises both radicals (hydroxyl, superoxide) and non-radicals (hydrogen peroxide) which both contribute to the response of DCFH2. The aim of the study [1] was to measure specific radicals formed by nanoparticles in contact with water.

Methods:
The method is based on spin trapping using diethoxyphosphoryl-5-methyl-1-pyrroline (DEPMPO) that traps radicals right after their formation. In short, nanoparticles (NPs) were dispersed in pure water with added spin trap, incubated over-night, and centrifuged. The supernatant was analysed with LC-MS and the “pellet” consisting of the NPs was analysed with MALDI-TOF-MS to directly measure the spin trap radical products adsorbed to the surface of the NPs.

Results:
It turned out that the major part of the spin trap radical products was adsorbed to the particle surfaces. Carbon black (Printex 90) showed by far the strongest radical generation as compared to silica NP (NM-200), with carbon nanotubes (NM-400) in between. The preliminary results showed that the Printex 90 produced mainly the hydroxyl radical (·OH) but also the hydroperoxyl radical (·OOH). NM-400 produced only ·OH and NM-203 produced no radicals. The total radical formation potential (sum of ·OH and ·OOH from both supernatant and “pellet”) of Printex 90 was 13-46 nmol/mg NP and 8-20 nmol/mg NP of NM-400 depending on the amount of NP dispersed in the water.

Conclusions
When studying the specific radical formation of NPs using spin traps and MS techniques it is necessary to be aware, that a substantial part of the spin trap radical products may be adsorbed to the surface of the NPs and has to be measured independently. The preliminary results show that radical formation by different types of NP may range from no radical formation of silica NP and up to about 50 nmol/mg NP of carbon black. All mechanisms behind ROS production are not known.

Novel Aspect:
Quantitation of radicals formed by nanoparticles in their dispersion media and on their surface using spin trapping and MS has to our knowledge not been reported previously.

1. The present study was part of the Danish NanoSafety Centre.
A NOVEL NANOCOMPOSITE COATING BASED ON SOL-GEL TITANIA/HYDROXYAPATITE FOR SOLID-PHASE MICROEXTRACTION COUPLED TO GC-MS

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Keywords: Solid-phase microextraction; Sol-gel technology; Hydroxyapatite; Titania; GC-MS.

Introduction:
The underlying principle of SPME technique is based on the analyte distribution between the sample matrix and the extraction phase[1,]. Titania and hydroxyapatite (HAP) have received great interest in analytical chemistry due to very good physical and chemical properties [2]. HAP can improve the surface area, grain size and thermal stability of TiO2. Addition of TiO2 changes the surface morphology as well as an increase of the mechanical and chemical stability of HAP [3].

Methods:
A non-aqueous organic/inorganic sol-gel rout was applied for synthesis of HAP sol and then a transparent stable titania sol was prepared. After repeating steps including; dipping, drying and sintering, a sol-gel coating was formed on the modified stainless steel (SS) wire. Finally, applicability of the prepared fiber was assessed for the HS–SPME of BTEX as model analytes from water samples followed by GC–MS.

Results:
The thickness, morphology and composition of the prepared sol–gel coatings; TiO2, HAP and HAP/TiO2, was investigated and analyzed by using of FESEM and EDX. The coating thickness was estimated below 1µm when the coating process was performed 5 times. This is much less than the thickness of the thinnest conventional coating. The FESEM images showed a fully cracked and quite non-uniform coatings in terms of porosity and particle size by use of pure TiO2 or HAP. Addition of HAP sol to the pure TiO2 sol, considerably changes the morphology of HAP/TiO2 coating; the coating is perfectly uniform and the size of the particles in HAP/TiO2 nanostructure is increased compared to the pure TiO2 coating. Nanoparticles (<50 nm) and pores (less than 100 nm) have been spread uniformly throughout the wire, providing a high surface area and a high sample loading capacity.

The obtained chromatograms of BTEX after HS–SPME by prepared TiO2, HAP and HAP/TiO2 coatings in optimum extraction conditions testify that HAP/TiO2 coating exhibited high extraction capacity and have better peak shapes.

Conclusions:
The proposed sol–gel method for the synthesis of HAP/TiO2 and the coating process on SS wire are very cost-effective, simple, fast and structurally controllable. The obtained chromatograms of BTEX after HS–SPME showed that HAP/TiO2 coating exhibited high extraction capacity. Very LOD and LOQ values were obtained for BTEX.

Novel Aspect:
A novel titania/hydroxyapatitenanocomposite coating fiber was prepared for first time through a relatively fast sol-gel technique.

References
Mass Analysis of Surface Immobilizations of Phenol-Containing Molecules through Chemical Modifications

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Keywords: Immobilization, Mannich reaction, Phenol-containing molecules, Self-assembled monolayers, Surface chemistry

Introduction
To construct applicable materials incorporated with biologically active phenol-containing molecules, strategies for immobilization of phenolic molecules on materials are required [1]. Although a number of immobilization methods have been accomplished and reported—mostly harnessing the phenol functionality, the practical use as a general immobilization method has been hampered due to their complicated chemical reactions and low reaction yield on surfaces[2-3].

Methods
Chemical functional groups, carboxylic acid, thiol, and azide, were introduced to phenolic molecules using Mannich reaction followed by immobilization to self-assembled monolayers on gold via various surface chemistries, carbodiimide coupling reaction, Michael addition, and ‘click’ reaction. As a practical application, we performed sulfotransferase enzyme assay on immobilized 2-naphthol [4]. The surface reactions were analyzed by MALDI-TOF MS and XPS.

Results
Naphthol, tyrosine, and hydroxyflavone were modified by Mannich reaction to possess carboxylic acid, thiol, and azide group, which were confirmed by MALDI-TOF MS. These functionalized phenolic molecules were then immobilized to the monolayers on gold that presented amine, maleimide, and alkyne via carbodiimide coupling reaction, Michael addition, and ‘click’ reaction, respectively. The immobilization of the synthesized molecules to the monolayers were analyzed by MALDI-TOF MS and XPS. The mass spectracalearly showed that all the surface conjugation reactions proceeded as expected, although the fragmented final products were observed. In addition, comparison of C1s-XPS indicated that the molecules were immobilized on the SAMs successfully. As a practical application, the 2-naphthol immobilized monolayers were exposed to sulfotransferase SULT1A1 and 3’-phosphoadenosine 5’-phosphosulfate, which yielded 2-naphthol sulfate.

Conclusions
We describe a simple, fast, and reliable method for the surface immobilization of phenol-containing molecules such as flavonoids by introducing chemical functional groups using Mannich reaction while maintaining the hydroxyl functionality so that their biological activities were not compromised.

Novel Aspect
Our method can be a general and practical platform for the immobilization method of various phenol-containing molecules on surfaces of various materials.
References

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Journal of Industrial and Engineering Chemistry 35 (2016) 1–7
Introduction
The interaction of nanoparticles (NPs) with cells and tissues has become a growing field of interest in consumer care and safety, nanotoxicology and medical applications e.g. as theranostic or diagnostic agents. It will be shown that laser ablation inductively coupled plasma mass spectrometry (ICP-MS) is a new analytical tool which can visualize the distribution of NP and which provides quantitative data at cellular levels.

Methods:
In our work we have used laser ablation coupled to ICP-MS in a differential line scanning mode to study and image the up-take and distribution of metallic nanoparticles (Au, Ag, TiO2) in single cells. Different types of adherent cells have been grown on microscopic slides and were incubated with different types of metallic nanoparticles at different time frames and concentration.

Results:
We have studied the effect of concentration, incubation time, nanoparticle diameter and surface coatings in fibroblast cells. After development of a calibration strategy using nanoparticle suspensions of known particle concentrations we could convert integrated intensities measured for a cell into the number of particles per cell. A similar strategy has been applied to convert measured intensities in tissue samples for Fe2O3-nanoparticles applied as contrast agents for magnetic resonance imaging (MRI) to enhance the signals in atherosclerotic plaques. By doping the oxide particles during synthesis by lanthanide elements we could easily differentiate and quantify natural (endogenous) from exogenous iron in the tissue.

Conclusion:
We have optimized a laser ablation system coupled to ICP-MS to achieve sub-cellular resolution. By application of a matrix matched calibration method we can convert the measured intensities of NPs directly into the number of particles up taken by cells. The sensitivity is sufficient to detect medium sized NP as single particle or smaller once (<20 nm) as agglomerates in cells and tissue.

Novel Aspect:
We discuss the concept and present the technical features of a novel quantitative elemental microscope based on inorganic mass spectrometry.
449 - SUPRAMOLECULAR NANOREACTORS STUDIED BY ION MOBILITY MASS SPECTROMETRY

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Keywords: Supramolecular chemistry, catalysis, nanoreactors, ion mobility mass spectrometry, CID

Introduction:
Cucurbiturils (CB[n]) are pumpkin shaped macrocycles of glycouril [1]. They are water soluable compounds which can host small molecules in their cavity and operate as nanoreactors [2]. Inclusion complex formation and catalytic activity of CB[n]s in dissociation reactions of azoalkanes have been studied using MS/MS and ion mobility MS experiments.

Methods:
Complex formation and MS/MS have been studied by ESI-Q-TOF MS (QSTAR from ABSciex) and Agilent 6560. Azoalkane inclusion inside nanoreactor have been followed by DTIM-MS using Agilent 6560 and modified Waters Synapt. Collision cross sections (CCS) have been compared to DFT calculated structures.

Results:
Complex formation between CB[n] (n=6, 7 or 8) and azoalkanes was followed by ESI-MS. The inclusion (endo) complex formation was verified by DTIM-MS and it mainly depended on steric factors. Instead of endo complexation, formation of exocomplexes was observed with smaller CBs. Size of CB[n] (n=6, 7 or 8), size of azoalkane and protonation state of the formed complex greatly affected the products formed during thermal activation of isolated complexes. In fact, different reaction pathway resulting in different end-products was observed for azoalkane complexed in nanoreactor on +1 and +2 charge states and for uncomplexed azoalkane.

Conclusions
CB[n] based nanoreactors are not only catalyzing reactions, but change substrate reactivity and enable formation of new reaction products. DTIM-MS is a valuable tool in analysis of structural features and especially endo-complexation in nanoreactors.

Novel Aspect
We have recently discovered that nanoreactors don’t only catalyse reactions, but also enable formation of completely new products. DTIM-MS used for structural analysis of nanoreactors.

References

450 - NEW BINDING PROPERTIES OF PYRIDINE[4]ARENE CAPSULES STUDIED BY IM-MS AND IRMPD

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Keywords: Supramolecular chemistry, Pyridine[4]arenes, Ion mobility mass spectrometry, IRMPD

Introduction
Pyridine[4]arenes [1] are macrocycles, which form dimeric and hexameric capsules via hydrogen bonding [2]. This type of capsules are widely studied in supramolecular chemistry as hosts, which can bind guest molecules inside the capsular cavity. We have recently discovered new binding properties of pyridinearene capsule, which was previously assumed to encapsulate only anionic guests [3-5]. The structures of new complexes have been studied using IRMPD and IM-MS.

Methods
Complexation of pyridine[4]arene with cationic guests was studied by ESI-Q-TOF MS (QSTAR from ABSciex). The structural features of observed complexes were studied in IRMPD experiments (performed with Varian FT-ICR) and DTIM-MS. DFT calculations have been used to visualize the gas phase structures.

Results
Complexation properties of pyridinearenes were tested with different alkyl ammonium guests. In (+)ESI-MS experiments, complexation was observed only with tetramethylammonium cation (TMA). In (-)ESI-MS spectra ternary complexes with TMA cation and two anions were observed to bound to pyridinearene dimer. These ternary complexes were studied in infrared multiphoton dissociation (IRMPD) and drift tube ion mobility experiments. Gained results supported the encapsulation of cationic guest while two anions were bound outside the capsule. The gas phase structures were visualized by DFT calculations.

Conclusions
New type of supramolecular ternary complexes have been observed in gas phase. Different mass spectrometric techniques have been utilized to study their structure. Results were consistent and supported the encapsulation of cationic guest, while anions were bound outside the capsular cavity.

Novel Aspect
New type of supramolecular ternary complexes observed in gas phase. The structural features of these complexes were studied using IRMPD experiments and IM-MS.

References
Mass Spectrometric Analysis of Vicinal Diol-Containing Flavonoid Molecules via Enrichment with Boronic Acid-Functionalized Particles

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Keywords: Boronic acid-functionalized particles (BAPs), Enrichment, Flavonoid, Matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF MS)

Introduction
Detection and quantification of flavonoids which exhibit broad biological and pharmacological activity with little toxicity [1] has been hampered by their bad water-solubility and rapid metabolic elimination from human body [2]. This study concerns the development of an efficient enrichment method for facilitating the analysis of vicinal diol-containing flavonoid molecules using boronic acid-functionalized particles (BAPs) and MALDI-TOF MS.

Methods
Boronic acids on BAPs bind to vicinal diols to form boronate monoesters at basic pH. This complex remains intact during the enrichment process, and the vicinal diol-containing flavonoids are easily separated by centrifugation and subsequent acidic treatment. The selectivity and the limit of detection of our strategy were confirmed by MS analysis, and the validity was assessed by performing the detection and quantification of a model molecule quercetin in mouse organs.

Results
The selectivity of BAPs towards vicinal diol-containing flavonoids was evaluated in the presence of other flavonoids which have multiple hydroxyl groups and carbonyl groups that potentially interact with boronic acids. Next, we observed excellent enrichment feasibility for vicinal diol-containing flavonoids of our method in the presence of other flavonoids in highly excess amount. A calibration curve for quantification was constructed with various amounts of quercetin in the presence of an internal standard, and the limit of detection for quercetin was 75 pmol. The practical applicability was confirmed by quantifying quercetin spiked in fetal bovine serum with excellent accuracy and precision. Furthermore, we showed that our method can effectively enrich vicinal diol-containing flavonoids and facilitate the quantification of the targets present in animal tissues in low abundance. Finally, excellent recyclability of the BAPs with good reproducibility was confirmed indicating the practical usability and cost saving of our method.

Conclusions
We validated the selectivity and efficiency of BAPs towards vicinal diol-containing flavonoid molecules and constructed a calibration curve for quantifying quercetin. The feasibility and applicability of our method were assessed via the quantification of quercetin spiked in FBS and animal tissues. Furthermore, the recyclability of BAPs was confirmed. Taken together, this method is promising from the viewpoint of cost saving and practical applications.
Novel Aspect
Boronic acid functionality was harnessed for the analysis of flavonoids in spite of its low water-solubility and interference of other vicinal diol-containing biomolecules[3].

References
H2 detection in-situ by membrane introduction mass spectrometry

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Keywords: MIMS, energy, hydrogen, light rare gases, real-time analysis

Introduction:
Direct measurement of gases formed in hydrogen reactors based on nanomaterials [1] is often challenging as many molecules may be of interest, rapid response is necessary and small volumes are involved. We propose an instrument, based on mass spectrometry, for small inorganic gases analysis (H2, CO2, O2, NO, Ar,...) specifically adapted to the hydrogen production applications.

Methods:
Gas analysis in the reactor is processed by a membrane interface (MIMS) and a quadrupole mass spectrometer. As water from the permeat may interfere with the hydrogen signal [2], we added a cryotrap at -100°C before the vacuum cell. The pumping system was modified to increase compressibility of hydrogen. Calibration of H2 signal is managed by a gas diluter. The apparatus was tested in a H2 formation reactor.

Results:
The apparatus was tested in dry and in-water conditions. Headspace analysis was not possible as signal was not stable due to droplet formation on the membrane. For in-water analysis stirring of the solution is necessary to maintain constant signal.

The modifications we made on the instrument improved the detection and stability of the H2+ (m/z 2) signal. Signal had a linear response to increasing concentrations. Detection limit was 2 ppm. First, use of specific material for the vacuum cell decreased hydrogen and water adsorption and ultimately lowered detection limit. Second, memory effect due to low compressibility of hydrogen was solved by the use of a second turbopump. Blanks were constant before and after introduction of high quantity of H2 for a long period. Finally, addition of a cryotrap decreased drastically (more than 99%) water amount in the permeat, and therefore detection limit of hydrogen was greatly improved in particular in wet conditions.

Conclusions
The constructed prototype allowed for improved H2 detection in mass spectrometer. Signal is more stable and detection is made possible directly in the water solution, with no headspace required. Concomitant analysis of O2 and CO2 was produced in a hydrogen formation reactor.

Novel Aspect:
New instrument specifically adapted to analysis in real-time of H2 and different other gases directly connected to a reactor to monitor hydrogen production from nanomaterials.

References
1. Luna-Barron A. et al., Applied Catalysis B: Environmental, 191, 18-28 (2016)
Keywords: single particle ICP-MS; arsenic nanoparticles; spectral interferences

Introduction:
Analysis of nanoparticles (NPs) using single particle inductively coupled plasma mass spectrometry (sp-ICP-MS) is able to provide information on the size of NPs and their number concentration. However, the determination of some elements via ICP-MS is strongly hampered by spectral overlap from polyatomic species. Overcoming interferences of polyatomic ions, which can compromise results, is one of the challenges with ICP-MS.

Methods:
The use of ICP-MS with reaction cell enables interference-free conditions to be obtained, even in the most demanding applications. This approach has also been proven to allow analysis of NPs in matrices containing interference precursors [1]. This work is focused on the determination of arsenic NPs in biological matrices because the arsenic suffers a polyatomic interference from ArCl+

Results:
The As NPs were prepared by borohydride-reduction of sodium arsenite according to previously published paper [2]. The use of NH3 in reaction cell mode (FNH3=0.3 mL/min) was demonstrated to be the most suitable to overcome the interference affecting trace As determination up to chloride concentration of 5.46 g/L, which corresponds to physiological saline solution. By using reaction cell, As NPs >50 nm can be detected. Because the sensitivity of signal was reduced not only by the reaction gas but also by salt content in sample, it was possible to provide accurate results only for As NPs >70 nm in at least 10 times diluted saline samples. In the case of <70 nm NPs, the histograms show incomplete distributions as result of the presence of the background signal.

Conclusions:
Asp-ICP-MS method utilizing reaction cell that allows determination of As NPs in presence of high chloride concentrations was introduced. Despite the reduction of sensitivity and therefore increased particle size detection limit, this method promises possibility of determining As NPs in biological matrices (e.g. blood serum).

Novel Aspect:
sp-ICP-MS offers fast determination and characterization of As NPs. The applicability to biological matrices may have interesting uses in medicine.


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Nanostructured Tungsten Oxide Substrate with Oxygen Vacancies for Efficient Surface-Assisted Laser Desorption/Ionization Mass Spectrometry Analysis

Introduction: (Limit of 400 characters)
The development of matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) for characterizing molecules was a significant breakthrough that build upon the 2002 Nobel-prize winning studies of Tanaka on soft desorption ionization methods for mass spectrometric analysis. While the vacuum environment and matrix preparation make the method time-consuming and laborious, ambient ionization mass spectrometry is becoming increasingly attractive because of its simple sample pretreatment process. More than 40 ambient ionization methods have been developed since 2004 and surface-assisted laser desorption/ionization mass spectrometry (SALDI-MS) is a very important branch in the ambient MS analysis. The search for more sensitive, versatile, and robust substrate for SALDI-MS becomes particularly important.

Methods: (Limit of 400 characters)
Sea urchin-like W18O49 nanoparticles was synthesized as follows: WCl6 dissolved in ethanol followed by hydrothermal reaction at 180 °C for 24h, and a blue flocculent precipitate was collected and diluted with ethanol to obtain the suspension. All MS analysis were performed using a home-made SALDI-MS system, and capillary nanoESI was used as a post-ionization method. For each analysis, drop a spot of W18O49 suspension on the aluminum plate as substrate, and then 5 μL of sample solutions. The laser used was a 980 nm continuous-wave laser without lens for focusing. The relative distance and angel between components undertook a systematic optimization to acquire satisfactory MS signals.

Results: (Limit 900 characters)
Sea urchin-like W18O49 nanoparticles have been successfully prepared via hydrothermal process. A very large absorption tail present in the visible and near infrared regions of the absorption spectrum gives clear evidence that the W18O49 nanoparticles contain a large number of oxygen vacancies, which become less for W17O47 and even be missing for WO3. The characteristic structure showed that compared with other nanomaterials, this substrate has a maximum energy absorption from 980 nm laser and function as “energy converter”, realizing efficient desorption of analyte. For MS analysis, the “pulse” form total ion chromatogram signal profile occurred since the power of the laser came to 4 W/cm2. This characteristic property gives much higher MS response and better S/N than paper spray (PS) and nanoESI analysis of 5 μL, 20 ppb diethylhexyl phthalate (DEHP) solution in positive mode and perfluorooctane sulphonate (PFOS) in negative mode, respectively. We also noticed that there was about a 60-s delay between the signal excitation and laser irradiation, and it became shorter as the laser power increased. When the power came to 7 W/cm2, the delay was less than 10 s. The photothermal analysis of the W18O49 substrate dropped on the aluminum plate showed that the temperature increased faster and had a higher final value as the laser power increased. We propose that there would be a positive correlation between the signal excitation and heat accumulation but it is not a decisive factor, since the highest final temperature was about 120 ºC during testing, and such a low temperature was not enough for the thermal desorption. We compared WO3 as substrate for MS analysis and the results showed that neither oxygen vacancy nor instrumental signal. Also, for 60 min irradiation at 6 W/cm2,
the Raman spectrum showed a certain degree of oxidation for W18O49. Corresponding MS signal for W18O49 decreased.

Figure 1 SEM images of sea urchin-like W18O49 nanoparticles a) 500 nm and b) 5.0 μm; c) UV/Vis absorption spectrum of W18O49, W17O47 and WO3; d) TIC signal profile at different laser power; e) The photothermal analysis of the W18O49 substrate at different laser power; f) Comparison of the MS response for ESI, PS, nanoESI and this method; g) Raman spectrum of W18O49 during 60 min irradiation; h) The MS response and absorbance at 808 nm of W18O49 substrate during 60 min test.

Novel Aspect: (Limit of 150 characters)
Novel sea urchin-like W18O49 nanoparticles with oxygen vacancies was applied for SALDI-MS analysis with significantly increased desorption/ionization efficiency.

References:
INFLUENCE OF SILICA NANOPARTICLES SURFACE CHARGE IN MALDI-MS ANALYSIS OF PEPTIDES

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Keywords: MALDI-TOF, mass spectrometry, nanomaterials, functionalized silica nanoparticles

Introduction:
Nanomaterials have been widely used in laser desorption/ionization time-of-flight mass spectrometry as an alternative for matrix (LDI-MS) and as well for improving signal intensities in MALDI-TOF-MS [1]. In this regard, bare silica nanoparticles (SiO2NPs) have been successfully utilized for the signal enhancement of peptides [2]. Here, we report influence of the two types of functionalized silica NPs in the development of MALDI-MS signals intensity of peptides.

Methods:
The sulfobetainesilica NPs (SB-SiO2-NPs) and amino propane sulfonate silica NPs (APS-SiO2-NPs) were prepared according to the published procedure [3]. MALDI experiments were done by using the AB SCIEX4800 time-of-flight (TOF/TOF) mass spectrometer in reflectron positive mode, which is equipped with a 355 nm Nd:YAG laser. Typically, to acquire a single spectrum 800 laser shots were fired at a frequency of 200 Hz at 25% of the full laser power for samples.

Results:
Among the different methods for sample preparation, two methods were selected for MALDI-TOF-MS experiment. In this regard, 1 µL of functionalized SiO2-NPs was deposited onto the MALDI plate. Following that 1 µL mixture of sample solution, including peptide and matrix, was spotted on the top of dried silica NPs. In a parallel way we mixed 1 µL of functionalized SiO2-NPs with 1 µL mixture of sample solution and after 3 min vortex, 1 µL of NPs/sample solution was directly spotted on to the MALDI plate and air dried for MALDI-MS analysis. In all analyses, the concentration of peptides in MALDI samples was maintaining constant. The maximum signal intensity for peptides was obtained when we used NPs as additive to the sample solution. SB-SiO2-NPs had the most influence on analyte signal enhancement. Alteration the ratio of matrix and NPs in sample solution had a positive effect on signal enhancement. As well, concentrating sample with the coffee-ring effect during sample preparation, improved signals intensity.

Conclusions:
This work demonstrates that functionalized SiO2 NPs can be used for enhancement of ion signals in MALDI-TOF-MS analysis of peptides. This improvement on signals intensity is possibly related to the electrostatic interaction between NPs and peptides. Furthermore, alteration NPs surface charge have influence on this electrostatic interaction and change the amount of peptides ionization.

Novel Aspect:
New functionalized silica nanoparticles have been utilized for the first time, since improving signals intensity of peptides in MALDI-MS analysis.

References:
1336 - ELEMENTAL ANALYSIS BY AU-NANOPARTICLES ENHANCED LA-ICP-MS

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Keywords: Laser Ablation, Nanoparticles, LA-ICP-MS

Introduction
A preliminary study of nanoparticles-enhanced LA-ICP-MS is presented. It represents an analytical strategy to improve the analytical performance of the LA-ICP-MS in terms of sensitivity and LOD, preserving its excellent characteristics (simplicity, rapidity, etc.), without any changes in the experimental set-up.

Methods
Some drops of Gold Nanoparticles (AuNPs) colloidal dispersion were deposited on the sample’s surface, the solvent evaporated before the analysis. Spherical AuNPs dispersions in aqueous citrate buffer (Sigma Aldrich Co.) or naked PLAL (Pulse Laser Ablation in Liquid) [1] AuNPs of different size were used on certified standard target (Copper, Titanium, Bronze, Brass, Silicon and Glass).

Results
A considerable increase of the measured signal intensity was observed in the presence of AuNPs. By analogy with results already obtained with Laser Induced Breakdown Spectroscopy [2,3], when a critical number of NPs are deposited on the target surface, the laser pulse electromagnetic field induces the collective oscillation of the conduction electrons of the NPs that in turn results in a strong enhancement of the field. The improvement is probably induced by the best quality of the nanoparticles’ agglomerate generated during the laser ablation. Metallic elements show enhancement also in non-conductive matrices. Different metallic elements show different enhancement in the same matrix, as well as the same element shows different enhancement in different matrices. It should be noted the AuNPs can be completely removed after the analysis.

Conclusions
The existence of an improvement of sensitivity in LA-ICP-MS signal via AuNPs allows to identify and quantify elements without damaging significantly the sample’s surface unlike conventional LA-ICP-MS.

Novel Aspect:
The AuNPs allow to improve the sensitivity of LA-ICP-MS technique almost in a nondestructive mode, which can be extremely useful in many fields as forensic sciences and cultural heritage.

References
Introduction:
To characterize covalently functionalized graphene, several analytical methods are commonly applied. Raman spectroscopy, for example, allows to detect the conversion of sp2-hybridized carbon from the graphene lattice to sp3-hybridized carbon, giving insight in the degree of functionalization of the nanomaterial. However, it cannot provide any information about the molecule bound to the graphene, making it hard to conclude if the target molecule is attached or not.

Methods:
The nanohybrid was characterized by laser desorption/ionization time-of-flight mass spectrometry (LDI-ToF MS) using a Bruker Reflex IV mass spectrometer equipped with a N2-laser (λ = 337 nm). Further, the hybrid was washed with trifluoroacetic acid in DCM:MeOH (1:2, V:V) five times and each of the green filtrates was analyzed via electrospray ionization (ESI) quadrupole ion trap (QIT) MS (esquire6000, Bruker).

Results:
In this study, graphene was covalently functionalized with 5,10,15,20-tetraphenylporphyrin (TPP) via a diazonium salt intermediate. For the first time our investigation employed LDI-ToF MS. LDI revealed the presence of several interesting species on the graphene sample. The molecular-ion peak of the TPP was observed together with other TPP-related signals occurring from side reactions, such as a TPP dimer. Washing the hybrid system several times yielded green filtrates with the color fading from the first to the last washing cycle. ESI MS of the filtrates resulted in signals of TPP together with the byproducts already detected in LDI, decreasing in intensity after each washing cycle. The precipitates from the washing cycles were then investigated again by LDI to check whether TPP is still present on the graphene or not. TPP was still observable in the LDI mass spectrum whereas the signals for the byproducts were significantly reduced, supplying strong evidence that the TPP is indeed covalently attached to the graphene lattice.

Conclusions:
LDI-ToF MS was successfully introduced as a new analytic method for the study of covalently functionalized graphene. LDI-ToF MS allowed for the first time the identification of large molecules on a graphene lattice, enabling the detection of products and byproducts, which is not possible with currently applied methods, such as Raman spectroscopy. Furthermore, a combination of ESI-QIT and LDI-ToF MS gave strong evidence that the TPP is indeed covalently attached to the graphene lattice.

Novel Aspect:
LDI-ToF MS was introduced as a new analytic method for covalent graphene samples and provided insight into the composition and chemistry of the molecules on the surface.

References
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Keywords: ESI, LC-MS, ferrocene, oxidation, picrate

Introduction:
The oxidation reaction of ferrocenes is one of the typical reactions of ferrocenes. Ferrocene is readily oxidized to the ferrocenium ion under acidic conditions. The oxidation reaction of ferrocene derivatives by air under acidic conditions has been investigated by several researchers. However, the formed ferrocenium ions is not recognized by NMR due to its paramagnetic property. In this paper, LC-MS with ESI was used to detect and quantify the ferrocenium ions.

Methods:
The LC-MS was measured using a Shimadzu LCMS-QP8000A. The electric potential was 4.5 kV for positive mode, and -3.0V for negative mode. An OOS column was used under MeOH-H2O eluent conditions.
The oxidation reaction was proceeded by air in 1,2-dichloroethane in the presence of trichloroacetic acid [1]. After the reaction, the solution was washed by water to remove the acid. Picric acid was added to the solution as anion source.

Results:
The ESI(+)-MS of ferrocene showed molecular ion peak (m/z=186) as base peak, and a fragment ion peak of m/z=121. After the oxidation reaction, the mass chromatogram showed two peaks; one is for the unreacted ferrocene, and another is a new peak. The ESI(+)-MS for the new peak showed only molecular ion peak (m/z=186). The ESI(-)-MS showed only one peak for picrate anion (m/z=228). Therefore, this chromatogram’s new peak would be assigned as ferrocenium picrate.
The ESI(+)-MS of ethylferrocene showed molecular ion peak (m/z=214) as base peak, and a stable fragment ion peak of m/z=199. After the oxidation reaction, the mass chromatogram showed only one peak. The peak was assigned as the oxidized form by ESI(+)-MS and ESI(-)-MS. This is attributed to the high oxidation reactivity of ethylferrocene. For acetylferrocene, which has low reactivity, small peak was found in the mass chromatogram. The peak was assigned as the oxidized form by ESI(-)-MS.

Conclusions:
Based on the LC-MS with ESI, the oxidation reaction of ferrocenes was measured. The oxidized forms were assigned by the ESI(+)-MS, and the picrate ion as the counter anion by ESI(-)-MS. This ESI(-)-MS measurement is useful for the low oxidation reactive ferrocenes such as acetylferrocene.

Novel Aspect:
The LC-MS with ESI using picrate ion as the counter anion was an effective method for the measurement of the oxidation reaction of ferrocenes.

References:
Introduction:
Synthetic polypeptides and proteins are particularly useful tools in biochemical research. The synthesis on solid support is limited to only relatively short sequences, while the method of producing recombinant proteins' main restriction lies in incorporating post-translational modifications and unnatural amino acids [1]. A native ligation approach requires cysteine residues in peptide chains and therefore limits the choice of synthetic strategy [2].

Methods:
Substrates for the ligation reactions were synthesized on the solid support. Products of synthesis were analyzed using ESI-MS and MS/MS Methods: Ligation reactions were performed on the solid support and in solution. Products of ligation were investigated by LC-ESI-MS analysis.

Results:
Omniligase is characterized by its unspecific activity. It allows coupling almost without any limitation (except Pro in active center) peptides [3]. Our results show that it is possible to performed ligation on the solid support. One of the most important factors to achieved a product of ligation is chosen appropriate resin. We tested selected solid supports, swellable in water, such as ChemMatrix resin, PEGA resin and TentaGel. The best results were obtained for PEGA resin which was used to the further experiments. Preliminary results indicate the relatively high efficiency of solid phase enzymatic ligation.

Conclusions:
In my poster I will compare the results of Omniligase ligation on the solid support and in solution. I will also discuss the factors influencing the efficiency ligation under various conditions.

Novel Aspect:
Evaluation of mass spectrometric methods (LC-MS, MS/MS) as a tool for monitoring the micro scale chemoenzymatic peptide synthesis on solid support.

References
Keywords: TASP, Dynamic combinatorial libraries, LC-MS, synthetic helical proteins

Introduction:
TASP molecules are artificial protein models formed by attaching peptide chains to a cyclic scaffold. We present preparation of TASP dynamic combinatorial libraries (DCLs) basing on a new scaffolds, which are created by formation of disulfide bonds between N-(2,6-dimercapto-1,3,5-triazin-4-yl)peptides. DCLs were analyzed by LC-MS.

Methods:
We synthesized our peptide conjugates using the standard SPPS protocol. Libraries of TASP molecules have been created from N-(2,6-dimercapto-1,3,5-triazin-4-yl)peptides (or their dimers with a disulfide bridge) via oxidation by atmospheric oxygen or CuCl2 in 2-propanol. Selective formation of a cystine residue has been performed by adding I2. We used LC-MS and IM-MS to characterize TASP libraries. Identity of the isolated compounds has been confirmed by HR-MS.

Results:
Oxidation of N-(2,6-dimercapto-1,3,5-triazin-4-yl)peptides by atmospheric oxygen in aqueous solution does not occur. The peptides incubated in a more hydrophobic environment of 2-propanol produced TASP molecules by formation of disulfide bridges between 2,6-dimercapto-1,3,5-triazine moieties. Subsequent oxidation of the peptide conjugates containing an N-terminal Cys residue with an attached dimercaptotriazine moiety allows controlling the size of the macrocyclic scaffold. Similarly to systems proposed by Otto et al. [2], our peptide conjugates gave mainly trimers and tetramers. Formation of disulfide bond between Cys residues attached to triazine ring affects the size of oligomers. Used peptide sequence were designed as amphipathic α-helices. Dynamic combinatorial libraries of the oligo helical bundles were analyzed by LC-ESI-MS and IM-MS. Some of them were purified and analyzed by CD spectroscopy, showing stabilization of α-helical structures by intra-chain interactions.

Conclusions
We developed a new type of scaffold based on triazine moieties connected by disulfide bonds into cyclooligomers. Our method is useful in designing dynamic combinatorial libraries of TASP. The libraries of synthetic proteins may be simply characterized using LC-MS and IM-MS. Fixing of the relative positions of the two triazine rings by subsequent formation of disulfide bonds between attached Cys residues affects the size of the macrocyclic scaffold.

Novel Aspect:
We developed first dynamic combinatorial libraries of TASP molecules with scaffolds based on oligo triazine disulfides, which were fully characterized by LC-MS and IM-MS.

FAST ANALYSIS OF MODERN OIL PAINTINGS AND DYES IN TEXTILES BY SURFACE ACOUSTIC WAVE NEBULIZATION-MASS SPECTROMETRY (SAWN-MS)

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Keywords: SAWN-MS, oil paintings, dyes, textiles

Introduction:
GC-MS and LC-PDA are the methods of choice to study the binding media of paintings and dyes in textiles [1,2]. Relatively large samples are needed and long sample preparation and analysis time. In GC-MS, limited information is obtained due to the hydrolysis and derivatization steps, the high temperatures and ionization energies used.
SAWN-MS maintains the integrity of fragile molecules due to mild ionization, greatly reduces the sample sizes and the analysis time.

Methods:
The SAWN device was placed in front of the API inlet of a triple-TOF 5600+ MS (Sciex) and operated through a connection with a frequency and power controller. 1 µL of liquid sample was loaded on the chip and the SAW regulated by applying a power to the electrodes (≈5 W). This step was repeated if needed (up to 1 min) and the data accumulated into a single file. The interface heater temperature was 150 °C, the inlet/outlet and curtain gasses were 0 psi and 10 psi respectively.

Results:
Herein, we describe the SAWN-MS technique and optimised experimental procedures for the analysis of modern oil paintings and dyes in textiles for conservation studies.
SAWN-MS analysis revealed a rich representation of molecular ions that were used for the identification of mixtures of colourants present in dyed textiles [3] providing information on the colouring procedure and the state of degradation.
The technique was also applied to study levels of oxidation and hydrolysis of 20th century oil paints, by monitoring the extracted free fatty acids and diacids, both as glycerides and in the free form. The formation of diacid functionalities play a significant role in the development of water sensitivity and paint instability/failure [4]. A correlation with the pigment type and additives used in paint formulations was obtained. SAWN-MS also enabled the detection of short chain fatty acids, which cannot be easily detected by GC-MS.

Conclusions:
SAWN-MS is an excellent analytical tool for conservation studies on oil paintings and textiles. It offers unique advantages in terms of ease of implementation, simplifies the sample extraction procedures, significantly reduces the required sample sizes and overall analysis times are typically few minutes. Moreover, due to the new mechanisms and efficiency of ionization it provides additional molecular information compared to other ionization techniques.

Novel Aspect:
Novel ionization technique for fast MS analysis of organic components in paintings and dyes in textiles.

References

551 - MASS SPECTROMETRY ANALYSIS OF MEDIEVAL ARCHAEOLOGICAL JEWELS

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Keywords: mass spectrometry, archaeological findings, SERJs, medieval jewellery

Introduction:
The history of jewellery is long and goes back many centuries. Slavic women had worn characteristic metallic S-shaped end ring jewels (SERJs) and those jewels were usually found near their temple skull bones. Laser desorption/ionization mass spectrometry (LDI-MS) allows to effectively study organic compounds profile on the SERJs surface and helps us to understand the way they were used - whether as a part of a headdress or knitted directly into braids of hair.

Methods:
Synapt G2-S high resolution tandem mass spectrometer (Waters, USA) equipped with vacuum MALDI ion source was used for all experiments. Organic matter of unknown origin adhered to SERJ surface (from Dětkovice archaeological locality, Moravia region, Czech Republic) was analyzed directly on SERJs surface. LDI-MS and mass spectrometry imaging experiments were done without application of matrix.

Results:
MSI of fiber residues and fiber-like imprints present on SERJs surface revealed the saccharide chains in the form of sodium adducts. Strong signals at m/z 201.0024, 363.0647, 525.1129, 687.1681, 849.2052, 1011.2562 and 1173.2977 were found in positive ionization mode and ascribed to cellulose. Besides, signals at m/z 437.0921, 599.2493 and 761.1912 were observed with lower intensity. The latter can be explained by a cross link cleavage of glucopyranose ring[1] in sugar chain and further confirm the presence of polysaccharides. On the other hand, no signal of animal origin was observed on SERJs surface.

A distinct group of signals (i.e. m/z 242.0504, 445.1452, 648.2383, 851.3283, 1054.4178) corresponding well with N-acetyl-glucosamine chains was found in MS spectra obtained from a small piece of organic matter hidden in corroded surface of another SERJ. N-acetyl-glucosamine is a common part of chitin present also in beetle elytron. We hypothesize that those signals correspond with remains of a corpse-eating insect.

Conclusions:
SERJs were attached to a headdress by a plant thread (morphology and MS data)
N-acetylglucosamine signals revealed presence of insect body remains
Systematic analysis is now focused on characterization of fibers with animal origin (e.g. residues of animal skin)

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Novel Aspect:
Laser desorption ionization mass spectrometry allowed characterization of the organic residues deposited in corroded surface of medieval metallic jewels.

References:
Determination of the wine and food traces from archaeological materials by GC/MS methods

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Keywords: Archeology, Ceramics, organic residues, GC/MS, molecular markers,

Introduction: The organic residues of the ceramic objects contain biomarkers produced by biochemical transformations of their original content [1-4]. The characterization of the lipid fraction in archaeological material is of great importance to obtain information on uses and dietary of people who used this material. For obtaining precise results it is important to identify presence of molecular markers in ancient materials. The aim of this study is to analyze archaeological materials with sensitive and selective methodology involving also optimal extraction Methods: The method of this study allows the identification of markers of food, wine and resins.

Methods: The identification of the organic residues in archaeological materials was performed following few stages: a) extraction of lipid; b) compounds derivatization and c) instrumental analysis. The lipids were extracted using chloroform–methanol (2:1) mixture and then the extracts were derivatised by N, O-bis (trimethylsilyl) trifluoroacetamide (BSTFA). The analysis of the samples was carried out using a GC/MS system. The mass spectrometer was operated in the electron ionisation mode (70 eV). The mass range was scanned in the range of m/z 50-650.

Results: A robust analytical method for determination of markers of wine, food and resins from archeological materials has been developed. The mass spectra of molecular markers are registered and discussed. The method proposed here will allow that the technique GC/MS to can be used extensively for the identification of lipids and wine markers, understanding the structure of the residues preserved in the samples.

Conclusions: Chemical characterization of the organic residues of ceramic vessels, contributes to a better understanding of their uses of the ancient people. Identification of chemical markers, in conjunction with a traditional study of the ceramics, plays a relevant role in establishing what the original content of a specific vessel was.

Novel aspect: The method was used for characterization of ceramic samples from centre of Transylvania, near city Turda which was built by Romans under name Potaissa. The samples are originated from Roman period. This investigation is made for first time in Transylvania region. The analyses allowed us to differentiate between archaeological material devoted to the production or preservation of wine and those used to store the vegetable oil or animal food.

References:
Introduction
Rapid Climate Changes (RCC) that occurred during the Holocene are very important to understand climate changes currently underway (1). The characterization of these abrupt events allows the evaluation of ecosystems and ancient populations from a resilience point of view.

Methods
Here we investigate variations in $\delta^{18}O$ and $\delta^{13}C$ of Mesolithic archaeo-malacofauna (Pila sp.) found in Central Sudan (2) at high-resolution intervals along the growth axis of shell. Furthermore, in order to correctly interpret the data on the fossil specimens and validate the use of the molluscs in paleoclimate studies, modern molluscs were collected from two locations along the White Nile valley.

Results
Comparison of $\delta^{18}O$ and $\delta^{13}C$ in archaeological shell and in modern specimens revealed significant differences. The $\delta^{18}O$ archaeo ranges from -10.97‰ to -6.23‰ whereas $\delta^{18}O$ modern ranges from -1.17‰ to +5.13‰ suggesting climate change. Moreover, the lower $\delta^{13}C$ values for modern shells suggest a variation of carbon source in the aquatic system.

Conclusions
This study demonstrates that a drying process occurred over time along the Nilotic area. The low oxygen isotope values of Mesolithic samples indicate significantly less evaporation of the Nile water and/or a different rainfall amount and seasonal distribution in comparison to the current river. (3)

Novel Aspect:
This study provides the first application of the use of Pila sp. as a proxy for paleo-climatic information.

References
MOLECULAR CHARACTERIZATION OF THE PREPARATIVE LAYERS OF XXIInd SARCOPHAGUS, A MASS SPECTROMETRY APPROACH

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Keywords: cultural heritage, restoration, extraction, separation, mass spectrometry

Introduction:
The molecular characterization of historical artifacts is becoming an increasingly important aspect for cultural heritage [1]. Nowadays all the developments in extraction, separation and analytical methodologies can be helpful for challenging tasks, such as the chemical reconstruction of the natural compounds used by artists [2]. Any of the informations about the chemical composition of binders, oils, varnishes, etc., play a fundamental role both for restoration purposes and for the reconstruction of historical informations.

Methods:
The first step of our workflow is represented by an ammonia extraction of chemical compounds from two micro samples from the preparative layers and two microsamples from external layers of a wooden sarcophagus, which can be dated towards beginning of the XXIInd Dynasty.
The methodology used for the characterization of extracted molecules are GC-MS (for monosaccharides and lipids analysis) and high-resolution MS (for proteins identification).

Results:
According to extraction procedure, we divided analytes in three categories: monosaccharides, lipids and proteins. Monosaccharides analysis led to the identification of binders extracted from all the layers. We identified Galactose, Glucuronic Acid, Ramnose and Arabinose, which are the main constituents of Arabic gum.
We could identify palmitic, stearic and oleic acid. The relative ratio of identified fatty acid is for sure affected by aging; their co-presence though is compatible with the putative presence of egg [3]. This hypothesis is strengthened by proteins identification. We could in fact identify in the external layers (the ones connected with the decoration of the sarcophagus) Lysozyme, one of the most abundant protein in egg. Altogether, our data suggest that the painting techniques adopted was tempera.
Moreover, in the preparative layers, we could unambiguously identify Collagen; the presence of this protein can be related to the technique used for the building of the sarcophagus itself.

Conclusions
Numerous approaches are focused on single classes of compounds; some others [4] suggest the possibility of using a single extraction procedure followed by different analytical strategies. The rationale at the basis of our approach starts from the latter assumption. However, the interesting aspect of our approach is represented by the heterogeneity of unambiguously identified molecules, which led to the characterization of the different layers sarcophagus.

Novel Aspect:
The ingenuity of our methodology relies in the use of powerful analytical methodologies (i.e. high resolution MS) in the workflow thus improving the quality of obtained results.

References
517 - MASS SPECTROMETRY STUDIES OF THERIAC FROM “SPEZIERIA DI SANTA MARIA DELLA SCALA” (ROME): MODERN TECHNOLOGIES TO LEARN FROM THE PAST.

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Keywords: Theriac, composition, analysis, mass spectrometry

Introduction
Theriac, a thick sticky liquid medicine, until the mid 18th century was regarded as universal panacea and was used to treat infectious diseases or poisonings. Theriac contained up to seventy different often exotic ingredients. The production of a proper Theriac took months and it was supposed to be left to mature for years [1] [2]!

Here the first approach to study the composition of an ancient Theriac sample from the “Spezieria di Santa Maria della Scala” (Rome) is presented.

Methods
Different chromatographic methods principally based on mass spectrometry techniques have been used to achieve a comprehensive Theriac characterization. The organic polar compounds were analyzed by UHPLC -qTOF-MS, in high resolution (2GHz). The volatile organic compounds were analyzed by means of GC-TQ-MS, using Head Space sampling or direct injection after sample derivatization with BSTFA/Pyridine. The metal part of inorganic compounds was analyzed by means of ICP-MS, the anionic part by IC-ConD.

Results
Very sharp chromatographic fingerprints of the Theriac sample have been taken. After have studying molecular mass characteristic and the fragmentation pattern of the various molecular species, by matching data with a “in-house” specific library, different compounds have been identified. In several case high resolution accurate mass have been crucial. Obviously, the identification step has been very intriguing! Some marker compounds specific of medicinal plants have been correctly identified but many other are very difficult to recognize, as consequence of the transformations due to Theriac preparation as well as to the aging of the sample. Afterwards, using external standard regression curves, the identified compounds have been quantified. Glucose and fructose, typically should be from honey, are the most abundant compounds, followed by glycyrrhizin, and salicylic acid. Atypical flavonoid liquiritin have also been detected, confirming the presence of liquorice. Calcium, potassium, sulfur and iron at concentration of 0.1-0.5% (w/w) have been found, while chloride has been found at a lower value.

Conclusions
More than thirty organic compounds and twenty elements have been identified, but further studies have to be done to better elucidate the composition of Theriac. The preliminary results showed how potent is the analytical approach used. By means of data obtained from a comprehensive characterization it is possible to suggest the presence of interestingly ingredients in the old Theriac sample. The rational study of Theriac is a challenge but can be convenient to suggest new solutions for the modern human beings health care.

Novel Aspect
For the first time the composition of Theriac has been investigated at a molecular level using different mass spectrometry techniques, including HR-MS.

References
MOLECULAR SIGNATURES OF PROTEIN AGING IN ANCIENT HUMAN BONES FROM THE ERUPTION AREA OF VESUVIUS.

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Keywords: Paleoproteomics, Mass Spectrometry, Cultural heritage, Protein Degradation, Ancient proteins

Introduction:
In search of markers for protein aging, we use the singular case of collagen from human bones from the excavations sites in Pompei, Herculaneum, Oplonti, S.Paolo Belsito, and Scalandrone Bay as control reference site. Deamidation of glutamine and asparagine is considered as aging molecular marker [1]. Moreover, other chemical modifications that are a likely signature of diagenetically derived alterations were considered.

Methods:
Specific proteomic protocols and different sample preparation were adapted to improve protein extraction and sequence coverage yields. The analyses were performed with the use of LC-MSMS. The exploitation of bioinformatic tools such as the error tolerant search in the Mascot suite [2] or the open mass modification utility in the Protein Prospector database search [3] were used for data processing in search of unexpected chemical modifications.

Results:
Ancient human bones (100) have been analyzed by proteomic approach following an adapted protocol based on the already developed one reported in [4]. From the raw LC-MSMS data and the use of Mascot platform, COL1α1 and COL1α2 were detected with a protein sequence coverage up to 80% in all the samples. All the deamidation sites were listed and their occurrence and deamidation state were statistically evaluated. The statistical analysis is suggestive of the most detected positions, that were considered the best candidates for the quantification of the deamidation level as protein’s aging biomarkers in ancient human bones. Similar approach was used to evaluate also other chemical modifications.

Conclusions:
The analysis of the deamidation in the different samples suggests that the deamidation level in the samples from Pompei is low in respect to an extensive deamidation observed in the samples from Herculaneum and Scalandrone (our control reference). Our preliminary results seem to suggest that the low deamidation level could be linked to the peculiar burial conditions and to the “lower” temperature experienced by Pompei inhabitants in comparison to Herculaneum ones.

Novel Aspect:
This set of samples constitutes an extraordinary test case to evaluate deamidation and other chemical modifications as molecular signatures of protein aging.


Wine production and consumption in pre Roman times: residue analysis of a production structure an ceramic vessels from the Western Mediterranean

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Keywords: organic residue analysis, tartaric acid, wine, gas chromatography – mass spectrometry

Introduction:
Wine was an important product in the Western Mediterranean. Organic residue analysis was carried out on samples from pottery and a production structure in four pre - Roman archaeological sites in Italy and Spain, aimed at identifying the presence of wine. At most of the sites the production and consumption of wine is compatible with other archaeological evidence (mainly botanical evidence).

Methods:
Samples were recovered both from the archaeological materials and the earth of the stratigraphic unit to which it belonged, in order to check possible contamination. The samples were mechanically cleaned in the laboratory and powdered. Samples were extracted following the extraction method proposed by Pecci et al. [1], derivatized and analysed by gas chromatography coupled with mass spectrometry.

Results:
The analyses indicate the presence of tartaric acid in some of the analysed pottery samples from the different sites in Italy and in a sample from an Iberian production structure at Coll del Moro (Catalunya, North East Iberian Peninsula).
Although other fruits, for example tamarind, may contain tartaric acid [2], on the basis of the residues identified [1,3,4], and the presence of grape pips or pollen in the area it is possible to suggest that wine or its derivatives was in contact with the sampled materials. The fact that no tartaric acid was identified in control samples, nor in some of the archaeological samples indicates that it does not derive from post depositional contamination.
In some of the samples also other acids such as succinic, fumaric, isocitric, malic and maleic acids, are present. Although they are not exclusive markers of wine, they are present in the beverage.

Conclusions

The data in our possession allow to confirm the hypothesis on the production of wine in the Iberian structure at Coll del Moro where abundant grape pips had been identified and the storage and/or consumption of wine or its derivatives at Etruscan and Bronze Age sites in Italy.

Novel Aspect:
Investigation on wine production and consumption, not only in ceramic vessels, but also in a pre-Roman production structure.

References


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Characterization of isobaric species and photo-degradation products of triarylmethane dyes within textiles

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Keywords: LC-ESI-Q-ToF, Triarylmethane dyes, accelerated ageing, photo-oxidation, degradation pathways

Introduction:
Since mid-19th century, triarylmethane dyes were widely used for ink production and dyeing textiles [1]. These dyes, characterized by bright hues, are one of the first classes of synthetic dyes produced and marketed [2]. Nevertheless, their analysis is highly complicated due to their poor light fastness and their complex composition constituted by homologous species, often differing only for the presence or position of the same substituent on the aromatic rings.

Methods:
Liquid Chromatography coupled with tandem high-resolution mass spectrometry (LC-ESI-Q-ToF) or Diode Array and Fluorescence detectors (HPLC-DAD-FD) were used to separate and identify the several miscellaneous components contained in standard triarylmethane dyes, unaged and aged wool yarns and historical samples of dyed specimens. The samples were pre-treated using an oxalic acid solution to obtain the highest extraction yields for all the components.

Results:
The analysis of standards and reference materials by LC-ESI-Q-ToF allowed us to identify specific fragmentation pathways for several triarylmethane dyes such as Fuchsine, Crystal/Methyl Violets, Methyl Blue and Diamond Green. Both positive and negative modes and collision energies were tested to optimize the MS conditions for each dye and to obtain complementary information on fragmentation pathways. The analysis of wool yarns dyed with triarylmethane compounds, artificially aged for different time intervals, allowed us to investigate and highlight different degradation pathways, through the identification of kinetic trends of different compounds in relation to the accelerated aging times. HPLC-DAD-FD was fundamental to provide further information on the minor compounds. Analysis of selected historical samples from 19th C dyeing manuals from Glasgow’s ‘Dye-versity’ project [3] highlighted the compositional complexity of early synthetic dyes, and revealed several isomers and different profiles depending on desired hues.

Conclusions:
The proposed analytical method, integrating HPLC-ESI-Q-ToF and HPLC-DAD-FD analyses, allowed the full characterization of the references and historical samples under study and to hypothesize the degradation pathway of several triarylmethane compounds. The detection of specific fragmentation patterns enabled distinction between different classes of compounds, while that of specific fragment ions distinguished isobaric species belonging to the same series.

Novel Aspect:

We used for the first time a solid silica core analytical column coupled with HRMS to fully characterize minor and degradation components in reference and historical samples.

References
1185 - ART AND CULTURAL HERITAGE NATURAL POLYMERS BY BOTTOM UP AND TOP DOWN APPROACHES

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Keywords: Cultural Heritage, Top down proteomics, Lipidomics, Metabolomics, Crosslinkings

Introduction:
Natural organic polymers represent the main organic components of the most of the historic samples. However, Cultural Heritage material is challenging to study[1]: (i) The sample size typically obtained from a museum- or archaeological artefact is on the order of tens of micrograms. (ii) Most pieces are made of more than one material, each containing many organic/inorganic components. (iii) The material is constantly evolving due to ageing or external conditions.

Methods:
Top down experiments are used for protein studies; particular focus is given to sample preparation fitted to handle trace compounds. Soft depolymerization and HRMS analysis of multimers are used for lipids; Kendrick and Van Krevlen plots help to decipher the high complexity of spectra. Polysaccharide analysis combines filter aided sample preparation and nanoLC-HRMS analysis of fingerprints; methods are fitted both to linear and highly branched structures.

Results:
This conference will show how organic chemistry combined with HRMS (orbitrap, FT-ICR) address the current challenges for analysis of trace amounts of Cultural Heritage samples. For example, structural elucidation of biopolymers of unknown structures and/or their chemical modifications will be shown (e.g. historic art paintings and watercolors [2]). Focus on protein crosslinkings inside paintings will be done. Another example is the combination of soft depolymerization experiments and HRMS to unravel the 3D networks formed by insoluble lipidic films (e.g. oil-paintings). The very first application of top down approach to the study of protein remains in archaeological ceramics will show impressive results both in terms of protein sequence coverage (up to 100% coverage) and identified sequences that show particular degradation patterns. The conference will be illustrated by the study of various outstanding samples from art but also from archaeological and paleontological sciences from the most famous museums and institutes of the world.

Conclusions:
Applied to the study of artworks, the chemical decoding of the organic molecular network has a major impact on the understanding of painting formulation, biopolymers degradation and it helps in the survey of preservation conditions. Considering archaeological samples, the information obtained by the proposed methods provide new historical insights; for example it points out ancient methods for food processing.

Novel Aspect:
Deciphering Art and Cultural Heritage natural polymers using unexplored developments in chemistry and top down approaches to chemically decode the organic molecular network.

References:
Introduction:
Mass spectrometry (MS) of synthetic polymers most often uses single stage MS alone but characterization of polymers can remain ambiguous without complimentary tandem MS experiments [1,2]. UV-photo-activation creates new opportunities for the characterization of polymers [3,4]. The goal of this work was to evaluate the performances of wavelength-tunable UV photo-activation of synthetic polymers, combined or not with field asymmetric waveform ion mobility.

Methods:
Precursor ions produced by ESI (positive mode) from a variety of commercial polymers (polyethylene glycol (PEG), polymethylmetacrylate (PMMA), and polysiloxane (PDMS), Sigma-Aldrich), have been stored in a linear ion trap (LTQ Thermo), and then submitted to UV synchrotron irradiation during a variable time period and over a range of wavelengths. For the highest mass polymers, UV activation was combined with field asymmetric waveform ion mobility spectrometry.

Results:
By applying suitable compensation voltages in FAIMS, it was possible to specifically isolate certain (M+nCation)n+ adducts produced by ESI and to avoid the overlapping problems. The selected ions could be trapped in a linear ion trap, and then submitted to UV synchrotron irradiation during a variable time period, at different photon energies. For example, the activation of [PEG95+4Na]4+, produced different effects as a function of the activation method (low energy CID vs UV-Photoactivation 16eV, 10s). As low energy CID only led to the loss of one sodium cation according to the equation [PEG95+4Na]4+ -> [PEG95+3Na]3+, the UV irradiation [PEG95+4Na]4+ led to a wide series of fragment ions. Polydimethylsiloxanes also gave net differences. Series of UV–photoactivation mass spectra, recorded with variable photon energies (6 to 24eV), showed that the fragmentation started with photon energies slightly above the supposed ionization potential of the oligomer. Important fragmentations were observed at 16eV.

Conclusions:
In this work, we demonstrated the potential of UV photo-activation of ions, produced from polymers and stored in a linear ion trap. The most useful UV wavelengths were identified. The use of FAIMS made it possible the selection of certain precursor ions that were difficult to isolate (particularly useful for highly charged ions). UV photo-activation could lead to different and useful fragmentations compared to the low energy CID of the same precursors.

Novel Aspect:
The use of synchrotron UV beam for tandem MS experiments from polymers and the combined use of ion mobility for multiply charged precursor ions selection.

References
Introduction:
Developed in the eighties from a founding change of mass scale proposed by Kendrick fifty years ago, the Kendrick mass defect (KMD) analysis has been advantageously used for an easy graphical interpretation of high-resolution mass spectra from carbonaceous samples. Recently extended to the processing of data from polymers [1], the traditional KMD analysis is now enjoying a new lease of life with the introduction of several concepts for an unprecedented visualization experience.

Methods:
Regular Kendrick masses (KMs) are $KM(R) = \frac{m/z \times \text{round}(R)}{R}$ with $m/z$ the accurate mass-to-charge ratio and $R$ the exact mass of a repeating unit (IUPAC scale). “Advanced” KMs compiling a “resolution-enhancement” [2-4] and a “charge-dependence” [5] are $KM(R,Z,n) = \frac{Z \times m/z \times (\text{round}(R)+n)}{R}$ with $n$ and $Z$ being positive/negative integers (calculations using spreadsheet or dedicated programs). KMDs and the “remainders of KMs” [6] are $KMD = \text{round}(KM) - KM$ and $RKM(R) = \frac{KM}{\text{round}(R)} - \text{floor}(\frac{KM}{\text{round}(R)})$.

Results:
Beyond the simplest KMD plot (KMDs vs. m/z) from the mass spectrum of a singly charged homopolymer, a regular KMD plot from multiply charged (co)polymer ions displays the charge state distribution instantly via an isotopic split. To overcome possible isotopic misalignments, a new “charge-dependent” KMD plot clusters the ion series at a chosen charge state into a single cloud facilitating their filtering and assignment. The new resolution-enhanced KMD plot strikingly improves the separation of ion series taking full advantage of the spectral width. Using well-fitted parameters, mass spectra of copolymers become a set of elliptic clusters discriminating ions by their end-groups and co-monomeric content. Terpolymers are also easily turned into a series of copolymeric clusters in a first resolution-enhanced plot, further filtered and re-analyzed via a second resolution-enhanced KMD plot or a “degree of polymerization” plot. The new “remainders of KMs” allow mass spectral data of low mass accuracy such as MALDI-TOF/TOF spectra to be turned into informative plots (RKMs vs. m/z).

Conclusions
The “Advanced KMD analysis” broadens the scope of applications of a very simple data processing tool to low or high mass accuracy data, to singly or multiply charged ions and to homo- / co- / terpolymer ions with one appropriate KMD plot in every case. With a rationalized approach to help users choosing the well-fitted parameters, it may be now routinely used for a graphical and highly visual exploration of mass spectral data.

Novel Aspect:
From the historical definition of a Kendrick mass ($KM = \frac{m/z \times 14}{14.0157}$) to a resolution-enhanced and charge-dependent KM, we propose a deeply rejuvenated KMD analysis.

References
327 - POTENTIAL OF LIQUID EXTRACTION SURFACE ANALYSIS FOR MASS SPECTROMETRIC EVALUATION OF SAFETY AND FUNCTIONALIZATION OF POLYMER FOOD PACKING MATERIALS.

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Keywords: Surface analysis, liquid extraction surface analysis, polymers additives, degradation products, food packaging.

Introduction:
The migration of constituents of polymer packaging into food has two aspects. Additives, post-polymerization or degradation residues, Non-Intentionally Added species (NIAS) are a risk for the consumers but, at the same time, “intelligent” packaging releasing natural antioxidants improve food shelf life. Hence, it is essential to develop methods to evaluate the safety and functionalization of polymer food packaging.

Methods:
LESA-MS (liquid extraction surface analysis mass spectrometry) combined with nanoESIionisation was optimized for the direct analysis of polymer packing materials. The method does not require any sample preparation, is fast, eco-friendly and highly reproducible. It appears to have many advantages over other direct analysis techniques and is well adapted to the polymer analysis.

Results:
The method was successfully applied to follow the release of oligomers from additive-free low-density polyethylene (LDPE) during recycling. The increasing number of cycles resulted in the gradual appearance of clusters of species with m/z separated by 14Da (the monomer) thus proving polymer degradation. LESA-MS analysis also allowed the detection of a series of species corresponding to residual polyethylene oxide - a plasticizing additive used for PET synthesis or recycling. The efficiency of the natural antioxidants (vitamin E and C) on the polymer stabilisation was proven by the comparison of the LESA-MS spectra of pure and functionalized LDPE. Moreover, the release of these additives (meant to protect food packed using this “intelligent” material) could be quantified using laboratory prepared solid state standards.

Conclusions
LESA-MS was optimized to study the release of low molecular species from polymer food packing films and the monitoring of degradation products during recycling of additive-free LDPE and migration of antioxidants from functionalized LDPE. The use of solid state standards made possible quantitative monitoring of the antioxidant release and thus quality control of “intelligent” packaging.

Novel Aspect:
LESA-MS is a fast and reliable ecological tool with a high potential for the evaluation of safety and functionalization of polymer food packing films.
Introduction:
Polyisobutenes (PIBs) is an important class of non-polar hydrocarbon polymers produced by cationic polymerization of isobutylene [1]. Owing to their outstanding chemical resistance and biocompatibility, PIBs can be applied in many fields spanning from coatings to various biomaterials [1]. In this lecture, we present a method based on the adduct ion formation of PIBs with anions [2], called anion-attachment method [3] for the characterization of low molecular weight PIBs using electrospray ionization mass spectrometry (ESI-MS).

Methods:
Polyisobutenes with different molecular weights and end-groups were synthesized by living cationic polymerization. Measurements were performed with a MicroTOF-Q type Qq-TOF MS instrument (Bruker Daltoniks, Bremen, Germany). The critical (activation) energies for the dissociation of [PIB+anion]− into PIB + anion were determined by means of broadband MS/MS measurements using the survival yield (SY) method and supported by high-level quantum chemical calculations (DFT).

Results:
The [PIB+anion]− adducts were generated using electrospray ionization (ESI) by adding the corresponding salts to the PIB solution. It was found that independently of the end-groups, presence or absence of aromatic initiator moiety, the PIB derivatives investigated in this study were capable of forming adduct ions with various anions such as NO3- and Cl− ions, thus allowing the characterization of these compounds using the negative ion mode of ESI-MS. The dissociation of the [PIB+anion]− adduct was shown to yield neutral PIB and charged anion and that the collision energy necessary to obtain 50 % dissociation of the precursor ion (CE50) changed linearly with the number of isobutylene units. Furthermore, it was found that the binding enthalpies of the halide ions to PIB, determined by using broadband MS/MS and the SY methods, increased in the order of I− < Br− < Cl− < F− . This order is in line with that obtained by DFT calculations and correlate with the size of the halide ion.

Conclusions:
The anion-attachment under ESI conditions proved to be an appropriate method for ionizing the non-polar polyisobutylene derivatives. Furthermore, applying broadband MS/MS and the SY method information on the strength of the interaction between the respective anion and the non-polar polymer chain can be gained.

Novel Aspect:
Our work has revealed that polymers even with high hydrophobicity can be effectively characterized by ESI-MS which is known to be mainly capable of investigations of polar compounds.

Acknowledgement:
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References
1128 - STUDY ON THE SYNTHESIS AND CHARACTERIZATION OF SURFACE ACTIVITIES OF HYDROPHILIC DERIVATIVES OF \( \beta \)-SITOSTEROL

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Keywords: \( \beta \)-Sitosterol, Hydrophilic derivatives, Poly(ethylene glycol), Surface activity

Introduction
Phytosterol means all the alcohol compounds with steroid moiety found in higher plant life and at least 44 sterols have been identified [1]. The most abundant phytosterol is \( \beta \)-sitosterol (24-ethyl-5-cholestene-3-ol) which has the greatest potential for the production of steroidal drugs, cosmetics for healing effects on damaged skin and anti-inflammatory effects, and food ingredients that function as cholesterol-lowering agents. However, hydrophobic and lipophobic properties of \( \beta \)-sitosterol have prevented its widespread application.

Methods
A hydrophilic phytosterol derivative (HPS) was prepared by coupling a hydrophilic matrixcarboxyethyl-\( \beta \)-sitosterol (CES) to poly(ethylene glycol) (PEG), where CES was prepared by reacting \( \beta \)-sitosterol with succinic anhydride. The chemical structure and the distribution of isomers of HPSs were investigated by H-NMR and Maldi-Tof respectively. Surface activities of HPSs were also measured and discussed as a function of molecular weight of the parent PEG.

Results
DS (degree of substitution) was calculated from 1H-NMR data and the DS values of HPSs (product having one sterol moiety at one end of PEG) were close to 1.0 and 2.0 for Di (product having two sterol moieties at both ends of PEG). From the Maldi-Tof spectra of PEG, HPS and Di with different chain lengths, each peak has intervals of 44, which is equivalent to the mass of monomeric unit of PEG, and all spectra shows a narrow range in molecular weight. Spectra of HPSs displays identical pattern with those of corresponding PEGs, except that the molecular weight (m/z) of HPS shifts to the higher m/z to the extent of approximately 500 m/z compared to that of PEG. Spectra of Di also displays a displacement to the higher m/z to the extent of approximately 1,000 m/z compared to that of PEG. These results strongly indicate that the HPSs prepared are pure mono-type derivatives. The solubility of HPS in water increased as the molecular weight of the parent PEG increased. However, based on the \( \beta \)-sitosterol moiety in HPS, HPS-1000 prepared from PEG 1000 showed the highest solubility.

Conclusions
Three kinds of HPSs were synthesized from PEG by reacting CES with 5 molar excess of corresponding PEG and removing unreacted PEG by extraction. All HPSs were soluble in water and found to have excellent surface activity and the same cholesterol-lowering effect as \( \beta \)-sitosterol. Thus, the potential application of HPS as a surface active material with biological activities of \( \beta \)-sitosterol such as healing effects on damaged skin or anti-inflammatory effects is expected.

Novel Aspect
New types of HPSs having cholesterol-lowering effects was synthesized by coupling \( \beta \)-sitosterol with a hydrophilic matrix to improve water solubility of \( \beta \)-sitosterol.

References
Introduction:
The characterization and the assignment of individual peaks of copolymers are great challenges in the mass spectrometry owing to the huge number of m/z peaks. The Kendrick mass defect (KMD) analysis [1-2] partly solved this issue with limitations such as the high resolution and high accuracy. However, our developed method that is based on the mass remainder after dividing by the exact mass of one of the repeating unit works with less limitations.

Methods:
Measurements were carried out with an Autoflex type MALDI-TOF MS instruments (Bruker Daltoniks, Bremen, Germany). DHB (2, 5-dihydroxybenzoic acid) and dithranol (1,8,9-anthracenetriol) were used as matrix, while ionizing agents were sodium trifluoroacetate and silver trifluoroacetate (solvents: MeOH, THF). Ethylene oxide- propylene oxide triblock copolymers were investigated. Thioester type agent was used for the RAFT polymerization of polystyrene [3].

Results:
A new data handling method, the Mass-Remainder Analysis (MARA), was developed that use the mass remainder values for the identification of the peaks in copolymer mass spectra and correct the intensities with the defects originated overlaps and isotopic peaks. The mass remainder (MR) values are calculated by division of the measured m/z values by the exact mass of one of the monomer unit. The polymer chains differ only in the number of monomer unit (used for division) have the same MR values. Therefore, these are suitable for grouping, classifying and identifying the copolymers with different composition. For the assignation a defined mass list is used therefore, the deisotoping of the spectrum is also done during the assignation step. Then in the next step the intensities are corrected. The intensities for the assigned peaks and their isotopologues are calculated and subtracted from the measured ones if it is necessary. Furthermore, the intensities of the identified peaks are corrected with the intensity of corresponding isotopologues.

Conclusions
It was demonstrated that our method was capable of characterizing ethylene oxide- propylene oxide copolymers with higher molecular weight, to determine the number average-, weight average molecular weights, the bivariate distribution and the composition drift [4]. Furthermore, polystyrene was identified to investigate the polymerization mechanism of reversible addition-fragmentation chain-transfer (RAFT) polymerization.

Novel Aspect:
The Mass Remainder Analysis (MARA) is a new method which capable of characterizing higher molecular weight copolymers with higher efficiency then previously used Methods:

Acknowledgement:
The work was supported by the GINOP-2.3.2-15-2016-00041 project and by the grant K-116465.

References:
COMPOSITIONAL ANALYSIS OF HIGH MOLECULAR WEIGHT BIODEGRADABLE POLYMERS BY HIGH-RESOLUTION MALDI-TOFMS COMBINING ON-PLATE DEGRADATION WITH RESOLUTION-ENHANCED KMD ANALYSIS

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Keywords: on-plate degradation, resolution-enhanced KMD analysis, high-resolution MALDI-TOFMS, copolymer, polymer characterization

Introduction:
High-resolution mass spectrometry is notoriously limited to the low mass range (<3 kDa) while industrially important polymers are of high molecular weight (typically >10 kDa). We propose a “on-plate degradation” as an in situ sample preparation to reduce the molecular weight of polyesters using an alkaline solution. The low molecular weight degradation products are then amenable to a mass-analysis taking full advantage of the resolving power of the analyzer.

Methods:
Poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (P(3HB-co-3HV)) with >100 kDa has been chosen as a reference aliphatic polyester. A sample solution is deposited on the MALDI target plate followed by a drop of the isotope labeled NaOH-CD3OD. Removing the excess of alkaline solution with water, the matrix solution is finally added to make co-crystal. MALDI mass spectra are recorded using a high-resolution JMS-S3000 spiral TOF mass spectrometer (JEOL, Japan).

Results:
Following the on-plate degradation, the mass analysis of P(3HB-co-3HV) reveals a complex fingerprint in the low mass range (<3 kDa) hence validating the degradation procedure. However, the mass difference between HB / HV repeating units and between carboxyl/methyl ester chain-ends are identical at 14 Da (CH2 moiety). To distinguish the variation of HB/HV composition from the variation of end-groups, the peaks associated with the methyl ester termination are shifted of 3 Da via the on-plate degradation using the isotope labeled NaOH-CD3OD solution. Instead of a time-consuming peak-picking and manual assignment of data, ion series are conveniently filtered in a KMD plot computed from the complex mass spectrum using our innovative resolution-enhanced KMD analysis [1]. Peak lists are finally turned into a “degree of polymerization plot” [2] displaying the discrete co-monomeric composition at first glance. The average HV/HB content also computed from the plot is eventually in good agreement with the reference value provided by the supplier.

Novel Aspect:
The compositional analysis of a high-molecular weight polyester is made possible by combining in situ isotope labeled degradation with an innovative data processing.

References
Introduction:
Advanced polymerization techniques allow production of synthetic chains with a perfect control of the molecular structure and of the comonomeric sequence [1]. One key application of such sequence-defined polymers is information storage: a message can be chemically encoded in the chain by using a set of two comonomers and can be read using tandem mass spectrometry (MS/MS)[2]. These polymers can be used as molecular barcodes in anti-counterfeiting applications [3].

Methods:
Polyurethane (PU) oligomers were synthesized as previously described [2]. Reading of different sequence-defined polyurethanes was evaluated in electrospray tandem mass spectrometry from deprotonated ions using a Q-TOF instrument. Extraction procedures of PUs were optimized as a function of the nature of the membrane.

Results:
Sequence-defined polyurethanes (PU) are composed of carbamate-based comonomers in which the coded moiety is a small alkyl segment that contains (1-bit) or not (0-bit) methyl substituents. These are extremely easy to read in the negative mode due to i) single cleavage of the carbamate bond in each monomer and ii) unique location of the negative charge at the α chain-end. As a result, the message coded in these PU chains can easily be read by MS/MS as the distance between two fragment ions corresponds to the mass of one or the other comonomer. Due to such a high MS/MS readability, PUs were tested as molecular barcodes for the labeling of different membranes made of PS, PVC, PET, and PVA. Optimization of the PU extraction step for each polymeric substrate permitted to reach detection limits in the ppm range for MS/MS sequencing. Moreover, the tagging homogeneity of membranes was evaluated from ESI-MS experiments.

Conclusions:
Sequence-defined PUs were used as molecular taggants for the labeling of various polymeric materials. Thanks to the renowned high specificity and sensitivity of MS/MS, taggants were recovered and identified even at very low concentration levels. Selectivity of taggant extraction from the membranes prevented any matrix effects during the electrospray ionization step, allowing direct sample introduction and hence fast reading of the molecular code.

Novel Aspect:
PU barcodes were successfully recovered and identified by MS/MS from polymeric materials in which they were used as anti-counterfeiting labels.

References:
Introduction
Substrate-assisted laser desorption inductively coupled plasma mass spectrometry (SALD ICP MS) is a technique for inorganic analysis of liquid samples of sub-microliter volumes in the form of dried droplets on suitable polymer substrates.[1] Here we study influence of substrate surface aging effects on analysis of cadmium and indium in samples prepared on polymer substrates treated by diffuse coplanar surface barrier discharge (DCSBD).[2]

Methods
A DCSBD device (RPS400-Roplass plasma system 400 W, Roplass, Czech Republic) was used for treatment of polymersubstrates at 400 W. Contact angle of the deposited droplet with Cd and In was measured using See System E, Advex Instruments, Czech Republic. Samples were desorbed in ablation cell (UP213, New Wave Research, USA) with a 213-nm laser, and content of 111Cd and 115In analyzed by a quadrupole ICP mass spectrometer (7500ce ICP-MS, Agilent, USA).

Results
Four polymer substrates (polyethylene terephthalate glycol, polyethylene terephthalate, polycarbonate and styrene-acrylonitrile) were exposed to cold plasma for 1, 5 and 10 s. Droplets of Cd and In solutions in citrate, biological culture medium and cell lysate were deposited in five replicates immediately, 1, 3, 6 days, 1, 3 or 6 months after the treatment on the polymer substrates. Contact angles of droplets as well as size of the generated stains were characterized. Typically, a longer treatment of surface substrate resulted in lower contact angle. The stain diameter gradually decreased with time after treatment, sometimes showing a maximum at deposition 1–6 days after the treatment. Also, the longer the surface treatment was, the slower return to the original state (high value of contact angle) was observed. Citrate concentrations above 5 mM resulted in significant drop of 111Cd intensity. Using In as an internal standard for determination of Cd alleviated the observed matrix and surface related phenomena and reduced RSD by a factor of ~2.

Conclusions
Aging of surfacetreated by DCSBD has been monitored using physical methods and related to SALD ICP MS response of samples prepared on the surface. The initial experiments show significant changes in contact angle, stain size and SALD ICP MS response. Using an internal standard, RSD values were reduced to <10 %. A half-year monitoring is under progress; a procedure for surface treatment and sample preparation will be recommended after completing the entire set of analysis.

Novel Aspect
Substrate surface treatment by DCSBD for dried droplet preparation. Systematic characterization of surface aging and its consequences for SALD ICP MS reproducibility.

References

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350 - REMAINDERS OF KENDRICK MASS: KEEPING THE BEST OF A KENDRICK MASS DEFECT ANALYSIS FOR LOW-ACCURACY MASS SPECTRA OF POLYMERS

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Keywords: low mass accuracy, Kendrick mass defect, Remainders, MALDI-TOF/TOF, polymers

Introduction:
The traditional Kendrick mass defect (KMD) analysis [1] is restricted to high-resolution mass spectrometry (low mass range, high-accuracy analyzers) as it evaluates the difference between rounded and exact m/z of polymer ions. The new resolution-enhanced KMD analysis [2,3] makes data in the high mass range or recorded with low-accuracy analyzers interpretable in a KMD plot but point alignment remains of low quality. The “Remainders of KM” (RKMs) overcome this issue.

Methods:
Data were recorded using a JMS-S3000 MALDI mass spectrometer (JEOL, Japan) equipped with a high-resolution spiralTOF, a linear TOF with oligomeric resolution and a TOF/TOF device (unitary resolution). A Kendrick mass (KM) is KM=m/z*(round(R)+n)/R with R the exact mass of the repeating unit and n an integer (n=0 for a regular analysis, n=+-1,2,3… for a resolution-enhanced analysis). KMDs and RKMs are KMD=round(KM)-KM and RKM(R)=KM/round(R)oor(KM/round(R)).

Results:
The RKM has been found to consist in the difference between a regular KMD and a resolution-enhanced KMD [4]. In other words, RKMs take advantage of the improved separation of ion series from the resolution-enhanced analysis while errors are cancelled from the subtraction to the regular analysis. Point alignments of homologous species in a new RKM plot (RKMs. vs m/z) are perfectly horizontal with an unrivalled separation power. A RKM plot is compatible with a great variety of mass spectral data, starting from single stage mass spectrometry using a high-accuracy analyzer in the high mass range with isotopic resolution. Despite a decrease of the mass accuracy via a loss of resolving power, tandem mass spectra recorded using a TOF/TOF device with unitary resolution down to oligomerically resolved data recorded with a linear TOF are turned into clear RKM plots separating product ion series and distributions via horizontal alignments, respectively [5]. It greatly facilitates the visualization and assignment as well as any subsequent data processing.

Conclusions
The Remainders of Kendrick masses (RKMs) fit onto any sort of mass spectral data from polymeric samples as long as a minimal oligomeric resolution is reached. RKMs also greatly simplify the data processing of copolymeric data [6] and are totally compatible with multiple charging [4]. Linked to the Kendrick mass defects (KMDs) in a unified theory, they are now part of an “Advanced KMD analysis” available in commercial programs computing mass defect plots.

Novel Aspect:
Data from low-accuracy analyzers or in the high mass range are turned into informative compositional maps for an easy sement of polymeric ion series.

References
ANALYSIS OF POLYSTYRENE BASED MICROPLASTICS IN THE ENVIRONMENT

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Keywords: polystyrene, microplastics, seawater, HPLC-APPI/HRMS

Introduction:
Marine anthropogenic litter is a severe environmental problem[1]. One of the main issues is the extreme stability of plastic wastes[2], which can generate nanoplastics (NPLs) and microplastics (MPLs)[3]. The analysis of these plastic particles should consider a combination of different analytical tools for the quantitative/qualitative analysis of MPLs/NPLs. These studies have been carried out using polystyrene (PS) as a representative polymer.

Methods:
A standard of PS (MW~1.2kDa) was solubilized in toluene at room temperature. For the mass spectrometric analysis, the performance of different ionisation sources (ESI, APCI, APPI, DART, MALDI) were optimized and assessed under negative and positive conditions. The chromatographic separation was achieved by size-exclusion with a GPC-column and using toluene as mobile phase. Mass spectrometric analyses were carried out using a QExactive mass spectrometer.

Results:
The optimal results were obtained by HPLC-HRMS equipped with atmospheric pressure photoionization (APPI) source operating in negative mode. The ionisation of PS resulted in a characteristic profile of signals, each of them separated by 104 m/z. The addition of a chromatographic separation with GPC column showed a good performance for qualitative and semi-quantitative measurements in complex extracts. The APPI source was by far the most efficient ionisation source for this polymer in comparison with ESI and APCI.

Additionally, the potential of ambient air mass spectrometry techniques, such as DART, for the detection and identification of PS was assessed. The results suggested that MALDI and ambient air ionization techniques result in low ionisation efficiencies in comparison with APPI.

These results were combine with others different techniques such as TGA and FT-IR which are useful tools for the characterization and identification of polymers but not to quantify them.

Conclusions:
GPC-LC coupled to APPI-QExactive is a suitable approach for the analysis of low molecular weight PS (< 4000Da) and applies to real seawater samples. However, the use of LC-APPI-HRMS should be complemented by other techniques such as TGA, DSC and FT-IR, which allow obtaining qualitative and semi-quantitative information about high-molecular weight polymers, which may also be present in the environment.

Novel Aspect:
The environmental analysis of polymers should rely on a protocol, which combines different types of techniques for the complete characterization of polymers.

References
Introduction:
Polybutylene terephthalate (PBT), a thermoplastic engineering elastomer, is widely used in a variety of industrial fields. Due to its industrial applications, it is important to evaluate its degradation degree to facilitate the product development process. The purpose of this work is to evaluate the degradation degree during the initial degradation stage which hasn’t been detected by FT-IR, directly and rapidly.

Methods:
A new and degraded product made by PBT were used as the samples. Thermal desorption and pyrolysis combined with DART-MS (TDP/DART-MS) was composed of a compact (Bruker) Q-TOF mass spectrometer equipped with a DART ion source (IonSense) and an ionRocket TDP device (BioChromato). The samples were cut to ~1 square millimeter and put into the sample pot. Mass spectra were measured as the samples were gradient heated from room temperature to 600 °C at a rate of 100 °C/min.

Results:
Regarding from the total ion current gram (TIC), as adding the temperature gradient heating by TDP device, thermal desorption and pyrolysis reactions were detected, as shown by the change in intensity. Mass spectra of samples at ca. 220 °C (ca. the PBT melting point) were detected the pyrolysis products, monomer and dimer units of PBT. Comparing the extracted ion current gram (EIC) of the PBT monomers (m/z 221.08, [C12H12O4+H]+) between new and degraded product, there was a difference in the detected temperature and in the amount of PBT monomer. For the degraded sample, the PBT monomer was detected at a temperature much lower than the new sample. Moreover, for the degraded sample, it was detected at much lower than the pyrolysis temperature of the PBT (ca. 400 °C). Thus, the PBT monomer that was detected at much lower than the pyrolysis temperature of PBT was presumed a one of the degradation products.

Conclusions:
TDP/DART-MS enables detection the initial degradation state which hasn’t been detected by FT-IR. Moreover, TDP/DART-MS can be a useful method to evaluate the degradation degree using PBT monomer as a marker. Thus, this method is expected to be applicable to polymer failure analysis, quality control and so on.

Novel Aspect:
TDP/DART-MS is useful failure analysis method for thermoplastic engineering elastomers such as PBT.
Recycled thermoplastic resins are regarded as important materials for realizing sustainable society. Therefore, it is important to control and grasp the thermal history of polymer recycle, because recycled thermoplastic resins are weakened in physical properties by thermal history added. Thus, the purpose of this work was to evaluate the thermal history, directly and rapidly.

Methods: (384 /400 characters)
Polypropylene(PP) virgin pellet and 3× recycled pellet were used. Thermal desorption and pyrolysis(TDP) combined with DART-MS was composed of a compact (Bruker) Q-TOF mass spec. equipped with a DART ion source (IonSense) and an ionRocket TDP device (BioChromato). The samples were cut to ~1 square millimeter and put into the sample pot. Mass spectra were measured as the samples were gradient heated from room temperature to 600 °C at a rate of 100 °C/min.

Results: (832 /900 characters)
Regarding the total ion current gram of the virgin pellet and 3× recycled pellet from room temperature to 600 °C, no significant difference was detected, and similar thermal decomposition patterns were observed. However, regarding the mass spectra, the thermal decomposition reaction of polypropylene seemed to begin at 260 °C. An antioxidant additive (Irgafos168) and its oxidized compound (tris(2,4-di-tert-butylphenyl) phosphoric acid) were detected at this point, with differences observed between samples. In comparing the intensity ratios of the oxidized and pure between samples, the ratio of the 3× recycled pellet was higher than that of the virgin pellet. This confirmed that the 3× recycled pellet possessed increased oxidized Irgafos168 because of recycle. In addition, in comparing the extracted ion current grams for Irgafos168 between samples, the 3× recycled pellet showed less than the virgin pellet. This confirmed that Irgafos168 was consumed by recycle.

Conclusions: (384 /400 characters)
Using TDP/DART-MS, additives contained in PP and PP polymer matrix were detected independently and directly by gradient thermal heating. TDP/DART-MS can be a useful way to evaluate the thermal history of recycled thermoplastic resins quickly and easily, using the antioxidants contained in the thermoplastic resins as markers. Moreover, this method could be applied to quality control for thermoplastic resin products, such as containers and parts.

Novel Aspect: (111 /150 characters)
TDP/DART-MS is useful evaluation method of thermal history for thermoplastic resins using the additive marker contained in them.
Introduction:

MALDI-LID-ToF/ToF mass spectrometry is a technique used to aid in the characterization of proteins. While MALDI tandem mass spectrometry techniques have been applied extensively to homopolymer compounds, with some application to block copolymers. Herein we report on MALDI-LID-ToF/ToF as a powerful tool to give information of monomer distributions, and microstructural information, for both block copolymers and statistical copolymers.

Methods:

MALDI-ToF and MALDI-LID-ToF/ToF experiments were performed using an UltrafleXtreme MALDI-ToF/ToF analyser (Bruker, Coventry, UK). The MALDI-ToF experiments were performed in reflectron positive ion mode, with an acceleration voltage of 19 kV. MALDI-LID-ToF/ToF experiments were carried out in reflectron positive mode, with an ion source voltage of 8 kV, and a post fragmentation cell acceleration voltage of 19 kV. Polymers were synthesized using photo-RDRP.

Results:

MALDI-LID-ToF/ToF results for the homopolymers revealed that the lability of the bromine end group led to a fragmentation pattern heavily dependent upon a radical backbiting process. This in turn led to the MALDI-LID-ToF/ToF spectra only containing a single dominant fragment structure, which had both the alpha end group and an addition CH2 vinyl, as well as some smaller secondary fragments from the radical backbiting process. This was seen for both the methyl and ethyl acrylate homopolymers.

It is this simplification of the fragmentation pathway to a single fragment that sequencing the copolymers became much less complicated. Simply observing the overall makeup of the spectra granted simple confirmation of the diblock and statistical copolymer structure, as the diblock retained two distinct regions of monomer separation while the statistical copolymer kept a random structure throughout. Evidence of mixing in the diblock also showed the microstructure resolution the technique can obtain.

Conclusions:

MALDI-ToF and MALDI-LID-ToF/ToF can provide simple screening of polymer and copolymer compounds, as the techniques use the same sample spot to produce both results, full analysis can be carried out in under an hour. There are clear differences between the diblock copolymer and the statistical copolymer spectra, and, despite the techniques simplicity, determining the co-monomer arrangement of a copolymer is fully within the reach of MALDI-LID-ToF/ToF.

Novel Aspect:

Utilizing a technique for copolymer analysis, previously only used for proteins, for fast screening of microstructure determination.

References
235 - UNRAVELLING THE ARCHITECTURE OF BRANCHED POLYESTER OLIGOMERS BY COMBINED TRAPPED ION-MOBILITY SPECTROMETRY – TANDEM MASS SPECTROMETRY

Robert Voeten (1) - Bram Put, van de (2) - Jan Jordens (2) - Ynze Mengerink (2) - Ron Peters (3) - Rob Haselberg (4) - Peter Schoenmakers (5) - Govert W. Somsen (1)

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Unravelling the architecture of branched polyester oligomers by combined trapped ion-mobility spectrometry – tandem mass spectrometry

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Keywords:
Ion Mobility Spectrometry; Branched Polymers

Introduction:
Synthetic polymers have many uses due to their large variety of potential properties. As alterations of the molecular structure, e.g. branching, result in significantly different solid-state properties, it is essential to characterize the architecture of produced polymers. We demonstrate trapped ion-mobility spectrometry with quadrupole time-of-flight mass spectrometry (TIMS-QTOFMS) for structural elucidation of branched isomeric polyester oligomers.

Methods:
Polyesters of terephthalic acid (TPA) and propylene glycol (PG) with trimethylolpropane (TMP; branching agent) were synthesized. Polymer solutions (20 ppm) in THF were analysed using a Bruker timsTOF instrument allowing recording of mobilograms while continuously gathering high-resolution MS/MS data. Supercharging was realized by adding m-nitrobenzyl alcohol. 1H/13C-NMR was used to verify polymer branching.

Results:
Analysis under optimized TIMS conditions resulted in observation of multiple conformers per polymer mass. Mobility separation was achieved as branched polymers exhibit a reduced rotationally averaged 3D-projection. Tandem MS of mobility-separated isomeric polymer species allowed their structural assignment. However, more peaks than possible structures were observed implying influence of the adduct-ion location on the respective mobilities. In verification of the latter, localization of charge by a trivalent ion, i.e. lanthanum, was achieved. For comparison, supercharging by m-nitrobenzyl alcohol was used to achieve multiple sodium adduct formation. Supercharging also allowed simultaneous low and high mass monitoring, showing potential towards increasing separation power. The latter provided a more accurate evaluation of the peak areas in the mobilograms, providing a semi-quantitative tool for assessment of the branching content of the polymer samples.
Conclusions:
The complex and divers properties of industrially relevant polyesters provided an ideal sample to show the added value of TIMS for polymer characterization. Structural elucidation of the multitude of isomers is realized by combining mobility traces and MS/MS data. Supercharging allowed assessing the branching content semi-quantitatively, and enabled mass observation of three times the number average molecular mass.

Novel Aspect:
Use of TIMS on supercharged polyesters allows assessment of the branching content, structure and adduct location of polyester oligomers.

Acknowledgement:
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Introduction

Functional materials comprise synthetic macromolecules with well-defined structures, optimized to impart properties needed for specific biomedical, technological, or environmental applications. Multidimensional mass spectrometry (MS) methods can provide accurate and sensitive information about the molecular-level composition, structure, and architecture of such materials, a requirement for understanding and optimizing their applications.

Methods

MALDI- and ESI-MS interfaced with tandem mass spectrometry (MS2), liquid chromatography (LC) fractionation, and/or ion mobility (IM) separation were utilized to elucidate sequence and architecture. Mild thermal degradation with an atmospheric solids analysis probe (ASAP) was used to analyze crosslinked materials. Conversely, surface-layer (SL) MALDI-MS was employed to characterize and image the molecular composition and defects of solid film surfaces.

Results

MS2 via collision-activated dissociation and/or electron transfer dissociation has been successfully used to determine the sequence of poly(N-isopropyl amide-methacrylic acid) copolymers, read the sequence of monodisperse copolyesters, and elucidate the architecture of multi-arm polyethers. For more complex systems, such as branched polyglycerol conjugates, adding LC and IM separation was essential for deciphering branching architecture and degree of conjugation. Crosslinked hydrogels required mild thermal degradation via ASAP-MS; this method mainly breaks weak junctions in the polymer network leading to signature ions for both the crosslinks as well as defects such as unreacted ends. On the other hand, molecular information on the surface composition of solid films (<2 nm depth) could be gained by SL-MALDI-MS; this solvent-free technique allowed to image surface defects from foreign materials, material absence, mechanical scribing, and solvent perturbation on thin films made of poly(methyl methacrylate) and polystyrene.

Conclusions

Multidimensional mass spectrometry methods encompassing different combinations of mass analysis, tandem mass spectrometry, and orthogonal separation by polarity and/or shape enable sensitive and precise analysis of the microstructures of synthetic functional materials that are widely used in industrial, biomedical, and technological applications, but challenging to characterize by other analytical techniques due to their complexity, size, or topology.

Novel Aspect

Complete molecular-level analysis of functional materials for meaningful structure/property correlations and continuous improvement of their macroscopic properties.

Keywords

Synthetic polymers; polymer sequence; polymer architecture; branched polymers; surface analysis
Keywords: ultrasound assisted extraction, steroidal saponins, Dioscorea deltoidea plant cell culture, Latin square experimental design, HPLC-MS

Introduction
Many of steroidal saponins have anticancer potential and may be used for treatment of cardiovascular diseases. Cell biomass grown in bioreactors can be used to produce steroidal saponins. Dioscorea deltoidea plant cell culture contain an extremely complicated mixture of furostanol steroidalsaponins. Therefore, it is critical to control the composition of cell cultures for medical use.

Methods
For the extraction of steroidal saponins ultrasound assisted extraction (UAE), multiple successive extraction (MSEM) and reflux extraction (RE) methods were used. LC separation was performed on a reversed phase C18 column in gradient elution mode. MS detection was conducted in the positive electrospray ionization mode (SIM mode).

Results
Optimization of the UAE parameters was performed with use of Latin square experimental design[1]. The following parameters have been optimized: extraction time, organic solvent concentration in extraction solution and the ratio of solvent to sample. Optimal conditions for the extraction of steroidal saponins by the UAE method were: extraction time, 60 min; acetonitrile (water) concentration in extraction solution, 50% (50%); the ratio of solvent to sample, 400 mL/g. As a result, high degree of the extraction of steroidal saponins from the D. deltoidea plant cell suspension culture was achieved. The obtained results were confirmed by MSEM and RE. It was also shown that the UAE method is not suitable for isolation of steroidal glycosides from the D. deltoideaplant material.

Conclusions
For the determination of steroidal saponins from D. deltoidea cell culture comprehensive and representative pre-LC-MS extraction procedure was proposed. Low degree of the steroidal saponins extraction from the plant material is most likely due to the lack of mechanical tissues in the cell culture, in contrast to plant material. The UAE method was tested on a series of 18 cell culturesamples of different inoculation date, growing condition and growth period.

Acknowledgements
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Novel Aspect
Comparison of the different approachesperformance characteristics have been carried out with respect to finding an optimal pre-LC-MS treatment of D. deltoidea cell culture.

References

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Introduction:
Over 20 states in the U.S. have legalized the use of recreational or medical cannabis because of therapeutic benefits for ailments such as cancer, multiple sclerosis, and ALS. Since chronic exposure to pesticides in cannabis poses serious health risks, pesticide analysis in cannabis is important. Apart from Oregon state regulatory limits for about 65 pesticides in cannabis, the state of California has issued more stringent regulatory limits for 72 pesticides residues in cannabis flower and edibles. We analyzed all 72 pesticides (including very hydrophobic and chlorinated pesticides generally analyzed by GC/MS) spiked in cannabis flower extracts using LC-MS/MS with ESI and APCI source well below the action limits specified by both Oregon and California State.

Methods:
Ground cannabis flower (1 gm) was extracted with acetonitrile (5 mL). The extract was spiked with different levels of 72 pesticides and 10 deuterated internal standards. After that, extract was further diluted 1:1 with acetonitrile containing 0.1% formic acid. The diluted extract was analyzed directly using LC-MS/MS triple quad with electrospray and APCI source. A 20 min LC-MS/MS method using LX-50 LC, C18 Column and PerkinElmer Qsight 220 MS with electrospray source was used to detect 70 out of 72 pesticides regulated by California state. A 7.5 min LC-MS/MS method using APCI source and C18 column was used to analyze rest of 2 out of 72 pesticides controlled in cannabis by California state.

Results:
Normally, pesticide analysis in cannabis and other food matrices is done by both GC/MS and LC/MS since some non-polar and chlorinated pesticides are difficult to ionize with electrospray and APCI ion source used in LC/MS systems. Two different LC-MS/MS methods with electrospray and APCI source were used for low-level analysis of all 72 pesticides (including very hydrophobic and chlorinated pesticides analyzed by GC/MS) in cannabis. The overall sensitivity for most of the pesticides, including those that are normally analyzed by GC/MS was between 1-300 ppb in cannabis, well below regulatory limits set by the state of Oregon and California. The ability to screen and...
quantitate all 72 pesticides, including the very hydrophobic and chlorinated GC/MS amenable, in cannabis with LC-MS/MS only with dual ESI/APCI source makes this a novel way of screening and quantitation of pesticides in cannabis and different matrices with a single instrument. The ionization mechanism for analysis of chlorinated pesticides in cannabis using LC-MS/MS system with APCI source would also be presented. The recoveries of majority of the 72 pesticides spiked in grounded cannabis flower were estimated to be between 60-120% with RSD less than 20%.

Long term stability data for pesticide analysis in cannabis was collected using a triple quadrupole mass spectrometer fitted with dual electrospray ionization source and atmospheric chemical ionization source (APCI) and combined with a heated and self cleaning laminar flow interface. Long term stability data for pesticide analysis in cannabis showed that response RSD over 1 week for majority of pesticides was between 2 to 10%.

Conclusions

The results demonstrate the successful approach to screen and quantitate pesticides in Cannabis well below the governmental action limits. Very hydrophobic and chlorinated species are also amenable, eliminating the need for further (GC/MS) instrumentation. In addition to this, the maintenance needs of the system are substantially reduced by the heated self-cleaning/laminar flow interface, despite of the presence of challenging matrices such as cannabis.

Novel Aspect:

Analysis of GCMS and LCMS amenable pesticides in cannabis using a LCMSMS system with dual ESI/APCI ion source.

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Introduction:
A series of cis/trans esterisomers, that showed high efficacy to reverse the acquired resistance of cancer cells during chemotherapeutic therapy was studied as candidate drugs [1]. In order to evaluate their stability in human plasma, a liquid chromatography tandem mass spectrometry (LC-MS/MS) method was developed together with a mathematical algorithm, that is able to identify the isomers present in analyzed samples, without their chromatographic separation.

Methods:
The LC-MS/MS analyses were carried out using a triple quadrupole system equipped with an electrospray source (ESI), operating in positive ion mode. The employed chromatographic parameters were finely tuned to minimize the run time. Therefore, a short C18 Column (3 cm) and fast elution gradient were used. Furthermore, a matrix of linear equations (LEDA), based on abundance ratios of product vs precursor ions, was applied to allow the isomers distinction[2-3].

Results:
Using the reported conditions, a co-elution of the studied isomers was observed. Analysis of isobaric molecules, and especially isomers, by tandem mass spectrometry is often complicated by the similarity between their fragmentation patterns and it is common that the same MS/MS product ions are present in the spectra of all the isomers. Hence, to find some differences among cis/trans isomers, a series of experiments based on energy resolved MS/MS (ERMS) was carried out. Knowing the characteristic ratios of pure isomer, LEDA algorithm elaborates each MS/MS acquired spectra, assigning the correct abundance to the identified isomer. Therefore, in case of an isomers’ mixture, LEDA is able to resolve the co-eluting peak determining the relative concentration of each isomer eventually present. The LEDA reliability was checked during stability studies. The results proved the effectiveness of correct assignment of the isomer present in the sample. This ability was essential to verify the behavior of the studied isomers during stability tests.

Conclusions:
The proposed approach, combining a LC-MS/MS method with a post-processing mathematical algorithm (LEDA), was able to deconvolute a mixture of isomers from chromatographically unresolved peaks. The results demonstrated that this combination was suitable for the evaluation of isomers’ degradation profiles in human plasma samples. Indeed, the stability experiments have highlighted a different behavior between cis/trans isomers.

Novel Aspect:
The LEDA approach has the advantage that isomers can be quantified without the need of LC separation or additional specialized ion mobility instrumentation.

References:


Introduction:
We reported a series of amide-based hybrid compounds incorporating 6- and 7-substituted coumarins and some NSAIDs as agents for the management of RA.[1] These derivatives were proved to exert remarkable anti-inflammatory action in spite of non-significant amide-bond cleavage in vivo. [1-3]
As a rational development, we report herein all such derivatives ester bioisosters to investigate the contribution of a more cleavable linker in terms of plasma stability.

Methods:
The kinetic studies were carried out through a stopped-flow assay. [4] The LC-MS/MS analysis were carried out using a triple quadrupole system equiped with an electrospray source (ESI), operating in positive-ion mode. The employed chromatographic parameters were finely tuned to minimize the run time. Furthermore, a matrix of linear equations (LEDA), based on abundance ratios of product vs precursor ions, was applied to allow the isomers distinction [3].

Results:
The ester hybrids were shown to inhibit the target human CA isoforms more efficiently than the corresponding amides. Using the LC reported conditions, a co-elution of the studied isomers were observed. Knowing the characteristic ratios of pure isomer, LEDA algorithm elaborates each MS/MS spectra acquired, assigning the correct abundance to the identified isomer. Therefore, in case of a isomers mixture, LEDA is able to resolve the co-eluting peak determining the relative concentration of each isomer eventually present. The LEDA reliability was checked during stability studies. The results proved the effectiveness of correct assignment of the isomer present in the sample. The drug stability studies demonstrate that the amides derivatives resulted to be stable in both human and rat plasma. Likewise, most esters derivatives exhibited human plasma stability, with the exception of Ketoprofen and Diclofenac derivatives, showing that the ester bond can undergo hydrolytic cleavage in human plasma depending on the NSAID fragment.

Conclusions:
Docking studies displayed the capability of the overall hybrid to bind to COX-1 and COX-2. Unlike amides, the ester bond can undergo hydrolytic cleavage in human plasma depending on the NSAID fragment. In vitro functional investigations and in vivo studies are currently ongoing to finally determine the mechanism of action of such derivatives.
The proposed LC-MS/MS approach with LEDA tool, was able to separate the isomers eventually present from LC unresolved peaks.

Novel Aspect:
Introduction of ester/amide-linked NSAIDs-coumarins hybrids for the management of RA. The use of LEDA tool to isomers analysis without LC or ion mobility separation.

References
Introduction
Despite regulatory efforts; use of “Bonzai” or “Spice” has increased in Turkey due to psychiatric, medical and social features. JWH-015, a narcotic cannabinoid, is one of the active components of Bonzai. Intensive literature survey indicates that studies on the metabolites of JWH-015 are limited to in vitro experiments only [1, 2]. Thus, screening of probable in vivo metabolites of JWH-015 via a non-targeted analysis were aimed in the present study.

Methods
Female Wistar rats of the same age (n=6, ~200-250 g) were used in the experiments. JWH-015 was administered via i.p. route at the dose of 5 mg/kg [2]. Urine samples were collected at varying time points within a 24-hour period and subjected to analysis by using a liquid chromatography mass spectrometry-ion trap-time of flight (LCMS-IT-TOF) instrument (Shimadzu, Japan). Molecular weights of the probable metabolites were calculated and their chemical structures were identified.

Results
In order to determine the pharmacokinetic properties of JWH-015, the analysis of the biotransformation products by LCMS-IT-TOF system were performed. As a result of analyses, a novel in vivo metabolite of JWH-015 was identified for the first time. The novel compound was a glucuronide metabolite of JWH-015, with a chemical name of hydroxy-JWH-015-O-glucuronide. Seven other metabolites with the previously reported chemical structures [1, 2] were also determined in the LCMS-IT-TOF analyses.

Conclusions
Although in vitro metabolism studies give highly accurate results for prediction of possible metabolism pathways and metabolites, in vivo verification of the results is crucial to reveal the real-time reflection of non-living based experiments. Therefore, time dependent determination of novel metabolite, hydroxy-JWH-015-O-glucuronide is very important in terms of pharmacokinetic profile of Bonzai.

Novel Aspect
Discovery of a novel metabolite of JWH-015 gives valuable information about biotransformation pathway of cannabinoid type of compounds.

References
Introduction
Ergosterol is the major fungal membrane sterol that regulates membrane fluidity, plasma membrane biogenesis and functions [1]. The inhibition of ergosterol biosynthesis causes a damage in cell membrane of fungi. Hence, quantification of ergosterol level in fungi is essential for development of novel inhibitor candidates [2]. Thus, in the present work, we synthesized new benzimidazole-derivative compounds and developed an LC-MS/MS method for assessment of their effect on ergosterol content of C. albicans.

Methods
Sterol extract was prepared from C. albicans, incubated with/without inhibitor for 24h [3], and injected (1 µL) to LC-MS/MS system. The mass spectrometric analyses were achieved by employing Nexera XR UFLC system coupled to an LCMS-8040 triple quadrupole mass spectrometer (All from Shimadzu, Japan). MRM was optimized using ergosterol standard, and Labsolutions LC-MS software (Version 5.86, Shimadzu) was used to process the data.

Results
Ergosterol standard was used for quantification of ergosterol in both inhibitor-free (negative control) and inhibitor containing samples. Benzimidazole compounds and reference drugs (ketoconazole and fluconazole) were used at 0.78 µg/mL, 1.56 µg/mL, and, 3.12 µg/mL concentrations. Ergosterol quantity in negative control samples was regarded as 100%. All concentrations were analyzed in quadruplicate, and the results were expressed as mean ± standard deviation. Ergosterol quantification studies indicated that compounds 5i, 5s and reference drugs significantly reduced ergosterol levels at all tested concentrations. Compound 5i displayed 61.74%, 85.41%, and 93.71% inhibitions at 0.78 µg/mL, 1.56 µg/mL, and, 3.12 µg/mL concentrations, respectively. An inhibitions ratio of 62.05%, 84.48%, and 93.12% were observed for compound 5s at the same concentrations. Reference drugs indicated similar inhibition potencies to those of compounds 5i and 5s in the range of 63.71% to 94.46%.

Conclusions
We achieved highly acceptable results via developed LC-MS/MS method. Ergosterol quantification studies using C. albicans indicated that compounds 5i and 5s and reference agents cause a concentration dependent decrease in the level of ergosterol. Hence, it can be obviously suggested that compounds 5i and 5s have a role in the ergosterol biosynthesis pathway.

Novel Aspect
The developed LC-MS/MS method may hopefully be beneficial for researchers who study on novel antifungal agents as ergosterol biosynthesis inhibitors.

References
Optimized MSn workflow for improved structure elucidation of pharmaceutically relevant extractables and leachables

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1Thermo Scientific, San Jose, CA; 2Eli Lilly and Company, Indianapolis

Introduction

The identification of unknown small molecules, such as impurities, metabolites, degradants, extractables and leachables remains one of the most challenging workflows. Mass spectrometry-based chemical and structural characterization of small molecules greatly benefits from multi stage fragmentation (MSn) coupled with high resolution and high mass accuracy analysis. However, the use of these tools during the untargeted analysis of complex samples is often challenging, owing to the slow precursor interrogation rate and the large number of potential precursors. Here, we describe new data acquisition approaches for the characterization of small molecule extractable and leachable compounds. Building upon sophisticated methods that employ multistage Orbitrap analysis (FTMSn), we dynamically update inclusion and exclusion lists between LC analyses to enable efficient and deep interrogation.

Methods

All experiments were run on a modified Thermo Scientific™ Orbitrap tribrid mass spectrometer. Liquid chromatography separations were carried out on a Thermo Scientific™ Vanquish™ LC system using mobile phases composed of: A: H2O/0.1% formic acid, and B: ACN/0.1% formic acid with gradient on a Thermo Scientific™ Accucore™ C18 LC column (2.1X100 mm particle size: 2.6µm). A mixture of common plastic and elastomer additives was spiked into matrix and analyzed using LCMS to demonstrate the methodology. This was followed by IPA/water extracts of representative elastomeric components used in biopharmaceutical process. The data was processed using Thermo Scientific™ Mass Frontier™ 8.0 and Thermo Scientific™ Compound Discoverer™ 3.0 software.

Preliminary Data

In this study, a mixture of known additive standards was used to verify the data acquisition features. The IPA/water extract of representative elastomeric components used in pharmaceutical process demonstrates that using the new features significantly enhances unknown extractable chemical entity identification in a representative solvent extract of an elastomeric component used in pharmaceutical process. The instrument control and data acquisition software for the mass spectrometer was modified to enable automatic generation of inter-run inclusion and exclusion lists based upon the real time component detection in the LC-MS data. Using these inter-run inclusion and exclusion lists, the instrument can selectively trigger MSn scans on features detected in the sample of interest while excluding any distracting background ions. Samples are automatically re-injected to allow for exhaustive MSn data generation for the detected features of interest.
Within each data-dependent LC-MS analysis, we employed sophisticated mass spectrometer methods that are comprised of multiple levels of MSn fragmentation coupled with both high-resolution Orbitrap and low-resolution ion trap analyses. The initial workflow was tested on commercially available standards comprising of plastic and elastomer additives such as antioxidants, slip agents, plasticizers, UV protectants and processing aids. Structure elucidation was conducted based on a newly implemented substructure similarity searching algorithm in Compound Discoverer. Additionally, the MSn data generated for the small molecule compounds of interest was used for generation of a custom spectral library using Mass Frontier 8.0. In the next steps, we will apply this workflow for data analysis of complex unknown small molecule samples.

Novel Aspect
Intelligent data acquisition, processing and interpretation strategy to enable rapid MSn characterization of small molecule unknowns
Options:
A graduate student is presenting author on this abstract? No
A post-doc is presenting author on this abstract? No
An undergraduate student is presenting author on this abstract? No

Oral Choice:
MS in Extractable and Leachable Analysis
Second Oral Choice:
Synthetic Polymers
Poster:
Small Molecules: Qualitative Analysis

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1258 - IDENTIFICATION OF DIPROPYLAMINOPRETADALAFIL USING HIGH RESOLUTION MASS SPECTROMETRY UNDER MULTI-STAGE FRAGMENTATION PATHWAYS

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Keywords: Dipropylaminopretadalafil, Diethylaminopretadalafil, HRMS, multistage fragmentation

Introduction:

Phosphodiesterase type 5 (PDE-5) inhibitors and their analogues are commonly detected in illegal health products used to treat erectile dysfunction in men. The emerging health risks have become prominent with the first fatal case associated with intoxication by desmethylcarbodenafil reported in 2017 [1]. Therefore, it is crucial that the laboratory is equipped with the necessary equipment and knowledge to structural elucidate the structure of new analogues. In this study, a new tadalaraf analogue was detected in a routine analysis in the laboratory. The structure of this unknown was compared to that of a known tadalaraf analogue, diethylaminopretadalafel, using liquid chromatography mass spectroscopy (LC-MS) and high-resolution mass spectroscopy (HRMS).

Methods:

The unknown PDE-5 inhibitor analogue was isolated by liquid-liquid extraction and purified by recrystallization process. The isolated compound was then dissolved in acetonitrile for Liquid Chromatography-Mass spectroscopy (LC-MS). The sample solution was further diluted with 0.1% formic acid in water: 0.1% formic acid in acetonitrile = 4:1 for direct infusion using a Thermo Fischer Scientific LTQ Orbitrap XL FTMS. The infusion flow rate was 10 µL per minute. The MS2 fragmentation pattern under High energy Collision Dissolution (HCD) was acquired using the optimized normalized collision energy (NCE). The multistage fragmentation pathways MSn (n= 2, 3) were acquired by selecting ions of interest under Collision Induced Dissolution (CID). The fragmentation patterns obtained from both modes were compared to a known standard, diethylaminopretadalafel. The accurate mass measurement of each product ion was determined with the aid of Mass Frontier 7.0 software. Mass error of all product ion has been limited to within ± 5.0 ppm tolerance.

Results:

The fragmentation patterns for both unknown compound and diethylaminopretadalafelwere compared. Under MS2 experiment in HCD mode, the unknown compound showed an intense signal at m/z 492, which is more than diethylaminopretadalafel at m/z 464 by 28 Dalton, indicating the presence of -C2H4- to the known standard structure. Similarly, the difference has been observed for the fragment ion at m/z 393 of the unknown compound and the fragment ion at m/z 379 of diethylaminopretadalafel. The common ion at m/z 334 is a result of the cleavage of C-1, C-3 and nitrogen [2]. Under CID mode, the MS2 spectrum of the unknown compounds showed fragmentions at m/z 393 and 292, both were 28 Dalton higher than the fragment ions at m/z 379 and 278 derived from diethylaminopretadalafel. The most intense ions at m/z 393 and 379 for both compounds can be formed from the cleavage of amido bridge via N-C bond and followed by the new N-C bond formation with a propyl and ethyl respectively. This is a good indication to rule out the possibility of having asymmetrical dialkyl groups linked to terminal amine. Two common ions have been observed at m/z 336 and 264. The first one can due to the loss of the labile N-propyl and N-ethyl for the unknown compound and diethylaminopretadalafel respectively. Meanwhile, the
smaller ion at m/z 264, consisting of indole- benzodioxole substructure can be formed from the loss of the ethylmethyl ester-N-alkyl linked moiety.

Under MS3 experiment, the precursor ion at m/z 393 produced three major ions at m/z 336, 292 and 264. Similar fragmentation pattern had also been observed for ion at m/z 279 derived from diethylaminopretadalafil to yield fragments ions at m/z 336, 278 and 264. The difference of 12 Dalton between ions at m/z 292 and 278 supports the formation of N-C(alkyl) bond as explained above. The common fragmentation pathways for ions at m/z 336 and 264 had been observed in both MS3 experiments. The former produced an intense signal at m/z 130 due to cleavage of the ethyl methyl ester and benzodioxole from the precursor, forming the stable methylated indole. Lastly, the precursor ion at m/z 264 gave rise to ion at m/z 234 as a result of the removal of HCHO from the benzodioxole group. Subsequent neutral loss of one CO resulted in the ion at m/z 206.

Conclusions
Dipropylaminopretadalafil has been characterized by a series of comparison studies with the known diethylaminopretadalafil under High energy Collision Dissolution (HCD) and Collision Induced Dissolution (CID). The similarities and differences observed by these two compounds have been explained through their fragmentation pathways with the aids of reference ions.

Novel Aspect:
In the absence of NMR facility and limited sample amount, the application of HRMS with MSn capability is able to provide important information on the fragmentation pathways through comparison with a suitable known reference compound.

References


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Keywords: maytansinoid, anti-tumor, metabolites, microsomes, tandem MS.

Title:
Comprehensive metabolite identification of DM4, a maytansinoid anti-tumor agent, in S100 fractions of Human Liver microsomes, by UPLC-QTof Mass Spectrometry

Authors:
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Introduction:
Maytansinoids are highly cytotoxic molecules; several of them are investigated in cancer therapy, as conjugates with antibodies (ADCs) targeting specific cells. This study describes the identification of the metabolites of the maytansinoid DM4, after incubation with S100 fractions of human liver microsomes (HLM) [1]. Particularly, we wanted to gain better understanding of truly metabolic processes from chemical degradation of the molecule.

Methods:
After 1-h incubation, the samples were injected onto a RP C18 UPLC column, and the mass spectrometer was operated in positive ESI alternating low- and high-energy full-scans. Molecular masses of all the DM4-related molecules were obtained by extraction of the masses of typical fragment ions from the high-energy scans (Product Ion Filtration, PIF), and mass defect filtering (MDF) from the low-energy scans. Fragment masses were used for structural elucidation.

Results:
Given the reactivity of DM4, the drug-related molecules in HLM incubates were matched with those occurring in control experiments, i.e. HLM incubates without the NADPH activator and DM4 incubates in incubation buffer only. The results are as follows.
Incubation in buffer only: DM4 underwent side-chain hydrolysis (N-methylglycyl maytansinol), degradation of 1,3-oxazinan-2-one ring of the core structure, SH oxidation to sulphinic and sulphonic acid, hydroxylation of the core ring.
S100 incubation without NADPH: besides the above molecules, the DM4 conjugates with cysteine and, at trace level, with glutathione, were observed.
S100 incubation with NADPH: Sulphinic and sulphonic acid were more abundant. In addition to the above, a metabolite with the side chain SH group replaced by an OH was observed, and a pair of products from Michael reaction of DM4 with 1,4-dihydropyridine-3-carboxamide, resulting from interaction with NADPH or one of its impurities. Investigations are ongoing to ascertain the nature of the Michael adducts.
Conclusions:
The experiments performed on DM4 HLM incubates allowed to distinguish true DM4 metabolites from products arising from merely degradative processes. To the first category belong a sulphinic and sulphonic acid, also partly generated by spontaneous oxidation, cysteine and glutathione conjugation, and side chain SH to OH substitution.

Novel Aspect:
Observation of novel DM4 metabolites, first discrimination of P450-dependent processes from other biotransformations and chemical degradation.

References:
63 - IMPACT OF CENTROIDING AND DIFFERENCES IN DATA PROCESSING ON QUANTIFICATION USING HIGH-RESOLUTION MASS SPECTROMETRY

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Janssen R&D, Discovery Sciences, Beerse (1)

Keywords: High-Resolution MS, quantification, data processing, mass extraction window

Introduction:
The improved performance of High-Resolution MS (HRMS) systems triggers more (bio)analysts to switch from triple quad MS to HRMS for quantitative analysis. The impact of differences in data handling by different software packages, the impact of centroid or continuum data as well as the use of theoretical or measured accurate mass on the selection of the optimal narrow mass extraction window was studied.

Methods:
Calibration curves of 8 pharmaceutical drugs in plasma were analyzed in 5-fold on a Synapt G2-S QToF system. Data were acquired in continuum mode and processed in four different ways: as centroid data, as individual profile data points and as full profile data using 2D and 3D processing. One compound was analyzed on 2 QTofs and one Orbitrap system and processed in six different software packages.

Results:
Centroid data provided advanced selectivity resulting in cleaner XICs for qualitative analyses but with a limited to no effect on quantitative performance (LOQ, precision, linearity). The optimal mass extraction window (MEW) selection showed to be solely defined by the mass accuracy of the system used. For profile data, the improved selectivity provided by narrower MEWs is counterbalanced by a loss in MS peak area affecting signal-to-noise. Therefore, the full width half maximum (FWHM) of the peak showed to be the ideal starting point for MEW selection in addition to mass accuracy that becomes gradually more important with increasing MS resolving power. 3D peak processing can overcome the challenges of MEW selection but comes with different challenges such as the difficulty in reviewing and adjusting bad peak integrations.

Conclusions:
The impact of centroid or profile data and different software approaches on the extraction of MS data to generate XICs were investigated. While there is an effect on the optimal MEW selection for which guidelines are given, the effect on quantitative performance was minimal.

Novel Aspect:
The impact of differences in data treatment by software packages
Optimization of processing parameters for quantitative HRMS performance

1221 - INVESTIGATION OF MONOAMINE OXIDASE INHIBITORY ACTIVITIES OF NEW CHALCONE DERIVATIVES: COMPARISON OF LC-MS/MS AND FLUOROMETRIC METHODS

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Keywords: Monoamine Oxidase, Inhibition, Chalcone, LC-MS/MS

Introduction
Monoamine oxidase (MAO), is a key enzyme that metabolizes monoamine neurotransmitters in peripheral tissues and in the central nervous system [1]. MAO involves two isoforms, i.e. MAO-A and MAO-B. MAO-A inhibitors constitute a well-known class of antidepressant drugs, whereas MAO-B inhibitors have importance towards age-related disorders such as Alzheimer’s and Parkinson’s diseases [2]. In the present work, we applied both fluorometric and LC-MS/MS assays to observe MAO inhibitory potency of new chalcone derivatives.

Methods
MAO enzyme inhibitory effects of newly synthesized chalcone derivatives (2a-2i) [3] were tested in the concentration range of 10⁻³ to 10⁻⁹ M via a fluorometric method using MAO-A/B Inhibitor Screening Kit (BioVision, CA, USA) according to the manufacturer’s guidelines [4]; in addition, a new LC-MS/MS assay method was also developed based on the measurement of tyramine and both methods were compared statistically with each other.

Results
In the fluorometric microplate method, a non-significant inhibition was determined against hMAO-A. In contrast, all compounds indicated higher inhibition profile towards hMAO-B isoform. Compounds 2c, 2e and 2i displayed good inhibition against hMAO-B with lower IC₅₀ values than 1 μM. In order to support the results of enzyme inhibition assay, quantification of substrate (tyramine) level was performed using LC-MS/MS via MRM. Effects of the compounds 2c, 2e and 2i on tyramine level were in agreement with those of compounds observed in fluorometric assay. It was observed that lower inhibitor concentration causes a decrease in the substrate level.

Conclusions
Inhibitors usually react with the enzyme and change it chemically. Hence, in the presence of an inhibitor, the enzyme-substrate complex does not form routinely. Thus, the quantification of tyramine level was performed by an LC-MS/MS method. It was found out that tyramine level increases in the higher concentrations of compounds 2c, 2i and 2f. Consequently, the results of fluorometric kit assay was supported/verified by a more sensitive LC-MS/MS method.

Novel Aspect
Newly reported LC-MS/MS method is very helpful to quantify substrate level in MAO-substrate reaction and to compare results with those of known fluorometric assay.

References
Fluorometric MAO-A (Catalog No: K796-100) and MAO-B (Catalog No: K796-100) Inhibitor Screening Kit, Data sheet BioVision, USA.
LC-ICP-MS AS AN ALTERNATIVE TO RADIOACTIVITY IN PHARMACOLOGY

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Keywords: ICP-MS; Peptide; Quantification; Pharmacology; Labeling

Introduction
Measuring the affinity of a ligand for its target is an issue of central importance in drug development. At present, radiolabeling is the reference method in pharmacology, allowing a highly sensitive detection of the molecule of interest but with many drawbacks (user safety, environmental issue). In this context, we developed an alternative methodology using mass spectrometry due to its intrinsic capabilities in terms of specificity and sensitivity of analysis.

Method
The cornerstone of this analytical methodology relies on the production of very abundant signals related exclusively to the peptide ligand of interest to be quantified by a generic mass spectrometry method, applicable to all receptor/ligand systems, while demonstrating performance equal to radioactivity. Among all techniques investigated [1-3], LC-ICP-MS associated with peptide selenium labeling provided extreme efficacy and robustness of quantification.

Results
This strategy was applied to quantify peptide ligands at very low concentrations from cell cultures in pharmacological experiments (saturation and competitive binding assays). Noteworthy, we developed a straightforward protocol that can be performed with a standard macrobore LC-ICP-MS system without any sample treatment or special instrumental set-up, while allowing efficient suppression of all mass interferences to reach the targeted sensitivity. Significantly, a quantification limit of 50 ng Se L-1 (50 ppt/63 fmol of injected Se) was achieved, the samples issued from the pharmacological assays being directly introduced into the LC-ICP-MS system. The proof of concept was illustrated on the vasopressin/AVP pharmacological model. The methodology was then successfully applied to a second ligand/receptor system (CCK-4/CCK) proving the generic aspect of the protocol. A very good correlation of all experimental data for the determination of Ki, Kd and IC50 by both ICP-MS and radioactivity demonstrated the reliability of the strategy.

Conclusions
We aimed to obtain a generic mass spectrometry method, applicable to all receptor/ligand systems, while demonstrating performance equal to radioactivity in order to convince pharmacologists to modify their practices by the eradication of the use of radioactive substances. The gathered results with two different ligand/receptor systems indicate clearly that the LC-ICP-MS-based developed method represents a viable alternative to radiolabeling.

Novel Aspect
LC-ICP-MS equaled radioactivity for peptide quantification from cell cultures which should convince pharmacologists to modify their practices.

References
Keywords: candesartan, micronization, HRMS, long term stability, degradation products

Introduction:
Water solubility is a rate-limiting factor for drug bioavailability. Micronization is the reference technique to increase solubility [1]. In spite of being an old technology, there is little published data about the degradation of pharmaceuticals during this process. Here, ESI-HRMS was used to identify degradation products (DP) of candesartan cilexetil (CC) micronized by milling and stocked for a long time period.

Methods:
CC samples was milled for 0.25, 2, 48 or 170 hours. Micronized CC was analyzed 24 months after milling using a ThermoQ-Exactivemassspectrometer at a resolution of 70,000 (@m/z 400). The formation of the DP was evaluated by plotting the ratio of the absolute intensity of the (DP) to that of the CC (IDP/ICC) versus time.

Results:
Different times of milling were applied in order to better correlates the micronization process and the formation of the DP of CC. The HRMS analysis of samples 24 months after the micronization process revealed the presence of O-Desethyl Candesartan Cilexetil (Impurity B) and 2-Ethyl-Candesartan Cilexetil (Impurity F). These impurities were identified both in positive (as Na adducts) and negative ion modes. It was verified two different profiles along the long term degradation study: for Impurity B, the ratio IDP/ICC increased seven folds after 48 hours of micronization and remained stable until the end of the experiment. Impurity F, however, showed a continuous growing of the IDP/ICC ratio revealing a close relation between the time of milling during the micronization process and its formation during storage.

Conclusions:
Our data indicates a strong correlation between the time of milling and the formation of DP. The kinetic energy transferred to the particles during the process, produces the DP in different extents according to their stability and kinetics of formation. For the first time, the profile of formation of these CCimpurities, especially impurity F, were described after a long term storage study.

Novel Aspect:
Original HRMS information about the long term stability of candesartan cilexetil after micronization by milling.

References:
Introduction:
Degradation products in pharmaceuticals may cause harmful side effects but the new substances generated may have different therapeutic activities. Ceftazidime is a β-lactam antibiotic, which polymerizes during normal storage conditions [1]. Ceftazidime polymer showed a high inhibitory effect on HIV-1 reverse transcriptase [2,3]. In this study mass spectrometry is applied with the objective of revealing the chemical structure of a cefazidime polymer.

Methods:
Ceftazidime (5 g) was heated to 60°C for 210 h in a covered container [2]. A methanolic solution of this polymerized material (1 µg/mL) was analyzed by direct infusion in a QExactive™ Hybrid Quadrupole-Orbitrap coupled to an HESI II source. MS and MS/MS spectra were acquired at a resolution of 70,000 (@ m/z 400) in the positive ionization mode. MALDI analysis was performed in an Autoflex III Smartbeam MALDI-TOF/TOF instrument using 2,5-DHB as matrix doped with NaCl.

Results:
The HRMS showed a distribution of chain length typical of a polymeric material, with ions distributed along 300 - 1700 Da mass range. The calculated average molecular weight from the HRMS result was 911.7500 Da. The ions were equally spaced at 74.018 Da units apart. For each oligomeric unit, three different isotopic patterns with mass differences of 5 and 14 Da were observed. MALDI spectra presented the ion clusters between 600 and 1300 Da split by a difference of 23 Da suggesting the presence of sodium cationized oligomers.

Conclusions:
For the first time, a mass spectrometry based study of ceftazidime polymer has been published. Our mass spectra data, together with other techniques, allows the complete characterization of this interesting degradation product.

Novel Aspect:
First mass spectrometry study of a ceftazidime polymeric degradation product.

References:
Introduction:
Volumetric absorptive microsampling (VAMS) is a novel sampling technique that allows the collection of an accurate volume of blood by dipping an microsampler tip. The purpose of this study is to validated the concentration of metformin using LC-MS/MS in VAMS. We demonstrate full validation to ensure that this method is accurate, precise, and stable in metformin.

Methods:
A high-performance liquid chromatography with mass spectrometry (LC-MS/MS) was developed and validated for the accurate determination of metformin in human using VAMS. The method was validated for accuracy, precision, sensitivity, recovery, matrix effect, specificity, linearity and stability according to the KFDA guideline.

Results:
Validation the selectivity of the optimized method was assessed to determine any interfering peak around the retention times of metformin. The calibration curve was calculated by using the linear regression equation of $y=ax+b$, which is the weight ratio of metformin to the peak area of the internal reference material. Calculations of deviations between the measured and theoretical values for the six standard concentrations used in the calibration using the back-calculation method showed that at least 85% of the standard concentrations were within the standard (within 20% of the lowest limit of quantitation, Concentration was within 15%). For standard concentrations that did not meet the acceptance criteria, they were excluded from the calibration curve. The coefficient of determination ($r$) was higher than metformin 0.9990.

Conclusions:
The LC-MS/MS method was used to establish the analytical method for analyzing the concentration of metformin in human blood, and the validity of the established method was verified. In the concentration range of 2 - 100 ng/mL, it showed good linearity and no interference from human blood. The fully validated LC-MS/MS method showed applied to clinical trials such as bioavailability, bioequivalence and pharmacokinetic evaluation of metformin.

Novel Aspect:
This is the first paper analyzed metformin by LC-MS/MS using VAMS, a novel technology of microsampling.

References
DETERMINATION OF AN ISONICOTINOHYDRAZIDE DERIVATIVE, A NOVEL POSITIVE INOTROPIC COMPOUND, IN MOUSE PLASMA BY LC-MS/MS AND ITS APPLICATION TO A PHARMACOKINETICS STUDY

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Keywords: isonicotinohydrazide derivative, LC-MS/MS, pharmacokinetics, mice

Introduction:
Heart failure (HF) is a growing public health problem with high mortality and morbidity [1-2]. The development of HF drugs is a pressing problem [3-5]. In previous works, we presented a new positive inotropic compound, an isonicotinohydrazide derivative (AF-HF001), which can directly effect on the myofibril and has a significant effect in the treatment of HF [6-8]. In this study, we employed LC-MS/MS for the qualitative and quantitative analysis of AF-HF001.

Methods:
Samples were prepared by one step precipitation with ethyl acetate and stored in acetonitrile. Chromatographic analysis was carried out on a Hypersil Gold C18 column with a gradient mobile phase consisting of acetonitrile and 0.1% aqueous formic acid. The analyte was detected by selective reaction monitoring (SRM) mode with target quantitative ion pair of m/z 292.1 → 148.2, using praziquantel as the internal standard (IS) m/z 313.1 → 203.2.

Results:
AF-HF001 is a novel positive inotropic compound, which has been reported very recently. The analysis of AF-HF001 in biological matrices is never published. We developed and validated this LC-MS/MS method for the quantitative analysis of AF-HF001 in ICR mice plasma. The one step extraction of ethyl acetate accomplished both drug extraction and protein precipitation, and provided better recovery, stability and simplicity. Then the chromatographic condition with both the composition of mobile phase and the column was also ameliorated to get good sensitivity and selectivity of drug in plasma. With the chosen ion pairs of drug and internal standard, we achieve the quantitative analysis with better precision and sensitivity of AF-HF001 in biological sample. Good linearity (r=0.995) was observed over a wide concentration range. The validation of method shows that this bioanalytical method we have developed is sufficient for the analysis of AF-HF001 in mice plasma and will be very essential for the further PK-PD studies of the drug.

Conclusions
A specific and sensitive LC-MS/MS method was developed for the determination of AF-HF001 in mice plasma for the first time. After a full method validation, it was successfully applied in the preliminary pharmacokinetic studies of AF-HF001 in mice after a single i.p. dose of 45mg/kg. The analyte was proved as a competitive candidate for the treatment of acute heart failure. Further pre-clinical and clinical pharmacological studies of AF-HF001 are also needed.

Novel Aspect:
A full validated LC-MS/MS method of AF-HF001 is described for the first time. It was successfully applied to a very first pharmacokinetics study of AF-HF001 in mice plasma.

References

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Introduction:
Ketamine is a well-known agent used in anesthesiology since 1960s. The analgesic properties of ketamine were discovered in 1990s. Surprisingly, quite recently this agent also was shown to display unique antidepressive properties. Researchers from Celon Pharma S.A. have conducted comparative analyses of antidepressive effects exerted by R- and S-enantiomers of ketamine and its metabolites in dry powder inhalation as a delivery method.

Methods:
Samples (rat plasma and brain) were purified by protein precipitation and centrifugation. Analytes separation was achieved on a chiral Lux® 3 µm Amylose-2 column with a mobile phase consisting of 10mM ammonium acetate, acetonitrile and 2-propanol. The flow rate was set at 0.5 mL/min. Mass spectrometric detection was performed with use of Agilent 6460 mass spectrometer in positive ionization mode. Ketamine-d4 was used as an internal standard (IS).

Results:
The aim of this study was to develop and validate LC-MS/MS assays for stereoselective quantification of R,S-ketamine and its metabolites, R,S-norketamine, (2S,6S)-hydroxynorketamine, (2R,6R)-hydroxynorketamine and R,S-dehydronorketamine in rat plasma and brain samples enabling the characterization of the ketamine pharmacokinetics after administration of a low intravenous (i.v.) or intratracheal (i.t.) dose. Due to very low volume (50 µL) of samples collected during preclinical studies only protein precipitation could be used as a sample preparation. As a result lower limit of quantitation (LLOQ) for all of compounds was determined to be 25 ng/mL. Analytes were separated in one 45 minutes run. Quantitation was performed using at least two MRM transitions per analyte and IS. The quadratic calibration curves for all analytes were obtained over the concentration range 25–5000 ng/mL. The intra- and inter-day precisions were <7.9% and the accuracy (relative error) was within ±5.3%.

Conclusions:
The study describes the development and validation of an easy, selective and reliable LC–MS/MS method for simultaneous determination of ketamineenantiomers and its metabolites in low-volume rat plasma and brain samples. The importance of the method is demonstrated by its ability to compare the ketamine metabolites concentrations in these matrices to healthy rats.

Novel Aspect:
LC–MS/MS method can differentiate enantiomers of ketamine and its three major metabolites in 45 min run using low volume complex matrix sample.
Keywords: Pharmaceuticals, Biologicals, Microsampling, HRMS, LC-MS

Introduction: (Limit of 400 characters)
The pharmaceutical industry is undergoing many changes. One challenge is to follow the 3R’s by reducing the number of laboratory animals needed in the new drug discovery process. One solution is to take smaller blood samples (microsampling) so fewer laboratory animals are needed [1]. Another challenge is the shift toward biologicals. Biologicals are more difficult to assay than small molecules.

Methods: (Limit of 400 characters)
For blood microsampling, either capillary microsampling (CMS) tubes or a volumetric absorptive microsampling (VAMS) device was used. For mass spectrometry imaging (MSI), various high resolution mass spectrometer (HRMS) systems are discussed. For biological assays, various MS systems are described including triple quadrupole systems and HRMS systems.

Results: (Limit 900 characters)
In one study, we used the 8-µL CMS blood sampling method [2]. In another study, we demonstrated the utility of both blood CMS and VAMS for getting samples from rat PK studies [3]. MSI has now become an important tool for understanding the distribution of a drug as well as its metabolites in laboratory animals. MSI can also be utilized for identifying disease and toxicity biomarkers as part of new drug discovery. Various strategies have been developed to measure therapeutic biological compounds, including monoclonal antibodies, in serum from mouse or monkey PK studies. One strategy is the “surrogate peptide approach”, where the large biological compound is cut into smaller pieces (typically by using trypsin digestion). Then one or more of the peptides is selected to serve as the analyte that is quantified (typically by LC-MS/MS) in order to then calculate the concentration of the large biological molecule. Another approach is to use LC-HRMS to measure the intact biological compound directly.

Conclusions (Limit of 400 characters)
Overall, these studies provide additional data that blood CMS is a valuable approach for serial blood sampling from discovery rat PK studies. Mass spectrometry continues to be a key system that is used throughout all stages of the pharmaceutical industry research paradigm, from early drug discovery to clinical studies. As MS systems continue to improve, then new applications become possible.

Novel Aspect: (Limit of 150 characters)
The talk will describe how MS has been utilized to provide solutions for the pharmaceutical industry during new drug discovery and development.

References
Luo Y., Korfmacher W., Ho S. et al., Bioanalysis, 7(18), 2345-59 (2015).
1256 - EVALUATION OF SMALL MOLECULE DRUGS RETENTION IN FLUORINATED SILICA NANOPARTICLE STABILIZED PICKERING EMULSION USING ESI-MINE(TM) PLATFORM

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Keywords: ESI-Mine™, Pickering emulsion, Picodroplet, Microfluidics

Introduction:
Microfluidic Water-in-Oil (W/O) picodroplet technology has been emerging as a useful tool for single cell and biological macromolecule characterizations. However, poor retention in polymeric surfactant stabilized picodroplets has limited its applications in small molecules related analyses.

Methods:
ESI-Mine™ is a novel, high-throughput, mass spectrometry (MS) based platform developed for analyzing the contents of microfluidic picodroplets. Fluorinated silica nanoparticle (f-SiNP) stabilized W/O Pickering emulsion is reported capable of retaining a hydrophobic fluorescent dye (resorufin) in picodroplets. Here, we profiled the retention potency of non-fluorescent small molecules with a logP range from -2 to 4 in Pickering picodroplets using ESI-Mine™.

Results:
Pickering emulsion picodroplets (600-700 pl) were generated on a flow focusing 60X60 Pico-Gen™ biochip. Af-SiNP (50 nm diameter) suspension in Novec™ 7500 (5%, w/w), prepared following Stöber's [1] and Pan's approaches [2], was used as the continuous phase, and an aqueous solution with/without 10 uM selected small molecule as the breaking phase. ‘Occupied’ picodroplets contain the selected small molecule, but ‘Empty’ do not. Picodroplet mixture of ‘Occupied’ and ‘Empty’ were reinjected onto a MS emitter microfluidic biochip interfacing with a Perkin Elmer Axion 2 mass spectrometer, i.e. ESI-Mine™. Total ion current (TIC) signal of the picodroplets was recorded as a series of peaks. Extracted ion current (EIC) signal only shows ‘Occupied’ picodroplet peaks if the selected small molecule is retained inside ‘Occupied’ picodroplets, but in both ‘Occupied’ and ‘Empty’ if the selected small molecule cannot be retained. Eight small molecules of a logP range from -2 to 4 were selected, and analyzed on ESI-Mine™ platform.

Conclusions
We employed and demonstrated ESI-Mine™ platform as a useful tool to evaluate the retention potency of non-fluorescent small molecules inside f-SiNP stabilized Pickering emulsion picodroplets. Improved retention potency of small molecules inside f-SiNP stabilized Pickering emulsion picodroplets was observed compared to conventional polymeric surfactant stabilized picodroplets.

Novel Aspect:
Novel aspects include MS analysis of Pickering emulsion picodroplets, and evaluating retention potency of non-fluorescent small molecules inside Pickering picodroplets.

References:
CHARACTERIZATION OF A NOVEL ZETAMICIN IMPURITY BY LC-MS AND NMR: AN ORTHOGONAL TECHNIQUES APPROACH FOR THE STRUCTURAL ELUCIDATION OF LOW-LEVEL BYPRODUCTS

Elisa Libralesso (1) - Elisa Ciuti (1) - Antonio Triolo (1) - Fabio Lamonaca (2) - Matteo Gentili (3)

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Keywords: antibiotics, impurities, LC-MS, NMR

Introduction:
Zetamicin is a wide spectrum antibiotic belonging to the aminoglycoside family. Its active principle Netilmicin is made up of a di-aminosaccharide unit (N-ethylgaramine) bound to an aminopyran moiety (6-(aminomethyl)-3,4-dihydro-2H-pyran-3-amine). A novel impurity was found in API Zetamicin, while performing a screening for potential related impurities; hence the need to fully characterize it in order to assess its potential toxicity.

Methods:
LC-MS analysis provided information on the m/z ratio, elemental composition and UV absorption maxima. The isolated impurity was then characterized by mono and bidimensional 1H NMR, thus refining the structure and assigning the correct isomer among the possible ones. Following the structural determination, the compound was synthetically produced.

Results:
Perfect correspondence of the 1H NMR traces, of MS and MS/MS spectra, and of LC retention times between the process and the synthetic impurities allowed to confirm the assignment to the correct isomer.

Conclusions:
This synergetic teamwork between orthogonal techniques, such as LC-MS and NMR, was tailored to exploit their unique strengths. In fact, the high sensitivity and separation capabilities of LC-MS allowed to detect and isolate the low-level impurity, and to sketch out of its general structure, while NMR characterization performed on the isolated compound succeeded in the punctual assignment of its structure.

Novel Aspect:
Combination of LC-MS and NMR proved effective in the case study, sensibly easing data interpretations, for the strengths of each technique covers the weaknesses of the other.
1259 - GENERIC METHOD FOR PARALLEL ARTIFICIAL LIQUID MEMBRANE EXTRACTION OF NONPOLAR BASIC DRUGS FROM HUMAN PLASMA

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Keywords: Drug analysis, liquid phase microextraction (LPME), sample preparation, automation, green chemistry.

Introduction:
Through parallel artificial liquid membrane extraction (PALME) it is possible to perform miniaturized liquid-liquid extraction in a 96-well platesystem without the need for solvent evaporation and sample reconstitution prior to LC-MS [1-3]. Thus, with PALME, liquid-liquid extraction can be downscaled, simplified and automated. The aim of this project was to propose, develop, and test a generic method for PALME of monobasic drugs from human blood plasma with log P > 2.

Methods:
The setup consists of a commercially available 96-well sample plate and filter plate. Samples of human blood plasma were mixed with carbonate buffer and methanol (total volume 250 µl) and werepipetted into wells in the sample plate. Liquid membranes of 3 µl organic solvent (1 % trioctylamine in dodecylacetate) were established by pipetting the mixture on filters in the filter plate. Acceptor solutions of 50 µl aqueous formic acid (20 mM) were pipetted in the filter plate. The two plates were clamped together and agitated for 45 minutes at 900 rpm. Acceptor solutions were analyzed by UHPLC-MS/MS.

Results:
Data on extraction of 46 analytes have shown recoveries >40% for most analytes. Preliminary work on linearity, precision and accuracy on a selected number of analytes complied with the requirements of international guidelines for bioanalytical method validation, and a full validation is currently in progress.

Conclusions
A generic PALME method was proposed for monobasic drugs with log P > 2, and tested for extraction from human blood plasma. The preliminary results show potential and comply with international guidelines for bioanalytical method validation. The generic PALME method provided a simple workflow (with potential for automation), efficient sample cleanup for LC-MS analysis [1], and reduced the consumption of organic solvent to 3 µl per sample.

Novel Aspect:
This study is the first attempt to develop generic methods for parallel artificial liquid membrane extraction (miniaturized liquid-liquid extraction in 96-well format).

References
Gjelstad, A., Rasmussen, K.E., Parmer, M.P., Pedersen-Bjergaard, S. 2013 Bioanalysis 5(11), pp. 1377-1385
Introduction:
Ionisation efficiency depends on the structure of the compound as well as solvent and setup used. Presently, the relative abundance is evaluated based on the peak area. We demonstrate that the accuracy of such analyses in biological matrices can be significantly improved by predicting ionisation efficiencies.

Methods:
All measurements were carried out in ESI negative mode. The logarithm of ionisation efficiencies (logIE) of 10, predominantly pharmaceutical, compounds were predicted in 6 biological matrices (blood, plasma, cerebrospinal fluid, urine, brain and kidney tissue). Charge delocalisation parameter WAPS and degree of ionisation $\alpha$ calculated via COSMO-RS were used as input parameters.

Results:
The logIE values in different biological matrices were in good correlation with the logIE values in a solvent. This good correlation suggests that ionisation efficiencies can be predicted in the matrices similarly to the procedure previously proposed for the solvent. To test this, WAPS and $\alpha$ were used to fit the models for predicting logIE values in biological matrices.
The best predictive power of the model was observed in liver tissue homogenate (the root mean square error of the models, sRMSE = 0.49) and the lowest predictive power in urine (sRMSE = 0.80). The correlation between measured and predicted logIE values over all matrices is high, R² = 0.83 and sRMSE = 0.67 logIE units. Therefore, on average, the mismatch between the predicted and measured ionisation efficiencies is lower than 8 times. This enables semi-quantitative LC/ESI/MS analysis by using a set of calibration compounds to adjust model coefficients for the biological matrix of interest.

Conclusions:
Until now equal ionisation efficiencies of all compounds in all matrices are assumed if authentic standards are lacking. For the compounds used in this study, this approach would lead to a mismatch of 660 times with reality. Correcting signals with predicted ionisation efficiencies improves accuracy by almost two orders of magnitude. Additionally, we demonstrate that ionisation efficiencies can be predicted in biological matrices.

Novel Aspect:
We present an accurate approach to predict ionisation efficiencies in ESI negative mode for complex biological matrices.
A NOVEL GC-MS METHOD FOR SIMULTANEOUS DETERMINATION OF SEVEN PARABEN DERIVATIVES IN PHARMACEUTICAL AND COSMETIC PRODUCTS CONSUMED IN TURKEY

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Keywords: GC-MS, parabens, isomers, pharmaceuticals, cosmetics

Introduction:
Due to official regulations on the use of Alkyl esters of p-hydroxybenzoic acid (parabens) [1], the presented study was developed for quantitative determination of methylparaben (MP), ethylparaben (EP), n-propylparaben (NPP), i-propylparaben (IPP), n-butylparaben (NBP), i-butyl paraben (IBP) and benzylparaben (BP) in some pharmaceutical and cosmetic products consumed in Turkey using gas chromatography-mass spectrometry (GC-MS) - isotope dilution method.

Methods:
GC was carried out using a highly inert 95% dimethylpolysiloxane, 5% diethylpolysiloxane phase column (Rxi®-5ms, 30 m × 0.25 mm × 0.25 μm film thickness, Restek, USA). The ion trap mass spectrometer was operated in the electron impact - positive ionization mode (+70 eV) using an external ionization configuration. In the full scan mode the mass range was varied from 35 to 500 m/z at 0.6 s scan−1.

Results:
Analyte concentrations were determined by isotope dilution method using 13C13-MP-EP-PP-BP solutions as an internal standard to quantitative the unlabeled paraben derivatives. The obtained results for linearity, precision, and accuracy of the proposed method support their potential use for simultaneous identification and quantitative determination of the MP, EP, NPP, IPP, NBP, IBP and BP in samples. The method was validated according to ICH Q2(R)1 regulations [2], and system suitability parameters were checked according to the USP.

The amount of each of paraben was calculated using the calibration equations in 17 pharmaceutical and cosmetic products. Among the samples, none of the above-mentioned parabens were found in 11 of the analyzed products; both MP and NPP were detected in 4 of the products.

Conclusions:
A simple, selective and highly accurate isotope dilution - GC-MS method was developed and applied to analyze the MP, EP, NPP, IPP, NBP, IBP and BP in cosmetics and pharmaceutical products. Further studies with more products of different compositions and different manufacturers have to be conducted to make a better assessment overall possible hazard from originating from pharmaceutical and cosmetic products.

Novel Aspect:
To the best of our knowledge, this is the first study in which pharmaceutical or cosmetics products from Turkish market were analyzed for parabens content using GC-MS.

References:
1223 - ANTICANCER ACTIVITY EVALUATION OF NEW PHORTRESS ANALOGUES AND IDENTIFICATION OF BIOACTIVE METABOLISM PRODUCTS USING LCMS-IT-TOF

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Keywords: Phortress, Benzoxazole, Anticancer, Active Metabolite, LCMS-IT-TOF

Introduction
Phortress, including benzothiazole moiety, is a prodrug displaying anticancer activity. The active metabolite of phortress is a potent agonist of the aryl hydrocarbon receptor and switches on cytochrome P450 CYP1A1 gene expression [1]. In the present study, new analogues of phortress were synthesized and investigated for anticancer activity. In order to identify a probable active metabolite, biotransformation studies were conducted and results were examined by mass spectroscopic analyses.

Methods
Target benzoxazole derivatives (3a-3p) were synthesized [2], and anticancer activity tests were performed on colon (HT-29), breast (MCF7), lung (A549), liver (HepG2) and brain (C6) carcinoma cell types [3]. Induction potential of the compounds 3m and 3n on CYP1A1/2 enzymes was additionally investigated. Biotransformation studies for 3m and 3n were evaluated by LCMS-IT-TOF instrument (Shimadzu, Japan). Moreover, molecular docking studies for compound 3n and its some metabolites were performed.

Results
Anticancer potentials of phortress analogs 3a-3pwere assessed by cytotoxicity, DNA synthesis inhibition, and apoptosis induction assays. Compounds 3m and 3n were the most cytotoxic agents, which inhibited DNA synthesis of carcinogenic cell lines, significantly. It was also determined that these compounds have an effect to induce apoptotic cell death. In the biotransformation studies, compounds 3m and 3n were incubated with HepG2 cell line for 24 h. Metabolite identification studies were performed using LCMS-IT-TOF system. Proposed biotransformation pathway displayed that there are metabolites (3mM1, 3mM2, 3nM1, and 3nM1), which undergo the same metabolic reactions as active metabolites of phortress. CYP1A1/2 enzyme induction and molecular docking studies gave similar results to those of phortress.

Conclusions
As synthesized compounds thought to be prodrugs, their metabolic pathways were evaluated by applying biotransformation studies. Identification of very similar metabolites to active metabolites of phortress recommended that 3m and 3n have same mechanism of action with that of phortress. Furthermore, results of CYP1A1/2 enzyme induction and molecular docking studies also supported this suggestion.

Novel Aspect
Two novel anticancer drug candidates, which have stronger activity than phortress, were developed along with their active metabolites determined by LCMS-IT-TOF analyses.

References
INVESTIGATION OF ISOMERIC METABOLITE STRUCTURES USING A COMBINATION OF IMS, MS/MS AND CHEMOMETRICS: APPLICATION TO BIOTRANSFORMATION.

Cécile Palaric (1) - Sébastien Rigaud (1) - David Mathiron (1) - Roland Molinié (2) - Jean-Yves Beaumal (3) - Tristan Renaud (4) - Serge Pilard (1)

University of Picardie Jules Verne (UPJV), Analytical Platform (PFA), Amiens (1) - University of Picardie Jules Verne (UPJV), BIOPI-EA3900, Amiens (2) - Technologie Servier, Synthesis, Orléans (3) - Technologie Servier, A.S.M.P., Orléans (4)

Keywords: Biotransformation; Position of substitution; MS/MS; Ion mobility; Chemometrics.

Introduction:
Biotransformation is used to build molecules, similar to active principal ingredient, in the drug discovery program [1]. The structural identification of metabolites remains challenging when isomeric substituted molecules are involved. In this work, we present a methodology which combines, travelling-wave ion mobility separations (TWIMS) [2], MS/MS fingerprints and chemometrics [3], to successfully assign a number of isomeric hydroxylated metabolites.

Methods:
A model molecule bearing an aza-bicyclo moiety and 6 of its hydroxylated metabolites, previously isolated, were used for our methodology development. Retention times (tR) from UPLC/HRMS separation, instrument measured drift times (tD) from TWIM separation of the protonated molecules, accurate m/z and intensity measurements of fragment ions from MS/MS spectra, were collected.

Results:
The tR, drift times and MS/MS data set of our standard molecules led to coherent information regarding hydroxylated metabolites substitution position, shape, polarity and stereochemistry. Statistical processing of MS/MS fragment ions intensities, using multivariate analysis (SIMCA-P), enabled a fast and unambiguous assignment of the metabolites structures, which could allow the transposition of our approach to investigate complex bioconversion mixtures.

Conclusions:
To validate our workflow, the UPLC/HRMS screening of 12 fungi stains followed by a principal component analysis (PCA) were performed. After the selection of the best candidate, the hydroxylated metabolites content was quickly elucidated with the previously described tools.

Novel Aspect:
Investigation of isomeric compounds using a combination of ion mobility, MS/MS and chemometrics.

References:
Introduction
For thousands of years, herbal medicines made from terrestrial plants were used by humans to prevent and treat diseases. Extraction is the crucial first step in the analysis of medical plants. Researches have shown that most of the therapeutic properties of D. deltoidea and T. terrestris are due to the presence of steroidal glycosides. The main objectives of this study were to develop a complete strategy of isolation and HPLC-MS determination of steroidal glycosides.

Methods
The HPLC separation was conducted on a C18 column. The separation was carried out in a gradient elution mode. Detection were performed by MS in positive ion (SIM mode). Ultrasound-assisted (UAE), refluxing (RE), low pressure refluxing (LPRE) and Soxhlet (SE) extraction techniques were developed for determination steroidal saponin content. The results were confirmed using the multiple successive extraction method and spiking with standards before extraction.

Results
Extraction parameters for UAE, RE, LPRE and SE methods were optimized by single-factor-experiment for isolation of steroidal glycosides from T. terrestris [1]. Extraction parameters for UAE method were optimized by Latin Square experimental design at 4 levels for 4 factors for isolation of steroidal glycosides from D. deltoidea plant material and cell culture [2]. Thermal decomposition of protodioscin was observed after long term high temperature extraction process. The accuracy for all studied extraction techniques was confirmed by spiking of the plant material with standards before extraction and HPLC-MS analysis and the analytical method was validated for linearity, limits of detection, limit of quantification, precision and accuracy. Also, a high degree of extraction was confirmed by multiple successive extraction method (MSEM) and RE Methods: At the same time, it was shown that the UAE method is completely inappropriate for a similar task when working with plant material. This may be due to the lack of mechanical tissues in the cell culture, in contrast to plant material.

Conclusions
In the course of the work cycle, a study was made of the patterns and features of extraction, chromatographic separation followed by mass spectrometric determination of the steroidal glycosides from plant material and cell cultures. The impossibility of using the UAE extraction method for working with plant material was also shown.

Acknowledgements
This work was supported by Russian Science Foundation (Grant No. 17-13-01146) for Moscow State University.

Novel Aspect
The method for HPLC-MS determination of steroid glycosides was developed and validated. The optimization and comparison of extraction techniques were performed.

References

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Introduction:
Tropane alkaloids are compounds with therapeutic properties naturally obtained from Solanaceae plants as Atropa Belladonna, which has been used in drugs and homeopathic products. The main concern related to homeopathic products is the scarce information about their composition as well as recent cases of secondary effects [1]. New methods are necessary to control these products, including untargeted analysis.

Methods:
The extraction method was based on solid phase extraction (SPE) for homeopathic products and then, the recomposed extract was analyzed by liquid chromatography coupled to high resolution mass spectrometry (Exactive-Orbitrap). For drugs (ointment) and Atropa Belladonna seeds only dilution was used prior injection. A homemade database with 41 tropane alkaloids was created and applied. Compound Discover 2.1 was used to search untargeted compounds.

Results:
The method was optimized using a SPE procedure to concentrate the compounds present in homeopathic products, as well as the dilution step for drugs and Atropa Belladonna seeds. The method was validated evaluating parameters as specificity, linearity, accuracy, precision and limits of detection (LOD) and quantification (LOQ). The proposed method allows the determination of a large range of tropane alkaloids in Belladonna seeds and ointment from Belladonna extract at concentration up to 337.5 mg/kg for atropine, while in homeopathic products only atropine, scopolamine, tropine and apoatropine were detected. When untargeted analysis was performed, other tropane alkaloids (benzoylecgonine) found in related plants [2] were also detected. In addition to tropane alkaloids, aminoacids, considered as precursors of tropane alkaloids (phenylalanine and ornithine), were confirmed in the seeds as well as sugars (glucose, fructose and lactose) in the homeopathic drugs.

Conclusions
The developed method provides the quantification of a large range of tropane alkaloids. High resolution mass spectrometry allows the retrospective analysis of the samples and the available software makes possible untargeted analysis, detecting other tropane alkaloids, precursors as aminoacids and other compounds present in the drugs.

Novel Aspect:
A suitable methodology for the comprehensive characterization of homeopathic products from Atropa Belladonna applying targeted and nontargeted approaches is provided.

References
Donato Squillaci (1) - Marta Menicatti (2) - Silvia Bua (2) - Claudiu Supuran (2) - Gianluca Bartolucci (2)

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Keywords: linear equations of deconvolution analysis, LEDA, energy resolved mass spectrometry, isomers resolution, ion trap

Introduction:
The separation of a mixtures of isomers by chromatography generally requires lot of time and it is isomers pair-specific. To overcome this issue, LC-MS/MS methods, together with a mathematical algorithm that is able to identify the isomers present in the sample without their chromatographic separation, were developed [1-2]. This approach was applied to MS/MS acquisitions obtained with both triple quadrupole and ion trap mass analyzers.

Methods:
The LC-MS/MS analysis were performed using both triple quadrupole (QqQ) and ion trap (IT) systems, either equipped with ESI source, operating in positive-ion mode. The chromatographic parameters employed to analyze the samples were finely tuned to minimize the run time. Moreover, a matrix of linear equations (LEDA), based on abundance ratios of product vs precursor ions, was used. This approach was verified to 6 isomers pairs of NSAID-coumarin hybrid inhibitors [3].

Results:
The study was carried out on standard solutions of analytes and their mixtures in the concentration range of 10 to 100 ng mL\(^{-1}\). Using the reported conditions, a co-elution of the studied isomers were observed. Therefore, in case of a isomers mixture, LEDA must be able to resolve the co-eluting peak determining the relative concentration of each isomer eventually present. However, some issues were occur in the QqQ results, that showed in some cases lack of precision. These problems were mainly due to the difficult evaluation of the abundance of the precursor ion before its decomposition. To avoid the early decay of the precursor ion, the MS/MS experiments in IT were carried out. The fragmentation mechanism in IT allows a better management of the ions (precursor/products) ensuring their correct abundance rating. Indeed, the different energy transfer during collision process, lead to the formation of product ions directly by precursor ion. However, this characteristic often produce a poor number of product ions, which contrasts the LEDA application.

Conclusions:
The quali-quantitative data obtained from both tested MS analyzer demonstrated the LEDA reliability in the isomers speciation.

Novel Aspect:
The LEDA approach has the advantage that isomers can be quantified without the need of LC separation or additional specialized ion mobility instrumentation.

References:
Introduction:
Polysorbate (PS) is a polyoxyethylene (POE) type non-ionic surfactant derived from esterification of sorbitan, made from a very complicated mixture of polymers, widely used as excipient. Analytical challenges are: structural heterogeneity caused by different sorbitan core structures esterified in different positions with a mix of fatty acids; small amount in formulations, potential interferences from excess of drug substance and excipients; no UV absorbance.

Methods:
All the information is obtained in one HPLC-MS run using a Q-Tof instrument. The method includes a low-energy survey MS scan, to obtain information on the intact components and the molecular weight distributions of the involved species; a data-independent MS/MS scan to detect characteristic fatty acids marker ions; a data-dependent MS/MS of selected precursor ions, triggered by detection of a given fatty acid marker in the data-independent scan.

Results:
We developed and validated an analytical method for simultaneous profiling and quantification of polysorbate 80 (having oleic acid as the main fatty acid component) either as neat excipient or contained in pharmaceutical and biopharmaceutical formulations.

The method provides identification of components, their apparent percentages, the fatty acids profile, the absolute quantification of this excipient in formulations, the detection and identification of its degradants.

The developed method finds different application in quality control, comparison of suppliers, monitoring batch-to-batch consistency, regulatory documentation, understanding preformulation and stability issues.

Conclusions:
The developed method is suitable for rapid characterization and quantification of neat polysorbate and polysorbate in pharma and biopharma formulations, yielding simultaneously components identity, their apparent percentages, the fatty acids profile and its absolute quantification in different kinds of solid and liquid formulations.

Novel Aspect:
First description of a suitable quali/quantitative method for comprehensive characterization of PS.

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RAPID HPLC-MS/MS EVALUATION OF THE BATCH-TO-BATCH CONSISTENCY OF THE PAYLOADS SUBSTITUTION OF A LYSINE ANTIBODY-DRUG-CONJUGATES (ADCS).

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Keywords: ADC; Conjugation site analysis; Lysine conjugation; Data-independent acquisition; Batch-to-batch consistency

Introduction:
The aim of this work is the determination of the drug substitution sites of a lysine antibody-drug conjugate (ADC) by a very specific LC-MS analysis of its peptide digests [1][2]. Conjugated peptides were detected with high sensitivity and specificity by monitoring the characteristic reporter fragment ions of the cytotoxic molecule. Moreover, the batch-to-batch consistency of the conjugation was studied to evaluate the reproducibility of the production process.

Methods:
The ADC was denatured and digested with a Trypsin/Lys-C mix (E/P ratio 1:25). The instrument was a Synapt G2 HDMS mass spectrometer interfaced with a Waters ACQUITY UPLC H-Class System, using an Acquity Peptide CSH UPLC column. The data acquisition was in MSE mode. Conjugated peptides were detected by extracting the payload fragment ions from the high energy trace. The results were elaborated by UNIFI software (Waters).

Results:
From the automatic data processing with UNIFI software, about 30 payload-substituted peptide were detected, which were then visually inspected to confirm their identity. The conjugated peptides were characterized by their accurate masses, retention times and by their MS/MS sequence, allowing unambiguous detection of the substituted residues [3]. Subsequently, eight batches of ADC were treated with the same experimental method and the extracted ion chromatograms of the payload reporter ions were compared: in this way it was possible to highlight similarities and differences among the obtained chromatographic profiles, and hence the similarity degree of the substitution sites among all batches. The evaluation of the obtained chromatograms showed that they were practically superimposable, demonstrating excellent batch-to-batch consistency of the payload substitution.

Conclusions:
The payload-substituted peptides in the digestion mixture were detected with high specificity, allowing substitution site determination based on sequence information from the MS/MS data. Moreover, the comparison of the profiles of the extracted chromatograms of the payload fragment ions allows to evaluate the reproducibility of the production process and to identify any differences, in the substitution sites, among several batches.

Novel Aspect:
Description of a quick and simple method to assess batch-to-batch reproducibility of the conjugation process in an ADC.

References
Introduction:
The activity of an Antibody-drug conjugate (ADC) is exerted after drug release from the ADC inside the cancer cells. Drug and its cellular metabolites are further transformed in vivo, generating a series of highly cytotoxic drug-related molecules which must be identified as a first step for their pharmacological and toxicological evaluation. Here we describe identification of the payload metabolites in serum of cynomolgus administered intravenously with an ADC.

Methods:
The deproteinized sera were analyzed by LC-MS/MS with a 100 x 1.0 mm RP column, using a Q Exactive mass spectrometer operated in positive ESI. Data acquisition consisted of a full-scan MS at FWHM resolution = 70000, alternated with a series of 30 MS/MS scans, at resolution = 17500 and HCD collision energy 20 V, acquired with precursors ranging sequentially from m/z 610 to 1161 in steps of 19 m/z units, and 20 m/z units quadrupole isolation windows. Scan time was 2.8 secs.

Results:
The above described method afforded full-scan sensitivity to sub-ng/mL levels, and sufficient selectivity to distinguish the metabolites signals in the presence of coeluting matrix peaks up to 3 degrees of magnitude higher in abundance. The payload-related metabolites were detected by extracting the accurate masses of selected fragment ions from the entire MS/MS data set, whereas their precursors were visually searched at the corresponding retention times in the MS data set, inside the 20 m/z-wide interval from which the fragment ions were generated. The method selectivity was verified by analyzing blank serum, and the capability of detecting unknown payload-related compounds by spiking blank serum with 5% v/v drug incubates in human liver microsomes. Cynomolgus sera treated with 5 and 1 mg/kg ADC showed the presence of the free drug, its cysteine conjugate, and the drug plus linker adduct. Each metabolite was characterized by the accurate masses of the precursor and its main diagnostic fragment ions.

Conclusions:
High-resolution MS and data-independent analysis with sequential windows MS/MS acquisition of a series of precursors spanning an about m/z 600 mass interval, allowed successful detection and identification of the payload-related metabolites in cynomolgus serum after treatment with an ADC at 5 and 1 mg/kg dose. The method features high specificity and full-scan sensitivity in the sub-ng/mL range, with mass accuracy typically below 2 ppm.

Novel Aspect:
First data-independent scanning application to the detection and identification of in vivo ADC metabolites.

For information please contact: scientific@imsc2018.it
MALDI TOF MASS SPECTROMETRY FOR THE CHARACTERIZATION OF ANTITUMOUR RUTHENIUM(II) POLYPYRIDYL COMPLEXES

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Keywords:
MALDI-TOF-MS
ruthenium(II) complexes
instrumental parameteres

Introduction

Ruthenium(II) polypyridyl compounds, such as Ru(II) terpyridine complexes, are among lead compounds in anticancer drug discovery [1]. Their characterization after synthesis is, thus, necessary, but also presents a challenge. To this end we used matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF-MS). Our main goal was to find how instrumental parameters affect the mass spectra of [Ru(Cl- Ph-terpy)(N-N)Cl]+ complex ions.

Methods

Experiments were performed on the MALDI TOF Voyager-DE PRO (Sciex, USA) instrument, equipped with a delayed ion-extraction device and a pulsed nitrogen laser (337 nm 3 ns pulse width) operating at 20.0 Hz laser repetition rate. Solution of dithranol in tetrahydrofuran was employed as a matrix. A 0.5 µL of the matrix was placed onto a stainless steel target plate and laid over by 0.5 µL of a sample solution.

Results

MALDI MS has been successfully applied for identification and characterization of transition metal compounds and organometallics [2-5]. In spite of this, data regarding the effect of matrix, laser intensity and analysis mode on mass spectra of transition metal complexes is limited [6]. In the study presented herein we optimised the values of input variables: the laser intensity, the extraction delay time, the accelerating voltage, the grid voltage and the number of laser shots for the characterization of the three complexes. The complexes have a general formula mer-[Ru(4'-chlorophenyl-2,2':6',2''-terpyridine)(N-N)Cl]Cl, where N-N is a bidentate ligand: 1) 1,2-diaminoethane (en), 2) 1,2-diaminocyclohexane (dach) or 3) 2,2'-bipyridine (bpy). The fact that Ru possesses seven stable isotopes aggravated the analysis of mass spectra of its compounds. Since the studied complexes contain an inner chloride, the isotopic distribution of Cl must also be taken into an account.

Conclusions

In the mass spectra of the studied Ru(II) complexes the peaks of molecular (nonfragmented) ions were visible only upon the careful selection of the instrumental parameters. Otherwise fragment ions dominated the mass spectra, which precluded the determination of the precise molecular masses of the complexes. The accelerating voltage, the grid voltage and the extraction delay time had the greatest influence on the mass spectra and isotopic resolution of the peaks.

Novel Aspect

Tuning of instrumental parameters of the MALDI TOF mass spectrometer for characterization of novel anticancer Ru(II) terpyridine compounds.
References
A Liquid Chromatography-Mass Spectrometry method (LC-MS) was developed, validated, and implemented in Quality Control (QC) for the identity testing of acellular pertussis combination vaccines. QC activities represent around 70% of product cycle time and are often considered as bottle necks. A single LC-MS method is able to replace several antibody based identity tests to enhance quality, increase efficiency, and reduce cost and cycle-time.

Methods
The vaccine samples are digested to generate peptides. The peptide mixtures are then separated using liquid chromatography and analyzed in real-time by the mass spectrometer. The mass spectrometer detects the antigen specific peptides or signature peptides using accurate mass and retention time.

Results
The LC-MS identity assay is able to not only detect the presence of each of the antigen specific peptides derived from Diphtheria Toxoid (D), Tetanus Toxoid (TT), Pertussis Toxoid (PT), Fimbriae (FIM), Pertactin (PRN), Filamentous Haemagglutinin (FHA), Inactivated Poliomyelitis Vaccine (IPV) but also the dosage of the vaccines for the identification and differentiation of the products with different formulations. The specificity of the method is validated by the positive detection of the antigen specific peptides in samples/products where the corresponding antigen is present and their lack of detection in samples/products where the corresponding antigen is absent. Robustness tests were performed to examine the potential sources of variation. This was done by selecting factors expected to affect method responses, and the effect of the factors were examined and statistically analyzed.

Conclusions
The identity testing for pertussis and related combination vaccines using LC-MS is specific and robust. The test method is validated and accepted by the Regulatory Agencies, such as FDA, Health Canada, and EU. Implementation of LC-MS ID test in QC replaces identity tests using traditional Western Blot, Dot Blot, Agar Immunodiffusion, and ELISAs, eliminates the use of more than 18 antibody reagents, and reduces the cost and cycle time.

Novel Aspect
Implementation of a LC-MS release test for vaccine identification in QC and obtaining regulatory approval represent breakthroughs in the vaccine industry.
Introduction:
Stable isotopes are a practical tool used to define animal movements and to delineate trophic structures in animal communities [1, 2]. A crucial aspect of spatial ecology is the definition of the degree of isotopic variability that can be measured locally in animal tissues, in order to carry out the correct probabilistic assignments to a geographic location, calibrating the isotopic discrimination between stable isotopes in trophic sources and animal tissues [3].

Methods:
Stable isotope ratios of bio-elements (\(^{2}H\), \(^{18}O\), \(^{13}C\), \(^{15}N\), \(^{34}S\)) were determined in feathers of Passerines from the Italian Alps. The feathers were sampled on an altitudinal gradient in a restricted region from juvenile and post-breeding generations. Stable isotope ratios were determined by Isotope Ratio Mass Spectrometry after preparation of the feathers according to Bontempo et al. (2014) [4].

Results:
Isotopic variations can be mainly related to species feeding habits [5], seasonal effect of regional climatic conditions [6], geographic and topographic patterns [7, 8], natural or anthropogenic inputs which perturbate the local isoscapes [9, 10]. Through this study we assessed the probable variability sources, taking into account the distinct stable isotope ratios separately. In particular, the local variability in the sampling sites within a specific area of Italian Alps was measured, comparing the isotopic composition in feathers of several common Passerine species sampled from different moult generations in molting and fattening sites within or close to the breeding locations. Using the derived information it was possible to determine the specific regional multi-isotopic variability, as well as to define seasonal isotopic variability related to trophic and habitat niches.

Conclusions
The local variability of the isotopic composition within the sampling sites, comparing different species and different feathers generations was measured. In particular, the regional multi-isotopic variability for Passerines according to trophic sources and animal tissues was defined. This information is crucial in depicting the isotopic niche in local birds of Central Alps, deepening the knowledge about isotopic variability related to topographic, environmental and seasonal perturbations.

Novel Aspect:
Isotopic variability in avian keratinous tissues was estimated with a multi-isotopic approach in a narrow Alpine region. The concept of isotopic niche was deepened.

1125 - POTENTIAL USE OF THE STABLE ISOTOPE RATIOS OF BIOELEMENTS AND ELEMENTAL COMPOSITION TO TRACE THE ORIGIN OF DAIRY PRODUCTS

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Keywords: stable isotope ratios, elemental composition, traceability, dairy products

Introduction:
There is an increasing demand for reliable analytical methods to verify the authenticity of the food we eat. This is also important for PDO dairy products, which can command significantly higher prices than their non-PDO competitors. Stable isotope ratios and elemental composition have been used for discrimination and can be used to verify the authenticity of marketed products. An overview of the use of this approach to trace dairy products is presented here.

Methods:
Isotope Ratio Mass Spectrometry and Inductively Coupled Plasma Mass Spectrometry methods were developed to characterise and determine the authenticity of different types of dairy products. In particular, the stable isotope ratios (SIRs) of five bioelements - hydrogen, carbon, nitrogen, oxygen and sulphur - were determined in defatted dairy products, whereas elemental composition was determined in the raw product after acid microwave digestion.

Results:
Geo-climatic and pedo-geological factors affect SIRs in nature and the isotopic variations are ultimately incorporated into animal tissue through eating, drinking, breathing and exchanges with the environment, and are memorised in the resulting dairy products. Similarly, the elemental composition of dairy products is affected by different factors related to the place of origin (e.g. geology and soil characteristics) and animal diet, but also depends closely on the conditions in which such products are made and processed. The combination of these two approaches has led to the development of models able to trace the origin of high premium products. The developed method was applied to test the traceability of PDO cheeses: Italian alpine cheeses (e.g. Fontina, Montasio and Toma) [1, 2], as well as buffalo Mozzarella from the Campania region, Grana Padano and Parmigiano Reggiano [3, 4, 5].

Conclusions:
This work considers a robust and effective method that can be used to guarantee the authenticity of dairy products to consumers, focusing particularly on cheese. The method is based on determination of stable isotope ratios and the elemental composition of products. Specifically, in the case of PDO Grana Padano and Parmigiano Reggiano cheese, the model has been officially adopted as a reference method for assessing the authenticity of grated and shredded products on the market. Furthermore, this method was recently officially validated and adopted by UNI (Italian Standardisation Body, UNI Regulation 11692:2017).

Novel Aspect:
The method applied and described has been officially validated and can be used before the courts in legal cases.

References
Development of novel compact carbon dating instrument based on Collinear Resonance Ionisation Spectroscopy (CRIS)

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Development of novel compact carbon dating instrument based on Collinear Resonance Ionisation Spectroscopy (CRIS).

Traditional carbon dating techniques based on isotope ratio accelerator mass spectrometry are expensive, experimentally complex and access to instrumentation is often associated with excessive lead times. The use of CRIS as an alternative method affords unparalleled interference suppression when compared to RIMS for example. This is achieved by effectively decoupling the inlet electron cyclotron resonance ion source from the resonance ionisation spectroscopy by means of a charge exchange cell. The instrument employs a variety of ion optics including initial multi-collector magnetic sector mass selection and a linear ion trap to increase duty cycle; the sample inlet is a flash GC nitrogen/carbon elemental analyser.

The CRIS technique was initially developed at CERN in the ISOLDE facility for the analysis of exotic nuclei. The instrument at the University of Manchester is configured for the analysis of carbon isotopes but can easily be tuned for other nuclei and offers a state-of-the-art alternative technique for various forensic, dating and food security applications.

Giles completed his Ph.D last year at the University of Manchester, advancing the field of Laser post-ionisation Sputtered Neutral Mass Spectrometry (L-SNMS) which also included some RIMS of organometalic biological agents.

He is an analytical chemist and works as a consultant for HPLC & LC/MS method development and also as a freelance mass spectrometer service engineer.

More recently Giles joined the Nuclear Physics group at Manchester as a part-time post doc working on the compact carbon dating system.
Keywords: metallomics, speciation, plasma source MS, Isotope ratio, instrumentation

Introduction:
Most ion sources for mass spectrometry are tailored for specific species such as molecules, atoms, or biomolecules, requiring instruments to be designed and constructed with limited flexibility. What would be desirable is a source that can ionize a broad range of species and sample types, including solids, liquids, or vapors. Such a source, termed the Solution-Cathode Glow Discharge (SCGD) is now being evaluated and will be described and evaluated.

Methods:
The Solution-Cathode Glow Discharge (SCGD) is a relatively low-power (~70W) ionization source that operates in the open atmosphere. Sample solution flows from a small glass capillary and serves as the cathode of a discharge that strikes to a metallic anode pin just above it. The electric field at the cathode surface is high enough that electrical spraying takes place. Thermal fragmentation or even complete atomization of incoming analyte species is also possible.

Results:
A broad range of sample types has been investigated with the SCGD. Metallic species in solution can be either fully atomized or partially fragmented, yielding alternatively atomic mass spectra or spectra reflecting original solution composition. Thus, the SCGD is useful for either elemental or chemical-speciation analysis. Further, sensitivity is sufficient that isotope ratios can be measured at precision levels dictated by the IAEA International Target Values for natural uranium (0.28% rsd), with an integration time of only 150 s. For somewhat labile molecules, fragmentation can be controlled merely by adjusting the operating current and voltage of the SCGD. For triethyl phosphate, for example, the survival yield can be varied from at least 25-80%. Under conditions that favor electrical spraying, the SCGD can be fed directly with a peptide-containing solution and yields fragmentation mass spectra that are unlike those from other ionization sources and are surprisingly easy to interpret.

Conclusions:
Although originally developed as an emission source for elemental analysis, the SCGD has proven to be an extremely versatile ionization source for mass spectrometry. It is simple to construct, operates on a low-power supply, requires no gas supply, and is simple to set up and employ. Most work to date has been with an Orbitrap® spectrometer; ongoing work will employ alternative mass spectrometers, some suitable for field use.

Novel Aspect:
A new, highly versatile but simple ion source has been developed and is being tested. It is useful for samples ranging from biomolecules to atoms.

References
113 - HIGH-RESOLUTION AND TANDEM MASS SPECTROMETRY TECHNIQUES CONTRIBUTE TO THE DISCOVERY OF NEW ARSENIC METABOLITES OF TOXICOLOGICAL IMPORTANCE

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High-resolution and tandem mass spectrometry techniques contribute to the discovery of new arsenic metabolites of toxicological importance

Qingqing Liu, Xiufen Lu, Hanyong Peng, Elaine Leslie, Martin Zuidfhof, Xing-Fang Li, X. Chris Le*

Keywords: Arsenic speciation; protein binding; toxicology; biotransformation; LC/MS/ICPMS

Introduction: Nearly 100 arsenic species, with diverse toxicities (1,2), are present in the environment and in biological systems. There is a tremendous analytical challenge in the identification and quantification of trace amounts of individual arsenic species present in complex biological sample matrix. This presentation will highlight our recent development of mass spectrometry techniques for studying arsenic species, including the oxygenated and thiolated, trivalent and pentavalent, and inorganic and organic arsenic species.

Methods:

Hybrid quadrupole time-of-flight, linear ion trap, and triple quadrupole mass spectrometers were used to conduct high-resolution and tandem mass spectrometry experiments. Effluent from high performance liquid chromatography (HPLC) separation was split 80% to inductively coupled plasma mass spectrometry (ICPMS) and 20% to electrospray ionization tandem mass spectrometry (ESI-MS/MS). Human primary hepatocytes and hepatocellular carcinoma HepG2 cells were treated with 0, 20, or 100 μM Roxarsone (3-nitro-4-hydroxyphenylarsonic acid).

Results:

The combined techniques enabled identification and quantitation of individual arsenic species present in a variety of environmental and biological samples. Roxarsone was metabolized to more than 10 arsenic species in human hepatic cells. Several new metabolites were identified (3,4). The 24-hour IC50 values of thiolated Roxarsone for A549 lung cancer cells and T24 bladder cancer cells were 380 ± 80 and 42 ± 10 μM, respectively. This thiolated arsenic metabolite was more toxic than Roxarsone, whose 24-hour IC50 values for A549 and T24 cells were 9300 ± 1600 and 6800 ± 740 μM, respectively.

Conclusions:

We have characterized the metabolism of Roxarsone in human liver cells and successfully identified the new phenylarsenical metabolites. The identification and toxicological studies of the new arsenic metabolites are useful for understanding the fate of arsenic species and assessing the potential impact of human exposure to Roxarsone.

Novel Aspect:

The complementary techniques enabled the discovery of new arsenic metabolites that have important toxicological implications. The study improved mechanistic understanding of arsenic biotransformation.

References

ISOTOPIC TECHNIQUES FOR STUDYING THE SOURCES AND PROCESSES OF POLLUTANTS IN THE ENVIRONMENT

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Keywords: isotopic techniques, sources, mercury, organic pollutants, environment

Introduction:
The use of isotopic techniques is in the midst of a remarkable period of innovation and discovery; the last decade has seen the emergence of stable isotopes of metals and new capabilities for measurements of organic compounds. These advances hold the potential to reveal new insights into the nature, sources and transformation processes of pollutants in the environment. The selected examples will include: polycyclic aromatic hydrocarbons (PAH), atrazine and mercury.

Methods:
The sources of PAHs were determined in lake sediments using isotopic composition of carbon, while isotopic composition of nitrogen and carbon in atrazine was used to characterize degradation processes. These measurements were performed by gas chromatography coupled to isotope ratio mass spectrometry through combustion unit (GC-C-IRMS). Stable isotopes of Hg determined by MC-ICP MS were applied to determine the Hg sources in Mediterranean deep-sea sediments.

Results:
The dominant PAHs signatures identified in sediments of Lake Bled were mainly attributed to a coal/wood burning source, but PAH from carsoot could also contributed. Retene (Re) and Perylene (Per) exhibit profiles and isotopic composition that are distinct from those of other PAHs suggesting their natural origin. However δ13C values of Re determined in anoxic part linked Re to both natural and pyrolytic origin [1]. Stable isotope analysis of atrazine brings forward two additional lines of evidence to assess the degradation state of atrazine and desethylatrazine in natural samples: (i) to distinguish different degradation pathways of atrazine and (ii) to detect further degradation of DEA [2]. Both mass-dependent and mass-independent fractionation processes influence the isotopic composition of Hg in the Mediterranean Sea. Positive Δ199Hg values are likely the result of enhanced Hg2+ photoreduction in the Mediterranean water column, while mass-dependent fractionation decreases δ202Hg values due to kinetic isotope fractionation during deposition and mobilization. At least three primary Hg sources of atmospheric deposition could be defined: urban, industrial and global precipitation-derived.

Conclusions
The results indicate that together molecular and isotope composition of PAHs are useful tools for obtaining more detailed identification about sources of PAH inputs into lacustrine sedimentary environments, while trends in multielement isotope data of atrazine may decipher different degradation pathways. Stable isotopes of Hg provide new insights into the sources and geochemistry of Hg in deep-sea sediments.

Novel Aspect:
Isotopic techniques such as GC-C-IRMS and MC-ICP-MS open new avenues for studying sources, fate and impact of contaminants in the environment.

References
Experimental evaluation of TIMS-based detection methods for isotopic analysis of uranium at ultra-trace level

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Keywords: TIMS, Isotopic Analysis, Uranium, Detection Method, Nuclear Safeguards

Introduction:
Thermal ionization mass spectrometry (TIMS) is one of the most accurate and precise techniques for isotopic analysis, and suitable for monitoring undeclared nuclear activities[1-3]. The analytical performance of the three TIMS-based detection methods was experimentally evaluated for isotopic analysis of uranium at ultra-trace levels, in terms of analytical accuracy, precision, and measurement uncertainty.

Methods:
Isotopic measurement of uranium at 1 ng, 100 pg, 30 pg, 5 pg, and 1 pg levels were performed by TIMS (TRITON Plus, Thermo.) using three detection methods; Dynamic, multi-dynamic, and static Methods: Analytical performance, such as accuracy, precision, and measurement uncertainty, was experimentally evaluated depending on the detection Methods:

Results:
The TIMS analysis for the ten replicated U samples with amounts of 1 ng and 100 pg showed no significant improvement in analytical performance irrespective of the detection method adopted, whereas slight improvement was observed for the analysis of 30 pg uranium using the multi-dynamic and static detection Methods: Considering the analytical performance and the easiness of detector calibration, the preferred detection methods for 1 ng and 100 pg U is the multi-dynamic method, while that for 30 pg U is the dynamic method. The static detection method offers the greatest accuracy and precision, and the smallest uncertainty for TIMS measurements of 5 pg and 1 pg of U due to the greater detection sensitivity of ion counters than faraday cups, the elimination of ion signal drift, and the large number of valid data sets in a measurement.

Conclusions:
This study deals with the evaluation of three detection methods (multi-dynamic, dynamic, and static) in the isotopic analysis of ultra-trace amounts of uranium using thermal ionization mass spectrometry. For samples containing 5 pg and 1 pg uranium, the static detection method provided the best results in terms of accuracy, precision, and measurement uncertainty.

Novel Aspect: (Limit of 150 characters)
This study shows that the static detection method of TIMS can be applied to particle analysis of environmental samples for nuclear safeguards.

References
C and H stable isotope ratio analysis using GC-IRMS for vanillin authentication

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Keywords: stable isotope ratios, vanillin, authentication, gas chromatography

Introduction:
Vanilla extracts are widely used as flavouring ingredients in foods and beverages and as aromatic compounds in perfumes and pharmaceuticals. Due to the high production cost of high-quality natural extracts from Vanilla planifolia, synthetic or natural identical biosynthetic vanillin (which derives from natural precursors such as guaiacol, ferulic acid, eugenol and lignin) are often used as a substitute for authentic natural vanillin [1].

Methods:
We combined analysis of 13C/12C (expressed as $\delta^{13}C$) values and 2H/1H (expressed as $\delta^{2}H$) ones using gas chromatography coupled to isotope ratio mass spectrometry (GC-IRMS) [2]. 16 authentic samples of Vanilla planifolia, 16 natural identical, 5 synthetic vanillin and 20 commercial extracts were considered.

Results:
The carbon isotope ratio values allow discriminating between natural and natural identical or synthetic vanillin (averagely -19‰ vs -30‰). Authentic natural vanillin from Vanilla planifolia and natural identical vanillin are characterised by $\delta^{2}H$ values much lower than synthetic vanillinones (averagely -50‰ vs +73‰). The isotopic values of all the commercial extracts declared to be from Vanilla planifolia (N=20), had $\delta^{13}C$ values within the typical range of natural vanillin, but $\delta^{2}H$ values outside the range and more similar to that of synthetic vanillin. Our results show how the stable isotope ratio analysis (SIRA) of 13C/12C is no longer sufficient to discover vanillin adulteration, due to the practice of adding 13C to the methyl site of synthetic vanillin [3], and the new analysis of $\delta^{2}H$ is mandatory.

Conclusions:
The combination of $\delta^{13}C$ with $\delta^{2}H$ GC-IRMS analysis of vanillin can therefore be proposed as a suitable tool to improve the detection of vanilla extract adulteration.

Novel Aspect:
Stable isotope analysis of 2H/1H can be a rapid and effective method to identify the natural or synthetic origin of vanillin.

References
Stable isotope ratio analysis for authentication of chitosan

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Keywords: stable isotope ratios, chitosan, authentication, animal or fungi origin

Introduction:
Chitosan is a linear polysaccharide with a number of possible uses (e.g. in medicine). It is produced by deacetylation of chitin, which is the structural element of exoskeleton of crustaceans. Due to the presence of allergens in seafood, its production from fungi has gained increasing attention. To identify its origin, residual glucans, viscosity and settled density are used, but often not effective. This calls to the development of new methods, such as the stable isotope ratio analysis.

Methods:
Following the prescription of OIV International Œnological Codex [1], residual glucans, viscosity and settled density were measured to identify the crustaceous or fungi origin of 20 chitosan samples. Moreover, the stable isotope ratios (SIRs) of four bioelements - hydrogen, carbon, nitrogen, oxygen - were analysed in the raw product using Isotope Ratio Mass Spectrometry.

Results:
The biosynthetic pathway of the chitosan's precursor chitin affects its isotopic composition. The samples identified by the OIV official methods as from crustaceans showed lower $\delta^{13}C$ (averagely -20.8‰) and higher $\delta^{15}N$ values (-2‰). This fits with the marine origin of the font (the exoskeleton of crustaceans) characterized by low $\delta^{13}C$ value (around -20‰) and high positive $\delta^{15}N$ value (around +7‰) [2]. The low $\delta^{15}N$ values here found are probably due to the addition to the yeast broth of nitrogen synthetic sources such as urea with low $\delta^{15}N$ values [3]. The samples identified of fungi origin based on the OIV prescriptions, are characterized by higher $\delta^{13}C$ (-13.8‰) and lower $\delta^{15}N$ values (-4.1‰). The higher $\delta^{13}C$ values of these chitosan samples are explained on the basis of the fact that sugar cane juice and molasses with typical $\delta^{13}C$ around -11‰ [4] are used as economic substrates in fungi chitosan production [3]. D/H and $\delta^{18}O$ are lower in chitosan from fungi probably due to the lower value of the fermentation water.

Conclusions:
This work considers a robust and effective method, based on the determination of stable isotope ratios of bioelement, that can be used to identify the origin of chitosan.

Novel Aspect:
Stable isotope analysis could be a rapid method to identify the crustaceans or fungi origin of chitosan.

References
1. Resolution OENO 368/2009, Chitosan, OIV International Œnological Codex
Laser ablation/ionization mass spectrometry is becoming again an attractive analytical technique for element composition determination of solids [1,2,3,4]. It offers several advantages: little sample preparation, high sample throughput, high spatial resolution and the possibility to analyze any kind of solid sample. We present a novel strategy using fs-LA at elevated pressure in combination with CD nozzle and Rf-only ion funnel for element imaging in solids.

Methods:
The laser ablation ion funnel ion source, described in this work, employs an Rf only ion funnel in combination with a convergent-divergent nozzle for collisional cooling of laser generated ions and their separation from the buffer gas. This configuration achieves a sufficient pressure drop between ion source and mass spectrometer while allowing for multiple collisions and consequent focusing. The “LAFU” source was coupled to a TOF-MS and different laser were employed.

Results:
The first results obtained with the coupling of ns-LAFU (3 ns, 532 nm) source with time of flight mass spectrometry (LAFUMA), showed that also low m/Q ions (\(^{12}\text{C}\), \(^{27}\text{Al}\)) can be successfully focused in the Rf-only ion funnel. The ion signal intensities could be increased by up to 300 times in comparison with ablation in high vacuum. Still the ion transmission through the ion funnel was found to have a pronounced dependence on m/Q, as a result of the pseudo-stationary fields created near the ion funnel electrodes. Compared to the previously used ns laser ablation, the fs laser (150 fs, 400 nm) ablation exhibits significantly smaller mass ablation rates and generally more comparable sensitivities. Transient evolution of the pulsed ion signals was longer than for ns LA regime and less affected by buffer gas pressure., indicating that a fraction of high energetic ions could not be thermalized completely in this case.

Conclusions
An ion funnel has been successfully combined with laser ablation for ion generation at elevated pressure, achieving a significant enhancement of ion transmission. The combination of the ion funnel with a CD nozzle for differential pumping shows promising features for the further development of laser ionization/ablation mass spectrometry in element and isotope analyses of solids.

Novel Aspect:
Collisional cooling of laser generated ions at elevated pressure in combination with an Rf only ion funnel for fast element image acquisition was successfully realized.

References
Overview of the volatile components of the Corsican liverwort Scapania undulata

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Keywords: Scapania undulata, essential oil, hydrosol, SPME

Introduction: (Limit of 400 characters)
S. undulata is a liverwort living wild on the rocks of deciduous broadleaved forests of Corsica [1]. To our knowledge, four studies deals with the chemical composition of S. undulata essential oil from Europe [2–5]. Some sesquiterpenes such as longiborane and himachalane derivatives were identified as main components. The aim of the present work was to determine and characterize the volatile compounds of S. undulata from Corsica using hyphenated Methods:

Methods: (Limit of 400 characters)
Plant material was collected in several Corsican forests and dried at room temperature. Essential oils and hydrosols were prepared by hydrodistillation as well as the volatile fraction was extracted by HS-SPME HeadSpace - Solid Phase MicroExtraction) under optimized conditions. Et2O extract was prepared by maceration from powdered plant material. The plant volatiles were investigated using an analytical procedure including LC, LLE, GC-FID, GC-MS, ESI-MS, tandem MS and NMR.

Results: (Limit 900 characters)
The present study focused on the volatile components of S. undulata from Corsica. The chemical investigations of the four extraction matrices allow determining different chemical profiles. Essential oil (EO), hydrosol (HY) and Et2O extract (EXT) was dominated by oxygenated sesquiterpenes (EO 63.5%; HY 82.0% and EXT 63.9%) while the volatile fraction (VF) extract by HS-SPME was dominated by hydrocarbon compounds (VF 61.2%). The main components of essential oil and hydrosol of S. undulata were respectively epicubenol (49.0% and 64.5%), β-Muurolol (5.0% and 7.2%) and longiborneol (3.0% and 7.2%) while epicubenol (44.4%) with longipinanol (11.9%) were the main components of Et2O extract. As previously reported, longipinanol is rather labile and was almost completely degraded during hydrodistillation, but was detected in considerable amounts in the ether extract [3]. In addition, epicubenol (27%), calarene (9.6%), were the main components of the volatile fraction sampled by HS-SMPE. As epicubenol, himachalol, longiborneol and logipinanol were absent to our spectral library, their identifications were ensured by NMR and ESI-MS experiments.

Conclusions (Limit of 400 characters)
The study provides an overview of the volatile components of S. undulata. Sesquiterpene compounds were the dominant natural products of the plant extracts obtained by hydrodistillation, HS-SPME and Et2O maceration. Oxygenated compounds characterized essential oil, hydrosol and Et2O extract while the volatiles emitted by the plant were largely hydrocarbons. The study confirms the necessity to use different sample preparation methods in order to study the complete chemical diversity of the plant.
Novel Aspect: (Limit of 150 characters)
The study reports for the first time the volatile components of S. undulata from Corsica. To our knowledge, the report of epicubenol as main essential oil component was original, as well as, the study of the volatile components emitted by S. undulata. HS-SPME analysis allows approaching of chemicals emitted by the plant and the identification provides information concerning the allelopathic properties of the liverwort.

References
1201 - EXPLORING FOOD AROMA RELEASE AND PERCEPTION USING DIRECT-INJECTION MASS SPECTROMETRY

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Keywords: DIMS, aroma, nosespace, olfaction, perception

Introduction:
Direct-injection mass spectrometry (DIMS) – in the forms of proton-transfer-reaction MS (PTR-MS), atmospheric pressure chemical ionization MS (APCI-MS), and selected ion flow tube MS (SIFT-MS) – has been increasingly implemented over the past two decades to explore food flavor release and perception. This keynote lecture will review novel food flavor insights that have been achieved using on-line DIMS techniques, focusing on applications in in vivo analysis.

Methods:
Food flavor perception is a complex process that depends on manifold parameters, including the physicochemical properties of the flavor compound, the food matrix, saliva, oral and nasal anatomies, as well as the mucosa lining the airways, amongst other factors [1]. The real-time analytical capability of DIMS techniques has opened up new opportunities for characterizing the fast dynamics of flavor release and relating this to perceived sensory impressions.

Results:
In vivo analyses using DIMS was pioneered around two decades ago by Taylor and Linforth using APCI-MS [2,3,4] and offers an opportunity to explore parameters affecting flavor release and perception systematically. Analyses involve sampling exhaled breath via the nose – termed nosespace analysis – to capture volatile aroma compounds that are released from the food (or beverage) matrix during oral processing and enter the nasal cavity during swallowing, eliciting perceived flavor impressions. This approach has been used, for example, to assess aroma perception in relation to taste attributes [5] and textural and physiological parameters [6], or olfactory acuity in general [7]. DIMS has also been applied to generate sensory profiles of food products based on non-destructive headspace analysis, as a means to characterize a product’s sensory attributes without the (explicit) need of a trained human sensory panel [8]. New developments and peripheral equipment have extended the range and versatility of DIMS systems in food flavor research [9,10].

Conclusions:
Nosespace analyses via DIMS techniques has provided many insights into flavor perception that would not have been achievable using conventional mass spectrometry in combination with classical gas chromatography (GC-MS). The shortcomings of DIMS techniques tend to be their lack of unequivocal identification, but this requirement is obviated when using a targeted approach to monitor fast processes of individual flavor compounds that are known a priori.

Novel Aspect:
On-line direct-injection mass spectrometry is a versatile and powerful tool for investigating the dynamic processes involved in flavor release and perception.

References


For information please contact: scientific@imsc2018.it
97 - EFFECT OF GERMINATION TIME, ROASTING LEVEL AND ALTITUDE IN THE AROMA RELEASED BY COFFEE POWDER MONITORED BY PROTON TRANSFER REACTION MASS SPECTROMETRY AND GAS CHROMATOGRAPHY MASS SPECTROMETRY

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Keywords: PTR-QMS; GC-MS; coffee powder; aroma; volatile organic compounds

Introduction
Coffee is one of the most consumed beverages all over the world, as well as in Thailand especially in recent years. Two species of coffee are harvested in Thailand, Coffea arabica in the northern part and Coffea canephora var. robusta in the southern part. Arabica coffee plantation in the north of Thailand cover about 9000 hectares, distributed in eight provinces. Arabica coffee is mainly grown in the cooler highlands, wet processed in order to obtain high quality green bean and used in roasted coffee for domestic use[1]. Wet-processed beans are characterized by more fruity and acidic notes and less bitter and burnt attributes, compared with dry-processed beans. Moreover, the wet method allows the preservation of intrinsic properties of the beans, leading to coffees with a more desirable aroma[2].

Coffee aroma is the main responsible for organoleptic properties and therefore appeal of coffee. It is developed during the roasting process and depends on operative conditions such as temperature and time, but also on variety, origin and composition in terms of aroma precursors in green beans. In particular, caramelization of sugars leads to the production of furans (responsible for burnt and caramel notes), Maillard reaction of sugars develops pyrazines (characterized by roasty notes, mainly responsible for the coffee aroma), trigonellines are the precursors of pyridines (which confer smoky notes to the coffee) and lipids metabolism leads to the production of carbonyls [2, 3, 4, 5]. The effect of three different factors, such as altitude of harvesting (900 and 1500 meters above sea level), germination time (0, 24, 28 and 72 hours) and roasting level (light, medium and dark) on the volatile profile found in 24 different types of coffee were analyzed by Proton Transfer Reaction Quadrupole-Mass Spectrometry (PTR-QMS) and by Gas Chromatography Mass Spectrometry (GC-MS). The best conditions to minimize undesirable odor and maximize the desirable ones were evaluated by Design Expert®.

Methods
Coffee beans (C. arabica) were harvested at 900 and 1500 meters above sea level in the Thai province of Chiang Rai in Doi Chang by Waweecoffee Co., Ltd. 1 Kg of green coffee beans, harvested at each altitude, was placed in boxes, soaked with 5 liters of water in a growth chamber at 28 °C, in dark, and let germinate for 24, 48 and 72 hours. 300 gr of green coffee beans harvested at 900 and 1500 meters were not induced to germinate. Germinated and not germinated coffee beans were roasted at 200 °C by Waweecoffee Co., Ltd. Three different roasting profile were achieved: light, medium and dark. Light-roasted coffee beans were roasted for 6 minutes, show 1-5% of weight loss and a light brown color; medium-roasted coffee beans were treated for 8 minutes, show 5-8% of weight loss and a medium brown color; dark-roasted coffee beans were roasted for 10 minutes, show 8-10% of weight loss and a dark brown color.

The headspace of coffee powders was measured by PTR-QMS. 35 mg of coffee powder were placed to equilibrate in 40-mL glass vials at 30 °C for 15 min. Five replicates for each sample were analyzed. Four empty vials were analyzed and considered as blanks. The measurement order was randomized to avoid possible memory effect. The instrumental conditions were the following: drift voltage 600 V, drift temperature 75 oC, drift pressure 2.10 mbar, affording an E/N value of 149 Td (1 Td = 10-17cm2 V-1 s-1).
SPME analysis was carried out with a Gas Chromatography-Mass Spectrometer. 2 g of coffee powder were weighted in a 20-mL septum-sealed gas vial and sampled through the SPME fiber for 30 min at 60 °C. Chromatographic conditions were the following: 40 °C for 3 min, from 40 °C to 200 °C (0 min) at 3 °C/min, then to 240 °C (1.2 min) at 16 °C/min. Multivariate data analysis methods were applied to visualize data and the optimization of factors was performed using Design Expert®.

Results
2 g of coffee powder from eight different samples (L900, D900, L924, D924, L1500, D1500, L1524 and D1524) were analyzed by SPME-GC-MS. 27 compounds were tentatively identified and five of them were found only in the high-altitude samples. Acetic acid and 2-furanmethanol were high in L900 and L1500 samples and decreased with roasting and germination. 2-ethyl-6-methyl-pyrazine is found to be more abundant in low-altitude samples compared to the high-altitude ones and show no significant changes related to roasting level and germination time. Dodecane undergoes a reduction in both low- and high-altitude samples after 24 hours of germination. The volatile profile of all 24 types of coffee was monitored by PTR-QMS. The 181 mass peaks obtained by each of these analyses were subjected to one-way ANOVA (p < 0.01); subsequently 89 masses were found at significantly different concentration between the three factors. Among these 89 masses, only the ones also found in GC-MS and tentatively identified (14 masses) were used in the optimization of the conditions. A 3-level factorial design was used to study the effect of altitude, germination time and roasting level in the composition of desirable and undesirable volatile compounds in coffee. The 14 masses found both in GC-MS and in PTR-MS were used in the model and the optimum conditions were found minimizing undesirable odor (m/z 61, 80, 99 and 101), and maximizing the desirable ones (m/z 95, 97, 109, 111, 117, 123, 131, 135, 137 and 148). The software proposed 23 solutions, with an index of desirability between 0.537 (best solution) and 0.256 (worst one). The two best solutions were the samples M924 (low-altitude, 24h-germination and a medium level of roasting) with an index of desirability of 0.537 and M1524 (high-altitude, 24h-germination and a medium level of roasting), with an index of desirability of 0.517.

Conclusions
Altitude of harvesting, germination time and roasting level have different impact in the volatile profile of coffee powder. Altitude of harvesting seems to be the factor contributing the less, while germination time and roasting level have a more important role in the development of aroma. The tentative identification of mass peaks identified by PTR-QMS and GC-MS allows the determination of desirable and undesirable odor and therefore the optimization of the three factors responsible for the volatile profile of coffee powder.

Novel Aspect
PTR-QMS and GC-MS has been used for the first time to evaluate the effect of altitude of harvesting, germination time and roasting level in the development of aroma profile in coffee powder. Moreover, it is the first time in which data from PTR-QMS and GC-MS has been used coupled with Design Expert® to optimize growing conditions and treatments of coffee beans to obtain a more desirable aroma in coffee powder.

References
980 - FEASIBILITY OF SELECTED ION FLOW TUBE MASS SPECTROMETRY (SIFT-MS) TO REAL-TIME MONITORING OF EXHALED BREATH VOLATILE ORGANIC COMPOUNDS (VOCS) IN CANCER PATIENTS.

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Keywords: Selected Ion flow tube mass spectrometry, Breath, Real-time analysis, Volatile organic compounds, Cancer.

Introduction: The VOCs in the exhaled breath represent promising biomarkers able to give useful information on the physio-pathological status of the individual [1]. Real-time breath analysis of VOCs is an attractive non-invasive approach for cancer disease diagnosis and monitoring. Here we report the feasibility study of the use of selected ion flow tube mass spectrometry (SIFT-MS) to measure targeted breath VOCs suitable to monitoring cancer patients in a clinical setting.

Methods: The analysis of 32 targeted VOCs has been performed by Voice 200Ultra™. The instrumental VOCs panel included: ammonia, acetone, isoprene, acetonitrile, dimethyl ether, aliphatic alcohols (n=5, C1-C5), cyanuric acid, low chain aliphatic acids and their corresponding aldehydes (n=12, C1-C6) including benzaldehyde, surfer ethers (n=5) and phenol compounds (n=3). The study involved (n=12) healthy controls and (n=17) metastatic cancer patients.

Results: Twenty two (72%) out of 32 VOCs targeted VOCs panel were detectable by SIFT-MS under the analytical conditions used. As compared to healthy group cancer patients present significative lower breath level of ammonia (p<0.001), acetone (p<0.05), carbon disulfide (p<0.01) and phenol (p<0.05) evaluated by t-test. Within the series investigated was possible to recognize qualitative and quantitative individual VOCs signatures for both cancer patients and healthy individuals. Specific VOCs signature associated with the disease progression was observed in a case of a patient with sarcoma.

Conclusions: The use of SIFT-MS in clinical setting is a feasible and appropriate approach for the qualitative quantitative analysis of a discrete number of exhaled VOCs. The study underlines the potential of the real-time VOCs profile analysis to identify specific patient pharmacological phenotype and supports the use of such breath metabolomics approach for longitudinal monitoring of cancer patients.

Novel Aspect: The SIFT-MS analytical instrumentation can be effectively applied for the real-time breath VOCs profile analysis in a clinical setting

References
Introduction
Vaccines are one of the most important products of biological processes which are generally less controllable and more susceptible to variation [1]. HS-SPME-GC-MS method, allows determination of biogenic volatile organic compounds (BVOCs) emerged from microorganism [2]. The identified volatiles can be considered as biomarkers for the identification and classification of organisms as well as for monitoring the bioreactor [3-5].

Methods
HS-SPME sampling was applied for extraction of VOCs, emerged from C. tetani in different culture media, followed by GC-MS analysis for identification and quantitation of BVOCs. Then, a portable dynamic air sampling (PDAS) device was constructed and used for sampling biomarker VOCs from the exhaust gas flow of bioreactor by the SPME fiber at the optimum condition. Other cultivation parameters such as pH and optical density were measured during the sampling time.

Results
About fifty volatile compounds released from C. tetani in different media were identified by the proposed method. The main detected compounds were sulfur compounds such as dimethyl trisulfide, dimethyl tetrathiol, and butanethioic acid S-methyl ester which specified as a biomarker for monitoring bacterial growth in a bioreactor. In the second step, sampling condition was optimized and the exhaust gas flow rate of 1 mLs⁻¹ and sampling time of SPME fiber in PDAS of 20 minutes were obtained. Sampling of bioreactor was performed 6 times per day during the incubation period. The results showed good correlation between conventionally control parameters of bioreactor and quantitated VOCs.

Conclusions
Dynamicsampling from off-gas of bioreactor by SPME fibers was used for sampling bacterial VOCs from bioreactor and analysis was done by GC-MS. Reliability of proposed method was performed using pH and optical density of the C. tetani cultivation as the conventional monitoring parameters. Results showed that, the proposed method can be used as a novel process monitoring method for microorganism cultivation.

Novel Aspect
HS-SPME followed by GC-MS method was developed for monitoring bacteria growth in bioreactor.

References
Introduction:
Aroma of dark chocolate depends on process and cocoa origin and variety. A sensory analysis of 206 standardized chocolates produced from various cocoa beans classified them in four sensory categories. These categories were confirmed in a PTR-MS analysis of the chocolates volatilome [1]. The objective here was to study the nosespace of a subset of chocolates simultaneously with their temporal profile to better explain the sensory categorization at a perception level.

Methods:
A Temporal Dominance of Sensations (TDS) evaluation of 8 chocolates (2 selected per category) was done in triplicate by 12 subjects while the aroma released in their nose were simultaneously collected and injected into a PTR-ToF-MS. The two sets of data were analyzed conjointly by defining an index of abundance of each detected aroma compound while a given attribute was dominant: the Abundance While Dominance (AWD) index [2].

Results:
TDS is able to dynamically capture multidimensionality of perception. The obtained TDS curves clearly differentiated the chocolates that were regrouped by sensory category as revealed by a principal component analysis (PCA). The dynamics of perception have been considered only scarcely in comparison to simultaneous in vivo aroma release over time measured by on-line mass spectrometry (nosespace). Computation of the AWD indices at individual level allowed to statistically assess the differences between the products over subjects and replicates and assessed statistically the relationships between the two sets of data. Through correspondence analyses (CA) some relationships between certain aroma compounds and the sensory attributes expected to be related to them were found. Although in previous studies pairing nosespace and TDS various temporal links could be proposed [3], no clear relationships could be safely established due to the fact that the conclusions were mainly based on a descriptive analysis of the data conducted at panel level.

Conclusions:
Pairing nosespace with Temporal Dominance of Sensations evaluation of dark chocolates categorized in four sensory groups provides meaningful data that can be analyzed at individual level thanks to the AWD indices. Descriptive multivariate analyses of these AWD indices gave interesting clues on the relationships between the aroma compounds released in mouth and their expected perceived sensory attributes.

Novel Aspect:
Pairing nosespace PTR-ToF-MS with Temporal Dominance of Sensations allows assessing the relationships between aroma compounds released in vivo and their perception.
References

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High-pressure photoionization time-of-flight mass spectrometry combined with dynamic purge-injection for rapid analysis of volatile metabolites in urine

Keywords: high-pressure photoionization mass spectrometry, dynamic purge-injection, urine analysis, volatile organic metabolites

Introduction:
Small molecule metabolites are widely used as biomarkers in the research field of metabolomics for disease diagnosis and exposure assessment. As a readily available biofluid containing plenty of volatile organic metabolites (VOMs), urine is ideal for non-invasive metabolomics analysis.[1] However, there is still lack of rapid analysis method for VOMs in urine.

Methods:
A dynamic purge-injection apparatus was designed by coupling of a thermostated water bath and a bubbling bottle. The urines were firstly acidized or alkalized by simply adding a small amount of H2SO4 or NaOH. The VOMs were released into the gaseous phase by bubbles bursting at the gas-liquid interface, which were taken into the high-pressure photoionization (HPPI) mass spectrometer for analysis.[2]

Results:
Various types of metabolites, such as ketones, alcohols, acids, sulfides, pyrroles and amines were detected directly by simple acidification or alkalization of urines. Nitrogen-containing compounds, especially polar amines, could be ultra-sensitively measured without any derivatization. Interestingly, a new biomarker 2,5-dimethylpyrrole was exclusively found in the smoker's urine sample besides toluene. Five valuable metabolites, trimethylamine, toluene, styrene, p-xylene, and 2,5-dimethylpyrrole, were well quantified with the limits of detection down to 0.02–0.1 μg L−1, and the relative standard deviation (R.S.D.) ranged from 0.42% to 14.4%. Being highly sensitive and humidity-friendly, the whole analytical procedure was easily operated in less than 6 min.

Conclusions:
The coupling of HPPI-TOFMS with dynamic purge-injection was demonstrated as a novel tool for rapid nontarget disease biomarkers screening or target monitoring of specific compounds through the investigation of volatile metabolites in urine. The highly sensitive and humidity-friendly system might aid in clinical diagnosis for development of diagnostic tools for disease identification.

Novel Aspect:
A powerful and sensitive direct mass spectrometric technique for high-throughput analysis of volatile organic metabolites in urine.

References:
3 - SPME-GC/MOX SENSOR SYSTEM: A NEW METHOD FOR THE EVALUATION OF HUMAN SEMEN QUALITY

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Keywords: volatile compounds, SPME, GC-MOX sensor system, human semen

Introduction:
The most used method for determining the volatile compounds of biological matrices is headspace solid-phase microextraction gas chromatography (HS-SPME-GC). Usually, the detector is mass spectrometer (MS) [1]. In this work, we propose a new technique based on the application of a MOX sensor [2], as additional detector after GC separation, to evaluate metabolome profile of human semen applied to infertility study.

Methods:
First, semen samples motility was assessed by standard semen analysis (SA). Next, volatile organic compounds (VOCs) of human semen were extracted by SPME technique and separated by gas chromatograph. The two detectors, MS and MOX sensor, work in parallel by a two-way splitter. The MOX sensor traces (resistance vs time) and physiological parameters of semen analysis were used for data analysis. Partial least-squares discriminant analysis (PLS-DA) was applied to the preprocessed sensorgrams to evaluate the ability of the GC/MOX sensor to discriminate sperm samples with different motility.

Results:
Seminograms allowed us to classify the 57 samples in two motility groups: 16 asthenozoospermic samples with sperm progressive motility <32% and 41 normozoospermic ones. Forty-nine total VOCs were detected by GC/MOX sensor system.

Overlapping of a chromatogram and the corresponding sensorgram (as function of run time) for each sample group showed that background contamination, such as column bleed, is not perceived by VOC sensor.

2D- and 3D- PLS-DA results obtained from sensor data were compared with data obtained by seminograms, considering 2 groups (asthenozoospermic and high motility samples). 2D-PLS-DA based on SA showed an accuracy of 85% in the discrimination in two groups. In three dimensions, accuracy increases up to 92%. Using exclusively sensor resistance profiles, accuracy is of 77% (in 2D) and 78% (in 3D) for discrimination in asthenozoospermic and normozoospermic samples.

VOC sensor has different affinity to organic compound classes, having a higher reactivity with aldehydes, ketones and acetamides.

Conclusions:
GC/MOX sensor system allowed us to develop a reproducible and novel method to detect human semen headspace VOCs. We have shown that the never-explored seminal VOC fingerprinting is a valid basis for discriminating samples with different motility. PLS-DA based on VOCs analysis by sensorgrams provided good classification between asthenozoospermic from normozoospermic samples, compared to PLS-DA based on sperm motility measured by semen.

Novel Aspect:
Setup of a dual system of volatile compound detection that allow to outline resistance profile that can discriminate asthenozoospermic from normozoospermic samples.

References
METHODOLOGY FOR QUALITATIVE AND QUANTITATIVE ANALYSIS OF VOLATILE COMPOUNDS FROM COMPOSITE MATERIALS AT ELEVATED TEMPERATURES

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Keywords: Volatile organic compounds, composites, gas sensors, TD-GC-MS.

Introduction:
The increasing use of composite materials on the aircraft structure led to the need to investigate their performance under on-board operating conditions and non-standard conditions such as the onset of fire [1]. The investigation of possible effects on the on-board air quality arising from the introduction of new composite materials on the aircraft structure, particularly at elevated temperatures where Volatile Organic Compounds (VOCs) may be released is evaluated here.

Methods:
The described methodology allowed the characterisation of composite materials inside an air-tight tube furnace operated up to 250°C. The real-time detection of released gases was accomplished using commercial off-the-shelf (COTS) gas sensors, in addition, stainless steel thermal desorption (TD) tubes were used and analysed by gas chromatography-mass spectrometry (GC-MS) combined with thermal desorption (TD-GC-MS) for both qualitative and quantitative analysis.

Results:
Real-time detection of released gases (CO, NO, SO2, NO2, CO2, O2) and volatiles was performed over a temperature ramp from 22 °C with dwell points at 70 °C, 150 °C, 200 °C and 250°C. The volatile thermal degradation products yielded by the chemical breakdown of carbon fibrebased epoxy composite at the glass transition temperature were measured, where a mixture of aromatic compounds and unsaturated compounds were identified and quantified.

Conclusions:
The sensors have demonstrated a good performance to real-time detection of gases, although cross-response of sensors to a range of volatiles might occur. It is important to accurately determine the characteristics of these compounds in aircraft cabins, and modelling is needed to link the emission rate in such tests with a possible concentration in the cabin.

Novel Aspect:
A methodology combining real-time data information and GC-MS data was set to investigate further on potential emissions of volatiles. To the authors knowledge, this work has not been previously reported.

References

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Introduction:
Increasing evidence suggests that the host-gut microbiota interactions and gut microbiota composition impacts strongly on the metabolic phenotypes of the host, although the underlying mechanisms are poorly understood [1]. Dysbiosis of the gut bacteria influences the production of the volatile fermentation products resulting on an altered metabolome and contributing to the onset of metabolic diseases [2].

Methods:
This research aimed to investigate longitudinal changes in volatile organic compounds (VOCs) present in the faecal headspace of Cushing’s syndrome mouse model of type 2 diabetes and once compared to their wild-type littermates. The volatile faecal metabolome was analysed using Selected Ion Flow Tube Mass Spectrometry (SIFT-MS) and Gas Chromatography-Mass Spectrometry (GC-MS) combined with thermal desorption (TD).

Results:
Univariate and multivariate data analysis revealed statistically significant differences in volatile metabolites between wild-type littermates and diabetic animals. Cushing’s mice developed obesity and showed significantly higher blood glucose concentrations and plasma insulin concentrations, supporting the hypothesis that Cushing’s mice develop a diabetic phenotype related to a gut microbiota interaction.

Conclusions:
The gut microbiota of Cushing’s diabetic mice seem to have a different composition once compared to wild-type littermates, and the statistical analysis revealed statistically significant differences in volatile faecal metabolites. Overall, the predictive model provided information on whether the volatile metabolome is able to discriminate groups according to the phenotype.

Novel Aspect:
The use of animal models can aid in the interpretation of metabolic pathways under controlled conditions. This work has never been done before.

References
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INVESTIGATIONS OF VOCS PRESENT IN BREATH USING PTR-MS: PRELIMINARY STUDIES OF KETONES AND ANAESTHETICS

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Keywords: breath, volatiles, proton transfer reaction mass spectrometry

Introduction:
The need for a non-invasive clinical diagnostics has grown over the past years. Breath research is gaining an important role in this field. More than 500 volatile organic compounds (VOCs) in breath can provide us with a wide variety of biomarkers. Most of these compounds are present in very low concentrations (sub parts per billion), therefore finding a sensitive instrument for analysing breath samples is crucial.

Methods:
Proton transfer reaction mass spectrometry (PTR-MS) has proven its qualities in many research fields including breath research.1,2 Its high sensitivity for VOCs, easy or usually no sample preparation and if used correctly, high selectivity, provide us with a strong analytical tool. Although PTR-MS usually uses H3O+ as a reagent ion (RI), it can also be used in modes to produce different RIs, e.g. O2+ and NO+, which react with VOCs of interest providing the reactions are exothermic.3

Results:
The investigation with the ketones is a major collaborative project involving 3 ESRs from 3 organisations. We measured and analysed fragmentation patterns while changing the reduced electric field for 21 compounds.4 A fast GC was coupled to the PTR-MS in order to separate isobaric compounds, which can have often completely different origins in the human body and therefore can be found in the breath. Some of these substances were measured also with different RIs and different humidity conditions, to provide us with an even wider database to allow ease of identification under different conditions.

In addition to investigating endogenous breath compounds, we have begun to investigate important exogenous compounds in breath. To date we have focused on the anaesthetics, isoflurane, sevoflurane, desflurane and enfurane. Initial studies have been undertaken to determine the product ions following reaction with H3O+, in order to determine which ions should be monitored. Detailed studies of product ions as a function of reduced electric field will be presented.

Conclusions:
We demonstrate that changing the reduced electric field aids in the identification of VOCs. This pre-study has generated a useful database of fragment product ions resulting from the ketones and anaesthetics for various reagent ions and under various conditions, which will be presented.

Novel Aspect:
In our work we are aiming to create a database of VOCs present in breath and their reactions with RIs, the product ions of which could serve as biomarkers for diseases.

References:
Sake is a Japanese national liquor, and many kinds are brewed for different flavor notes such as fruits, flowers, herbs, and spices. Additionally, sake can be served in a variety of vessels (cups): a choko, a Masu, a sakazuki, and a wineglass, allowing one to enjoy different scents, depending on the cup. The purpose of this work is to visualize the differences of sake fragrance depending on cups.

Hakutsuru Daiginjo (sake) and cups (Choko, Daichoko, wineglass, and Champagne glass) were used. Analytical system was composed of compact (Bruker) mass spec equipped with DART ion source (IonSense) and Volatimeship introducing device for volatile compounds (BioChromato). Data acquisition via the mass spectrometer were began, the cup added in sake were placed at the introducing tube of the Volatimeship, where the position of human nose, when the human is drinking.

Ethyl caproate (i.e., ethyl hexanoate) provides one of the characteristic fragrances of sake. As the cup of sake was placed at the introducing tube of the Volatimeship, ethyl caproate was immediately detected by mass spectrometer. The volatilization behavior of ethyl caproate for each cup were measured. The wineglass not only showed higher intensity at the moment of placing the cup, but it also showed better sustainability. These analysis results supported the results of organoleptic evaluation. Moreover, the detected compounds from sake was different depend on cups. As for wineglass and champagne glass, there were some compounds that were not detected from Choko. These analysis results also supported the results of organoleptic evaluation, too.

An introducing device for volatile compounds combined with DART-MS enabled continuous detection of the change in fragrance intensity directly from the sake vessel. Therefore, this method can be useful for objective evaluation of the duration and change of scent. Additionally, this method can be useful for visualizing the sensory evaluation, because it enabled visualization of the varying intensity, or quality, of the fragrance of sake depend on cups.

An introducing device for volatile compounds combined with DART-MS enables objectively evaluation the sensual sensation of fragrance.
1366 - EXPLORING INTER-INDIVIDUAL DIFFERENCES IN IN-VIVO FLAVOUR RELEASE BY SIFT-MS

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Keywords: nose-space analysis, in vivo flavor analysis, SIFT-MS, population screening

Introduction: (Limit of 400 characters)
Selected-Ion Flow-Tube Mass Spectrometry (SIFT-MS) allows on line accurate quantification of several volatile organic compounds down to the ppb range [1]. The technique has been applied to different cases of breath analysis [2]–[5] but to the authors knowledge it has not been applied to monitor in nose flavor release during food consumption for a large population.
In this work we describe a SIFT-MS based approach to measure in-vivo nose-space VOCs concentration during consumption of strawberry flavored candies on a large healthy population with the aim to investigate the inter-individual variability in flavor release: nose space analysis might disclose new information regarding the interaction between products and consumers [6].

Methods: (Limit of 400 characters)
In this study the effect of gender, age and BMI on flavor release was investigated. Ninety-two volunteers (60.9 % female) between 22 to 68 years old (mean= 40.9; SD= 11.9) participated in the study. Each subject, after filling in a socio-demographic questionnaire, consumed a strawberry candy following a specific bite-based procedure supported by a video tool. Simultaneously nose-space analysis with a SIFT-MS machine was carried out (SYFT VOICE 200 ultra, Syft Ltd, New Zealand). Each subject performed at least 3 replicates of the same sample.

Results: (Limit 900 characters)
With the set up used it was possible to monitor in real time 7 different aroma compounds released by the candy (ethyl maltol, 3-hexen-1-ol, ethyl 2-methylbutanoate, (Z)-3-hexenyl acetate, ethyl butanoate, ethyl hexanoate, 2-methylbutanoic acid) and acetone as marker of the participant breathing cycle. Principal Component Analysis (PCA) showed a possible effect of age and BMI on the flavor release signals. Both age and BMI were found to have a slightly negative correlation with in-nose aroma release for three of the monitored compounds (Pearson correlation, p<0.05). Significant differences were found in hexenyl acetate and acetone levels (Welch t-test p<0.01) between males and females: in both cases females presented higher in-nose levels. The study is part of a broader investigation that aims to evaluate the Italian olfactory function (COLFIT PROJECT) and here some preliminary results of the explorative data analysis are presented.
Conclusions (Limit of 400 characters)

Real time mass spectrometry analysis by SIFT-MS can detect and monitor in-vivo volatile organic compounds release during candies consumption. For the first time the analysis was applied to a large sample of the population with the aim to characterize how physiological factors like age, gender, BMI and eating behaviors affect in nose aroma release. The results suggest an effect of age on aroma release and an effect of BMI that might be used to study some aspects underlying the retronasal perception mechanisms in more detail.

Novel Aspect: (Limit of 150 characters)

Real time direct injection mass spectrometric analysis through SIFT-MS is a powerful tool to realize population screening and investigate flavor release mechanisms.

References


For information please contact: scientific@imsc2018.it
Introduction: (Limit of 400 characters)
Proton Transfer Reaction Mass Spectrometry has been described has particularly suited to provide “in vivo” on-line flavor monitoring during food consumption [1]. In the last years, the technique has been applied to different food matrixes [2-4]. Due to analysis rapidity and high sensitivity, the technique has become a reference tool for the investigation of the complex phenomena related to flavor perception [5].

In this work we describe a PTR-MS based approach to measure in-vivo nose-space VOCs concentration during chewing gum consumption coupled to discontinuous time intensity sensory evaluation.

Methods: (Limit of 400 characters)
Two different studies were conducted on chewing gums to monitor respectively differences in aroma release due to different gum ingredients formulations and the effect of physiological parameters (oral cavity volume, salivary flow and papillary count), gender and ethnicity in flavor perception. Sensory evaluation on flavor intensity and sweetness was performed simultaneously with nose-space analysis with two different PTR-MS machines (PTR-ToF-MS and PTR-QiTof-MS, Ionicon Analytik, Austria). A total of 40 panelists participated in the two studies.

Results: (Limit 900 characters)
In the first study, a significant difference in total aroma release (p.value < 0.05 ANOVA) was observed between the different chewing gum formulations. In particular, it was observed that these differences were compound dependent and that PTR-MS signals are associated to a high variability reflecting panelists’ physiological differences.

In the second study, ethnicity was found to have a significant effect on both in nose-space concentration and sensory perception. For different mass peaks associated to mint flavor compounds, Chinese panelists exhibited higher levels than European ones (p.value < 0.05 Welch’s t-test) generally after 90 seconds of consumption and after the gum was removed from the mouth. The same trend was found in sensory perception both for flavor and sweetness attributes.

Conclusions (Limit of 400 characters)
Real time mass spectrometry analysis by PTR-MS can detect and monitor in-vivo volatile organic compounds release during food consumption.

The results of the studies suggest a multimodal effect of aroma release on both flavour and sweetness intensity. Individual differences due to physiological, biochemical and physicochemical phenomena may have a relevant effect in aroma perception and thus in sensory flavor perception. Although, it cannot be excluded a cultural bias due to an ethnocentric experimental environment, result suggest that higher in-nose concentration in Chinese judges may explain their higher flavor and sweetness perception.

Novel Aspect: (Limit of 150 characters)
Real time direct injection mass spectrometric analysis coupled with dynamic sensory methods is a powerful tool to investigate flavor perception mechanisms and highlight physiological and cultural biases.

References


For information please contact: scientific@imsc2018.it
Wastewater-based epidemiology: Use of Adulterants to Estimate Cocaine Consumption Patterns in Brasília, Brazil by LC-QTOF-MS

Katyeny M. da Silva; Adriano O. Maldaner; Fernando F. Sodré

Keywords: wastewaterepidemiology, drugs of abuse, forensic toxicology, cocaine, adulterants

Introduction: Illicit drugs are a major public safety problem in Brazil. Findings of the Brazilian Federal Police concerning drug seizures show that levamisole (LEV) is the main adulterant in cocaine hydrochloride (mean of 13.2%), while phenacetin (PHE) is the mainly found in freebase cocaine – crack (mean of 15.2%). Thus, strategies based on quantification of LEV and PHE, together with the major cocaine metabolite, benzoylecgonine (BE), in wastewater may contribute to identify different forms of the consumed cocaine.

Methods: Composite (24 h) raw sewage samples were collected at the entry of the two main wastewater treatment plants (NW-WWTP and SW-WWTP) in the Brazilian Capital, Brasília. Aliquots of 50 mL (pH 2) were passed through solid phase extraction cartridges (StrataX) previously conditioned with MeOH:ACN (60:40) and H2O (pH 2). Analytes were recovered with 6 mL of MeOH, the extracts were spiked with isotopically labeled standards (BE-d3 and COC-d3) and concentrated to 0.2 mL. Analyses were carried out using a liquid chromatography system coupled to a quadrupole-time-of-flight mass spectrometry (LC-QTOF).

Results: Results revealed a cocaine consumption varying from 750±120 to 2470±410 mg/day/1000inhab in weekdays and 2340±1190 to 4580±750 mg/day/1000inhab in weekends. In terms of load, considering cocaine purity in Brazilian seizures (49 ± 29 %), we estimate an annual consumption of 2 tons of cocaine, in the region. PHE/LEV ratios found in seized cocaine street samples varies from 0.18 in hydrochloride cocaine to 15.2 in freebase samples, indicating the prevalence of crack consumption as PHE/LEV ratios becomes higher. Relationships between both adulterants in the samples evidenced a higher use of crack cocaine in the NW-WWTP region (PHE/LEV = 3.0±1.5) compared to SW-WWTP. The PHE/LEV ratio was the same in the SW-WWTP region (PHE/LEV = 1.5 ± 1.0) when compared to 2014 data, while the ratio for NW-WWTP region doubled suggesting an increase of crack users in this region.

Conclusions: The LC-QTOF-MS system allows the identification based on accurate mass measurements and quantification with multiple reaction monitoring. Regarding the forensic field, it is very important to know how a drug is consumed. The use of adulterants, such as PHE and LEV may be useful to provide novel information concerning cocaine consumption patterns and poliuse of drugs.

Novel Aspect: Alternative approach to differentiate crack and hydrochloride cocaine consumption based on the quantification of adulterants in wastewater samples.

References
218 - DUAL MICROPROBE CE-ESI-HRMS FOR THE CHARACTERIZATION OF PROTEINS AND METABOLITES IN SINGLE EMBRYONIC CELLS OF LIVE FROG EMBRYOS

Camille Lombard-Banek (1) - Rosemary Onjiko (2) - Sally Moody (3) - Peter Nemes (1)

University of Maryland, Chemistry & Biochemistry, College Park (1) - University of Maryland, Chemistry and biochemistry, College Park (2) - The George Washington University, Anatomy and Regenerative Biology, Washington (3)

Keywords: Single-cell, CE-ESI-HRMS, Systems biology, Proteomics, Metabolomics

Introduction:
Establishment of cell-to-cell heterogeneity is critical to the normal formation of different tissues in vertebrates. A system biology understanding of this heterogeneity with single cell resolution could provide important insights on how cell fate is established. We thereby developed a dual-microprobe sampling approach hyphenated to CE-ESI-HRMS to measure proteins and metabolites from the same identified single embryonic cells in the 8-cell frog embryo.

Methods:
A 10 nL volume was withdrawn from identified cells of the 8-cell X. laevis embryo using capillary microsampling. Metabolites were extracted in 40% acetonitrile/40% methanol solution. Proteins were delivered into 50 mM ammonium bicarbonate and trypsin-digested. All extracts were measured using our custom-built CE-ESI-HRMS platforms. Metabolites were quantified by using peak-area under the curve as a proxy of relative concentration. Proteins were quantified using MaxLFQ approach integrated in MaxQuant.

Results:
To advance the systems biology understanding of cell heterogeneity during embryonic development, we developed dual-microprobe sampling to collect two different portions of the cytoplasm from the same cell to measure metabolites and proteins. We used this approach on the D1 and V1 cells in the same 8-cell embryo. These cells are situated across the dorsal/ventral axis of the embryo. The approach is based on our previous microprobe-CE-ESI-HRMS protocol, whereby an ~10 nL volume of the cell cytoplasm is extracted, leaving the embryo essentially intact.1-3 We consistently quantified 335 proteins and 150 metabolite features. The different cells separated using PLSDA analysis based on protein and metabolites profiles. Overall, 6 proteins and 5 metabolite features were differentially expressed. Using these quantitative data, we performed pathway enrichment analysis. We identified the arginine and proline metabolism pathway as one of the most statistically significantly enriched, which suggests its importance in early embryonic development.

Conclusions:
We have developed a dual sampling approach enabling the analysis of proteins and metabolites from the same cell. The collected data represent the first example of proteomic and metabolomic analyses from the same single-cell in a live embryo, providing new information on cell heterogeneity in the 8-cell frog embryo. Moreover, the technology promises to deepen our understanding of the mechanisms involved in embryo patterning during embryonic development.

Novel Aspect:
Dual microprobe-CE-ESI-HRMS enabled proteomic and metabolomic analyses in the same single-cell of live frog embryos.

References:
Lombard-Banek, C.; Moody, S. A.; Manzini, M. C.; Nemes, P., Submitted

For information please contact: scientific@imsc2018.it
Title: Histology-Directed Proteomics: Combining Autofluorescence Guided Micro-Digestions with Liquid Surface Extractions

Authors: Daniel Ryan, Heath Patterson, Jessica Moore, Jeffrey Spraggins, Richard Caprioli

Keywords: Autofluorescence, LESA, Bottom-Up Proteomics

Introduction: (Character Count 400)
Liquid extraction surface analysis (LESA) has proven to be an effective approach for generating spatially-targeted, proteomics data from tissue.1,2 Current approaches enzymatically digest the whole tissue surface prior to extraction. When foci are smaller than the LESA spot size, signal from the area of interest is diluted. Herein, we demonstrate proteomic analyses of foci using histology-directed micro-digestions guided by autofluorescence (AF) microscopy.

Methods: (Character Count: 400)
Rat kidney tissue was sectioned and mounted on microscope slides. AF (fluorescence of endogenous molecules) microscopy images of a section were used to guide trypsin spotting onto foci of interest using a piezoelectric spotter. LESA samples from the individually digested foci were pooled and analyzed by LC-MS/MS on a Q Exactive Orbitrap mass spectrometer. Adjacent sections were digested using robotically sprayed trypsin to compare to traditional LESA approaches.

Results: (Character Count: 899)
Proof-of-concept experiments were completed using LESA to extract from undigested control sections, sections that were robotically sprayed with a homogenous coating of trypsin, and sections with trypsin spotted on foci guided by autofluorescence microscopy. The control experiments resulted in ~10 protein IDs per extract. Robotically sprayed tissue sections contained on average 250 protein identifications per mm2 sampled by LESA. The robotic spraying approach also suffers from some analyte delocalization during the spray process, as monitored by AF microscopy of the tissue following digestion. This approach is poorly suited for targeting foci smaller than the size of the LESA extraction. LESA samples from micro-digested kidney using AF-guided trypsin spotting provided upwards of 1500 protein ID’s per mm2 sampled by LESA, a 5-fold increase compared to the robotic spray method. The diameter of the trypsin spots approach 100 µm at its smallest, providing a 3-4 fold improvement of spatial resolution when compared to extracting via LESA alone.

Conclusions: (Character Count: 286)
LESA is limited to relatively large droplet diameters and is inadequate for studying small, discrete foci (< 400 µm) often of interest in biological tissues. The combination of tryptic micro-digestions guided by AF microscopy and LESA circumvents LESA’s spot size limitations by only producing digested peptides from the targeted foci.

Novel Aspect: (Character Count: 126)
Autofluorescence-guided tryptic micro-digestions combined with LESA allows for targeted proteomic analysis of small biological foci in tissue.
References
60 - COMBINING BIO-CONJUGATION AND MATRIX-ASSISTED LASER DESORPTION IONISATION MASS SPECTROMETRY FOR BIOMOLECULE DETECTION AND LOCALISATION IN TISSUES

Katherine Stevens (1) - Parul Mittal (2) - Andrew Abell (1) - Tara Pukala (1)

University of Adelaide, Physical Sciences, Adelaide (1) - University of Adelaide, Biological Sciences, Adelaide (2)

Keywords: MALDI, imaging, glycoprotein, cross-linking, bioconjugation

Introduction:
Membrane proteins are central nodes in the networks that allow cells to receive and transmit signals between themselves and their environment. These proteins comprise 30% of the proteome and their diversity is enhanced by post-translational modifications, such as glycosylation [1]. These proteins are involved in a range of diseases, including various cancers and inflammatory diseases [2,3].

Methods:
We have developed a matrix-assisted laser desorption ionisation (MALDI)-mass spectrometry (MS) approach that combines cross-linkers capable of targeting intact proteins within tissues, to enable their detection via MALDI imaging. This approach uses a novel photocleavable cross-linker that is released by excitation with a MALDI laser to produce a signature mass signal in the resulting spectra.

Results:
We have demonstrated a simple and versatile synthetic route for developing MALDI imaging cross-linkers, which contain a photocleavable moiety that is released by excitation using a MALDI laser and produces a signature mass signal in the resulting spectra. The mass signal can be easily modified as the design allows for a one-step addition of peptides of various lengths at the site of photocleavage within the cross-linker. Furthermore, the specificity of this probe can be modified to enable detection of different target biomolecules. The practical application of these cross-linkers has been confirmed by MALDI and electrospray ionisation (ESI)-MS experiments. We have found using MALDI experiments that we are able to detect the desired mass signal, which corresponds to the release of the expected photocleavage product.

Conclusions:
Based on our initial findings, we aim to apply this method for the detection of multiple glycoprotein targets simultaneously in tissue sections, as a potential new approach to cancer diagnosis. Additional ongoing research is focussed on optimising this technique for glycoprotein detection and developing similar approaches for targeting DNA oligos and glycan-binding proteins within tissues.

Novel Aspect:
This approach provides a method for detecting intact biomolecules using MALDI-MS. It has the potential for multiplexing by modifying the length of the peptide mass signal.

References:

For information please contact: scientific@imsc2018.it
Keywords: Target plate material, MALDI, anion formation

Introduction:
In MALDI mass spectrometry, initial charge separation and charge transfer processes remain elusive. To improve analyte anion signal intensity, enhance sensitivity, and gain more insight in the roles of the target plate material and matrix in MALDI anion formation, a systematic study was carried out. The influence of 21 different metals and alloy substrates, and a range of matrices, in the absence and presence of diphenyl phosphate (DPP), on anion yield, was studied.

Methods:
Molecular anion signal intensities were systematically analyzed and compared by varying laser fluence for substrates with varying melting points, thermal conductivity, electrical resistivity and work function. A comparison of DPP molecular anion signal intensity was made, based on the matrix UV-absorption (λ: 337.1 nm), electron affinity and crystallization. As many factors as possible were considered to explain observed anion signal intensity differences.

Results:
Metals with oxide layers that, upon surface ablation, show an intrinsically high anion formation in absence of a deposited sample, provide the highest DPP anion signal intensity. Using Ag as target plate material generally increases the analyte signal intensity 10-fold relative to the conventionally used stainless steel. Metals with high melting points (such as Cr, W, Mo, Ta) tend to underperform. For most metals, the homogeneously deposited fullerene-C60 performed best as a matrix, though anthraquinone and 9-cyanoanthracene performed best on Ag at the highest tested laser fluence.

Conclusions:
We conclude that when matrix and target plate material are chosen carefully, this can significantly enhance the ion signal intensity of an added analyte in MALDI. Hence, the commonly used stainless-steel target plates should be reconsidered. The target plate material acting as a catalyst, resulting in different anion signal intensities, should be considered, along with the potential of MALDI to predict catalysts for (photo-)chemical reactions.

Novel Aspect:
Target plate material strongly affects the outcome of the ionization process and a proper choice enhances ion yields significantly.
PLENARY LECTURE

1411 - LITHOGRAPHICALLY PATTERNED ELECTRODES FOR MINIATURIZED ION TRAP MASS SPECTROMETERS AND OTHER ION OPTICS DEVICES

Daniel E. Austin (1) - Stephen A. Lammert (1) - Aaron R. Hawkins (1) - Yuan Tian (1) - Ailin Li (1) - Trevor Decker (2) - Brett Hansen (1) - Qinghao Wu (1)

Brigham Young University, Department of Chemistry and Biochemistry and Department of Electrical and Computer Engineering (1) - Brigham Young University, Department of Chemistry and Biochemistry and Department of Electrical and Computer Engineering (2)

Lithographically Patterned Electrodes for Miniaturized Ion Trap Mass Spectrometers and Other Ion Optics Devices

Keywords: microfabrication, ion trap, planar electrode, charge detection

Introduction:
All mass and mobility analyzers rely on carefully shaped electric fields (sometimes combined with magnetic fields) to separate ions. These electric fields are traditionally established using shaped metal electrodes. We have demonstrated several types of devices in which electrodes are patterned onto circuitboard or onto rigid substrates (ceramic, glass). Advantages include miniaturization, novel devices, and complex electrode arrangements.

Methods:
We have developed quadrupole and linear ion traps, concentric dual ion traps, multi-stage image-charge detectors, charge detection mass analyzers, and electrostatic ion beam traps. Recent studies have focused on substrate misalignment effects, modeling the electric field, and microparticle analysis.

Results:
We have demonstrated the operation of a sub-mm linear ion trap with better than unit mass resolution for small organic molecules. We have also observed the effect of misalignment between electrodes in several degrees of freedom (corresponding to the possible orientations of the substrates), and compared the measurements to simulations of misalignment. Using angled electrodes printed on circuitboard material, we can measure the trajectory of microparticles passing through a vacuum system, such as the divergence from an aerodynamic lens or microparticle-surface impact and rebound. We are also developing a microparticle analyzer (mass, charge) for a future mission to study aerosols on Mars.

Conclusions:
These devices demonstrate the utility and flexibility of creating complex and miniaturized devices for ion analysis using electrode printing on substrates. Electrode alignment, which is critical for many applications, is easily explored using alignment between plates. Charge detection with printed electrodes has the same sensitivity as conventional metal electrodes.

Novel Aspect:
Novel approach to making ion optics devices including ion traps and charge detection analyzers. First study of misalignment effects in ion traps in multiple degrees of freedom.
MALDI Imaging Mass Spectrometry (IMS) has been employed in studies of a wide variety of biologically and medically relevant research projects. This technology has seen significant advances in both the instrumentation and in the fields of basic biological investigations and medically relevant applications including patient biopsies. Several of these applications will be presented in this presentation. In addition, new technological advances will be described, including sample preparation and instrumental performance to achieve images at high spatial resolution (1-5 microns) and at high speeds in order to minimize image acquisition time. Instrumentation improvements include those developed for both MALDI FTICR MS and MALDI TOF mass spectrometers and will be demonstrated by specific applications. Finally, new biocomputational approaches will be discussed that deals with the high data dimensionality of IMS, image registration with microscopy, and our implementation of ‘image fusion’ in terms of predictive integration of MS images with other imaging modalities.
The creation of a work of art is a process often depicted as untouched by material constraints. Pure, unfettered inspiration, without the limitations imposed by physics. It should be evident, however, that only with materials having defined physical properties a certain esthetic effect can be obtained. Works of art are complex technological objects: their appearance and their performance over time are the results of a careful selection of materials and techniques. Thus, artists’ choices can be interpreted not just in the classical framework of art history, but in a holistic approach encompassing esthetics and technology. In absence of direct testimony from their often anonymous creators, masterpieces of the past become key material evidence in our efforts to retrace the working practices of artists, the technical knowledge and esthetic preferences of ancient cultures, and the complex trade patterns linking distant empires.

The quest for color, for beauty is a fundamental constant in human history, as well as an economic engine, and an inspiration for art and technology alike. While we easily recognize that the desire to produce richer and more lively images is a clear driver to seek new materials, on the other side of the world, in mines or forests, or in the crucibles of the alchemist (or of his modern counterpart, the chemist), we often overlook how new materials lead to new esthetics.

Scientific investigations have expanded significantly our understanding of works of art, often upending traditional interpretations. A typical example is the rediscovery and investigation of the original polychromy of ancient Greek and Roman sculpture, once a contested topic and now an established research field. Many more cases highlight the importance of the work of cultural heritage scientists, not only in investigating works of art, but also in developing the techniques that make these studies possible. Examples include the discovery of the earliest organic pigment, in a 4,000 years old Egyptian leather fragment; new insights into the global trade of dyes in the Middle Ages; and closer to us, the introduction of synthetic pigments and dyes in the 19th centuries, from the craze for mauveine to Van Gogh’s unfortunate passion for geranium lakes, passing by Hokusai’s experiments with Prussian blue in the Thirty-six views of Mount Fuji, and the color revolution prompted by the arrival into Japan of the aniline dyes in the Meiji period.

Using advanced analytical chemistry, from x-ray fluorescence spectroscopy to Raman and surface-enhanced Raman spectroscopy, from electron microscopy to mass spectrometry, we can build a material history of art based on physical evidence gathered from masterpieces spanning four millennia, from ancient Egypt to Modernity, demonstrating that technological developments were not only readily embraced, and often prompted by artists and their audiences, but also that they in turn created new forms of expression, in a continuous dialogue between materials and meanings.
The ultimate goal in heterogeneous catalysis is to make use of each and every atom of supported (metal) catalysts, i.e. in the extreme to perform single-atom catalysis (SAC). While this arduous task constitutes a non-trivial, if not daunting challenge in ‘real-life’ chemistry, in the gas phase SAC can be achieved in a rather straightforward manner by conducting experiments with mass-selected species under (near) single-collision conditions. These mass spectrometry-based studies on isolated reactants, when complemented by state-of-the-art computational and spectroscopic work, provide an ideal arena for probing the energetics and kinetics of a chemical process in an unperturbed environment at a strictly molecular level without being obscured by ill-defined side effects. Thus, the concept of SAC can be explored or, more generally, the mechanisms of reactions and the active parts of single-site catalysts, the so-called ‘aristocratic’ atoms, can be identified.

Examples discussed include:
The room-temperature, cluster-oxide mediated redox reactions of the CO/N2O couple which, arguably constitute one of the most prominent oxidation processes, and novel metal-mediated C=H bond activation and C=C coupling of methane, which are regarded as one of the holy grails in chemistry.

References:
Introduction:
Proteins are the workhorses of the cell. They are the molecules that catalyze the reactions for the metabolic and catabolic activities of the cell, transmit signals and transport necessary molecules and metabolites. Proteins operate in systems, which can be pathways, organelles, cells or even tissues. The development of proteomic technologies allows the study of proteins in the context of cellular systems. This lecture will trace the development of some of the methods of proteomics and demonstrate their use to understand mechanisms underlying the disease Cystic Fibrosis.
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