ABSTRACTS INVITED SPEAKERS

Wolfgang Paul Lecture

Unraveling the Ocean's Influence on Climate and Health: Insights from Field and Laboratory Studies

Kimberly Prather

Director, NSF Center for Aerosol Impacts on Chemistry of the Environment.
Distinguished Chair in Atmospheric Chemistry, Scripps Institution of Oceanography,
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This presentation reveals insights gleaned from innovative laboratory experiments conducted at the NSF Center for Aerosol Impacts on Chemistry of the Environment (CAICE). Emphasizing coastal environments, scientists are exploring the composition, cloud-forming abilities, and ice nucleation properties of marine aerosols.

Using the Scripps Ocean-Atmosphere Research Simulator (SOARS), we investigate ocean-atmosphere exchange dynamics under varying conditions with control of winds, waves, microbiology, and temperature. Additionally, we are performing studies to better understand the transfer mechanisms involved aerosolization of bacteria, viruses, and gases from the ocean to the atmosphere.

This presentation will discuss the implications of human-induced environmental changes on human health, ecosystems, and climate. As we confront unprecedented warming, this presentation underscores the urgency of comprehensively understanding ocean-atmosphere interactions for effective climate mitigation strategies and public health policies..

Plenary Lecture II

Exploring Microscale Ecological processes: Insights from NanoSIMS

Ingrid Kögel-Knabner

Research Department Life Science Systems and Institute of Advanced Study (TUM-IAS), Freising, Germany

Nano-scale secondary ion mass spectrometry (NanoSIMS) offers an unprecedented tool to support classical quantitative measurements (e.g. elemental composition, isotopic ratio) by high resolution imaging of isotope and elemental distributions. NanoSIMS provides combined structural information with chemical composition as well as elemental and isotopic information. By allowing researchers to visualize isotopically labeled compounds within microscale hotspots, NanoSIMS facilitates the study of intact soil or plant structures and their individual components, including iron (hydr)oxides, clay minerals, bacteria, plant residues, or roots, with a remarkable resolution of approximately 100 nanometers. The versatility of NanoSIMS makes it a valuable asset in elucidating complex processes, particularly at interfaces such as the root-soil interface. By directly imaging processes at the relevant scale and identifying the key players involved, NanoSIMS supports and validates conceptual ideas in various fields of research. It enables, e.g., the tracing of complex plant-soil-microbe interactions, allowing for the resolution of processes at the microscale level. The presentation includes an overview of the technical fundamentals of NanoSIMS, followed by a brief discussion on sample preparation techniques. The main focus of the presentation will then shift to several exemplary studies, showcasing the potential of NanoSIMS in studying soil and plant microenvironments. These studies will highlight how NanoSIMS contributes to unraveling complex ecological processes and understanding the dynamics within intricate biological systems.

Plenary Lecture III

Nicole Strittmatter

Department of Bioscience, Technical University of Munich, Germany

Mass Spectrometry Imaging (MSI) is a powerful technique to characterise complex biological samples such as tissues or plant material, enabling the mapping of hundreds of chemical constituents simultaneously. In the Strittmater lab, we are using MSI predominantly to map changes in the metabolome of biomedical specimen ranging from organoids to preclinical and clinical tissue specimen using Desorption Electrospray Ionisation (DESI) MSI, an ambient technique operating under atmospheric conditions and enabling analysis without prior sample preparation. This makes DESI-MSI particularly suited for deployment in multimodal imaging studies, such as in combination with imaging mass cytometry, which allows the targeted, multiplexed detection of proteins. In this talk, I will highlight some recent applications that cover some of our dominant research interests such as host-microbe interactions, tumour biology and anti-tumour drug disposition and metabolism.

Session 2: Element and Imaging Mass Spectrometry

Plant Metal Homeostasis – Maintenance and Naturally Selected Variation

Ute Kraemer

Lehrstuhl für Molekulargenetik und Physiologie der Pflanzen, Ruhr-Universität Bochum, GErmany

Land plants meet the challenge of acquiring all mineral nutrients from their local soil, yet the composition of soils is never optimal and varies discontinuously across space, and in time. The performance of plants in maintaining their nutrition and balancing against the accumulation of non-nutrient analogues that are often toxic is critical for environmental and human health. Our research aims to understand the interactions of plants with the composition of their local soil, employing metal homeostasis as a model. We address the molecular physiological basis of how the genetic model plant Arabidopsis thaliana acclimates to shortages in available zinc, copper and iron levels in soil. In addition, to understand evolutionary adaptations of the metal homeostasis network, we focus on the heavy metal-hypertolerant zinc/cadmium hyperaccumulator species Arabidopsis halleri, a sister species of A. thaliana. Cross-species and within-species comparative approaches are increasingly revealing the genetic variants contributing to vast phenotypic alterations and the ecological roles of metal-related extreme physiological traits. Our results can help to design strategies for crop improvement, phytomining and phytoremediation.

Session 3: Food and Flavour Analysis

In Vivo-Stable Isotope Labelling in Food and Flavour Analysis

Matthias Wüst

Institute of Nutritional and Food Sciences, Chair of Food Chemistry, Rheinische Friedrich-Wilhelms-Universität Bonn, Germany

Stable isotopes of biochemically relevant elements, such as hydrogen, nitrogen or carbon, have been widely used as labels for metabolome analysis and general labeling strategies can be divided into *in vitro* and *in vivo* approaches.[1] *In vivo* labeling with labeled nutrients from the environment or growth medium takes advantage of the metabolic activity of the plants. As a function of growth rate, metabolic flux and labeling time, different degrees of labeling can be achieved and only minimal physiological perturbations are caused. Labeling of primary and secondary plant compounds with stable isotopes, like ^{13}C, is a powerful tool to investigate metabolic processes, because they are distinguishable from their unlabeled analogs by mass spectrometry. The combination of different labeling techniques with efficient separation and detection systems, like GCxGC-TOF-MS, provides the opportunity to increase the level of identification of bioactive relevant metabolites in crops and humans when authentic standards are not available. Two strategies, global labeling of whole plants with $^{13}CO_2$ and the administration of labeled key intermediates in plant tissues (see Figure 1), for improving metabolite identification are discussed by presenting numerous examples.

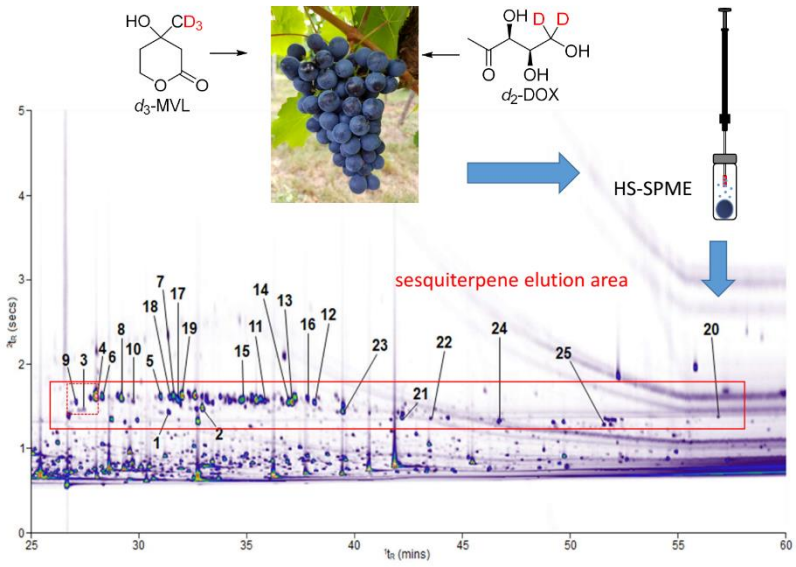

Figure 1: Contour plot of an HS-SPME-GCxGC-TOF-MS chromatogram (TIC) demonstrating the separation of labelled volatile compounds isolated from the headspace of grape berries of the red wine variety Lemberger (*Vitis vinifera*, clone 1Gm, exocarp) after *in vivo* labelling.

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Probing High Order Transient Oligomers using Ion Mobility Mass Spectrometry coupled to Infrared Action Spectroscopy

Iuliia Stroganova, Agathe Depraz Depland, Raya Sadighi, Sjors Bakels, Melissa Bärenfänger, Anouk M. Rijs

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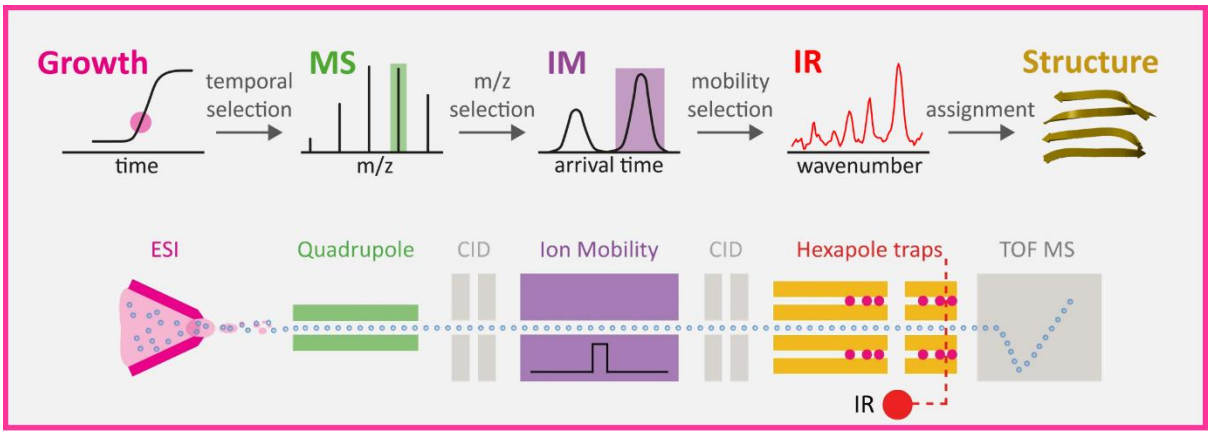
Neurodegenerative diseases, such as Alzheimer's, Amyotrophic Lateral Sclerosis and Parkinson's disease, are directly correlated with the development of protein and peptide aggregates on a molecular level. However, the mechanism of aggregation, and especially the transition from soluble oligomers to insoluble fibrils remains unclear. This is particularly important as neurodegenerative toxicity originates from the highly ordered transient oligomers that are formed along the aggregation pathway. The challenge is to obtain structural information of these elusive intermediate oligomers from their complex and heterogeneous environment. Therefore, we are applying a novel, hyphenated approach combining ion-mobility mass spectrometry with UVPD and IR action spectroscopy to isolate and to structurally characterize these transient oligomeric species.

To reveal key steps in the aggregation mechanism, we examine both the full proteins as well as on the aggregation-prone segments. Aggregation is initiated through incubation and sonication, and oligomer formation is probed over time using ion mobility-mass spectrometry. Furthermore, at the MS-LaserLab of the BioAnalytical Chemistry Division (VU Amsterdam), a novel, multidimensional spectroscopy- and mass spectrometry-based method has been developed allowing us to probe the structure and the kinetics of the initial steps of the aggregation process in a single measurement. To achieve this, an ion mobility mass spectrometer was modified to facilitate optical access, ion trapping and ion mobility slicing, resulting in the creation of the Photo-Synapt. Furthermore, the integration of small, table-top infrared and UV lasers with the modified mass spectrometer permits comprehensive structural investigations of the studied molecular systems of interest.

In this presentation, I will focus on our advances on studying the early steps of protein aggregation of alpha-synuclein, amyloid-beta, and the peptide segments of tau and TDP43. Our ion mobility mass spectrometry measurements provide more insight on the global picture of oligomer formation, while the IR signature reveal information on the local structure. The gain in molecular understanding of the aggregation process will lead to insights in the toxic nature of the observed intermediates and provide the basis for validating inhibitors during the aggregation process.



Session 5: Environmental Analysis

Rare Radioisotope Capabilities with HAMSTER - Astrophysics and Environment

Anton Wallner

Helmholtz-Zentrum Dresden - Rossendorf e. V., Institute of Ion Beam Physics and Materials Researchs, Dresden, Germany

Accelerator Mass Spectrometry (AMS) represents a single atom counting technique which allows the detection of radioisotopes at natural concentrations in our environment. Its high abundance sensitivity offers a variety of interdisciplinary applications in the fields of geosciences, environmental sciences or nuclear astrophysics. A new AMS facility, HAMSTER (Helmholtz AMS Tracing Environmental Radionuclides), will be installed at Helmholtz-Zentrum Dresden-Rossendorf (HZDR) in 2024. This facility will incorporate major recent technological developments which will open up new research areas.
With HAMSTER we will expand our measurement capabilities to new isotopes, including actinides, fission products and a number of additional new radionuclides. In this presentation, examples of our research portfolio will be presented, e.g. searches for traces on Earth of Supernovae and neutron star mergers, as well as new applications for studying past variations of solar and geomagnetic fields and also the distribution of releases of man-made radioactivity into our environment.

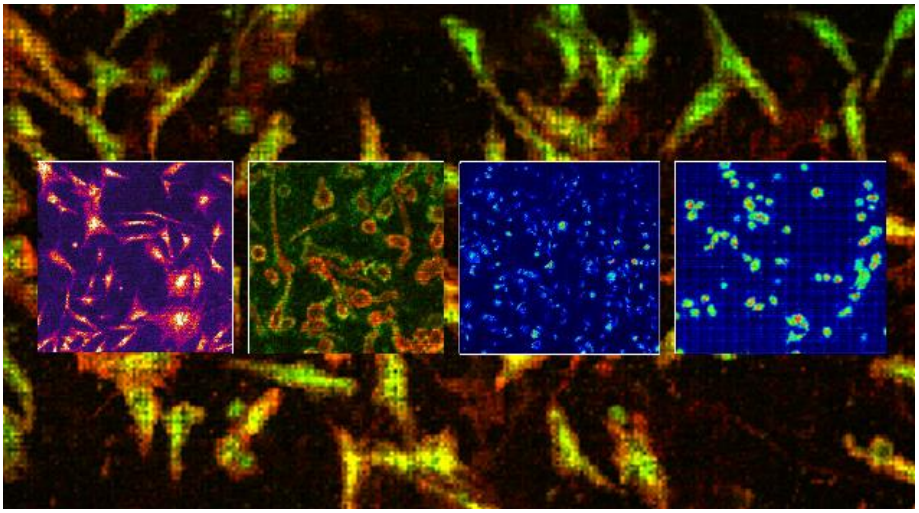
Session 7: Clinical Mass Spectrometry

Seeing is believing: Mass spectrometry in Spatial Biology

Ron M.A Heeren and the whole M4i team

Maastricht University, The Netherlands

Molecular analytical technologies in the field of spatial biology are rapidly evolving. New innovative technologies improve sensitivity, resolution, content and throughput at an ever increasing speed. Mass spectrometry is also undergoing a revolution in spatial biology. Innovative “omics” & imaging technologies, push the limits of single cell information. Contextual local metabolomics drives various applications in biomedical science and beyond. This lecture will focus on innovative analytical imaging MS, for a sensitive and selective molecular microscopy in the spatial study of distribution of molecules in cells and tissue. New insights in the spatial and molecular complexity of cellular metabolism help us to contextualize cellular function in health and disease. Innovations in mass spectrometry based chemical microscopes have now firmly established themselves in translational molecular research. One key aspect of translational success is the ability to obtain this molecular information on thousands of molecules on a process relevant timescale. Modern mass microscopes can now rapidly acquire images of metabolites, lipids, polymers, peptides and proteins, depending on the spatial resolution chosen. Combined this offers a truly precision multi-omics approach that reveals contextual molecular complexity of cellular phenotypes. The lecture will discuss the state-of-the-art and also provide an insight in what lies ahead in the future. The possibilities of high throughput targeted and untargeted MSI are seemingly endless, and will revolutionize the way we deal with spatial biology in the future.



Session 8: Computational Mass Spectrometry

SIRIUS and beyond: Turning tandem mass spectra into metabolite structure information

Sebastian Böcker

Institut fuer Informatik, Friedrich-Schiller-Universitaet Jena, Germany

In the face of unprecedented climate change, understanding the intricate interplay between the ocean and atmosphere is crucial. Human-induced pollution exacerbates environmental challenges, yet current models do not account for the impact of human pollution on the ocean and atmosphere. Particularly understudied are the impacts of waterborne microbes and pollution on coastal air quality and human health.

This presentation reveals insights gleaned from innovative laboratory experiments conducted at the NSF Center for Aerosol Impacts on Chemistry of the Environment (CAICE). Emphasizing coastal environments, scientists are exploring the composition, cloud-forming abilities, and ice nucleation properties of marine aerosols.

Using the Scripps Ocean-Atmosphere Research Simulator (SOARS), we investigate ocean-atmosphere exchange dynamics under varying conditions with control of winds, waves, microbiology, and temperature. Additionally, we are performing studies to better understand the transfer mechanisms involved aerosolization of bacteria, viruses, and gases from the ocean to the atmosphere.

This presentation will discuss the implications of human-induced environmental changes on human health, ecosystems, and climate. As we confront unprecedented warming, this presentation underscores the urgency of comprehensively understanding ocean-atmosphere interactions for effective climate mitigation strategies and public health policies..

Native top-down mass spectrometry for the study of RNA-ligand interactions

Kathrin Breuker

Institute of Organic Chemistry, University of Innsbruck, Austria

Top-down mass spectrometry (MS) of ribonucleic acids (RNA) is an emerging field of research with applications ranging from the characterization of posttranscriptional and synthetic modifications [1-3] to the determination of binding sites of proteins and small molecule therapeutics [4-6]. Here we show how data from top-down MS experiments on a Fourier transform ion cyclotron resonance (FT-ICR) mass spectrometer can be used to localize and relatively quantify posttranscriptional and synthetic RNA modifications, and how the same approach in combination with native electrospray ionization can identify RNA binding motifs and their occupancy with ligand. For the 40 nt aptamer of a neomycin sensing riboswitch functional in yeast and a 47 nt construct of rev response element (RRE) RNA from human immunodeficiency virus 1, we demonstrate that the binding of aminoglycosides to RNA is surprisingly intricate and can involve multiple binding sites and complex stoichiometries. We are currently extending our native top-down MS approach to other classes of RNA and ligands with the ultimate goal of establishing general principles of RNA recognition to guide the development of drugs that target RNA.

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Session 10: Molecular Imaging Mass Spectrometry

Advances in Nanospray Desorption Electrospray Ionization (nano-DESI) Mass Spectrometry Imaging

Julia Laskin

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Mass spectrometry imaging (MSI) is a powerful technique for molecular mapping of biological samples with high sensitivity and molecular specificity. Ambient ionization techniques enable imaging of biological samples with minimal sample pretreatment. We have developed an ambient MSI technique based on nanospray desorption electrospray ionization (nano-DESI). Nano-DESI is a liquid extraction-based technique, in which molecules are extracted from the sample into a dynamic liquid bridge formed between the nano-DESI probe and sample surface. The extracted analytes are transferred to a mass spectrometer inlet and ionized by electrospray ionization. The high sensitivity of nano-DESI enables imaging with high spatial resolution of 6-10 microns, which opens new directions for molecular mapping of individual cells in biological tissues. Furthermore, we have developed approaches for correlative imaging of lipids, metabolites, peptide, proteins, and glycans in biological tissues and used immunofluorescence microscopy of the same or adjacent tissue sections to extract cell-specific molecular signatures. We have also examined the effect of solvent composition on both the extraction and ionization efficiency, which provided significant enhancements in sensitivity and molecular coverage of nano-DESI MSI. These developments have established nano-DESI MSI as a powerful technique for studying biological systems.

Session 12: Analysis of Lipids, Carbohydrates, RNA and DNA

Electron activated dissociation for identification of lipids (and other small molecules)

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Metabolite and lipid identification is an important task in non-targeted analysis, but also represents one of the major bottlenecks. Identification of metabolites and lipids requires a multitude of parameters, e.g. matching retention times, m/z and fragmentation pattern, matched against reference standards for highest confidence. However, the number of commercially available reference standards is comparably small, and it is unlikely that a metabolomics/lipidomics laboratory can hold all standards they would require.
Especially in lipidomics, not for all possible lipids standards are available and therefore different approaches are required for the identification of lipid species. Electron activated dissociation (EAD) has been introduced as suitable alternative for lipid identification. EAD shows the advantage of diagnostic fragments for C-C single and double bonds allowing to locate double bonds in complex lipids. Here we report our current experience and results using EAD for the identification of lipids species as well as other small molecules.

Session 13: Instrumentation and Automation

A secret history of multi-reflection analysers

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Introduction

Development of mass analyser technology is increasingly dominated by industrial scientists, operating under conditions of commercial secrecy. Entire analyser concepts emerge from theory to generations of prototypes, only to be jettisoned with little impression saved to the scientific record of the day.

Multi-reflection and multi-turn mass analysers extend the flight path of ions by passing them between ion mirrors or electrostatic sectors respectively, enhancing the achievable resolving power of the analyser and relieving the painful focal quality constraints of prior time-of-flight (ToF) mass spectrometers.

The Thermo Scientific™ Orbitrap Astral™ mass spectrometer delivers a generational leap in sensitivity and speed, operating at up to 200 Hz, with extremely high ion transmission and destructive ion detection to produce hundreds of single ion sensitive MS/MS spectra per second. It was built upon decades of hidden technological developments, including multiple different mass analyser concepts. The secret history of these mass analysers and their constituent technologies is revealed and explained in the context of advances in the broader high-resolution mass spectrometry field.

Methods

A series of multi-reflection mass analyser concepts were envisaged, of which three were constructed, whereby ions oscillated between a pair of ion mirrors, either cylindrical, quasi-planar (Astral analyser), or shaped to generate an Orbitrap™-like quadro-logarithmic potential. The first of these operated in a "closed trap" arrangement, with ions admitted or ejected via a switched deflector, while the latter two incorporated an open multi-reflection ion track. The mass analysers were evaluated by a variety of measurements with electrosprayed calibration mixture, measuring performance in terms of resolving power, space charge tolerance, ion transmission, and spectral artifacts.

Results and Conclusion

All three analysers proved capable of reaching impressive resolving power, >70k, though shortfalls in other factors were notable. The closed trap analyser suffered substantial space charge effects due to its tightly focused ion packets, while the quadro-logarithmic system had poor ion transmission at high resolution caused by flawed injection optics. Although this latter shortfall was considered addressable, the competing quasi-planar Astral analyser had already leapfrogged it and was adopted for further development; a decision vindicated by the superior performance of the finalized Orbitrap Astral hybrid instrument.

Time-resolved ion mobility mass spectrometry to solve conformational changes in a cryptochrome

Nina Morgner, Rene Zangl Jonathan Schulte

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Tight control of the detailed interplay of proteins and their complexes is of high importance for correct cellular function. Small changes such as single mutations or post-translational modifications can be sufficient to alter protein conformation or binding efficiency and therewith the functional interplay between all participants. Investigating such systems requires instrumentation that can pick up differences based on such small alterations.

Cryptochromes, a widespread class of blue light photoreceptors, play a pivotal role in mediating diverse light-triggered biological functions. Here we employed a novel time-resolved ion mobility mass spectrometry approach to investigate light triggered conformational changes in the cryptochrome from Chlamydomonas reinhardtii (CraCRY). Light triggered chromophore excitation and an electron transfer cascade ultimately cause large conformational rearrangements which occur on a time scale in the msec – sec range.

We adapted a Synapt G2S ESI mass spectrometer to allow continuous or short pulse illumination of samples using a high-power LED prior to analysis. The setup allowed us to assess mass and ion mobility spectra over time, allowing us to explore the influence of specific amino acids in response to blue light activation. Our focus on the C-terminal domain of CraCRY revealed the impact of single mutations on the structural transition of helix α22 and the attached flexible c-terminal extension during lit-state formation. Notably, D321, a putative proton acceptor, proved essential for significant conformational changes, while D323 influenced the timing. These findings provide insights into the intricate mechanisms controlling such protein dynamics, if activated by blue light.

New opportunities for the discovery of novel bioactive natural products through the exploration of massive metabolomics and bioactivity extract datasets guided by computational methods

Jean-Luc Wolfender[1,2], A. Gaudrya[2], L. Quiros-Guerreroa[2], O. A. Kirchhoffer[1,2], L. Marcourt[1,2], B. David[4], A. Grondin[4], A. Rutz[1,2], E. Ferreira Queiroz[1,2], L.-F. Nothias[1,2], P.-M. Allard[1,2,3]

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Recent advances in high resolution mass spectrometry data dependent MS/MS analyses (HRMS/MS) have enabled the acquisition of increasingly precise data on plant and microorganism metabolomes. This allows mapping the composition of natural extracts at an unprecedented precision level [1]. This particularly permits the construction of virtual chemical libraries based on all putative annotation obtained. By combining annotation data sets generated from raw extracts, researchers can efficiently prioritize valuable natural products (NPs) for drug discovery, conduct compositional assessments of phytopreparations, or make correlations in eco-metabolomic or chemotaxonomic studies. In this context, to improve the annotation confidence of our molecular networking (MN) approaches using ultra-high performance liquid chromatography high-resolution mass spectrometry (UHPLC-HRMS/MS), we have integrated automated natural product (NP) class annotations and taxonomically informed scoring [2]. To further support this effort, we recently established an online resource for tracking NP structures and their occurrences in their respective source organisms [3]. We have applied this integrated approach to the investigation of a chemodiverse collection of 1,600 plant extracts from Pierre Fabre Laboratories which holds one of the largest plant samples library worldwide with over 17,000 samples (Collection registered in 2020 before the European Commission). For chemical space exploration of such a massive metabolite profile dataset, we developed novel computational tools to efficiently prioritize and target the isolation of high-value bioactive NPs [4,5]. We have also started to use knowledge graph database that contains interconnected, spectral, structural, taxonomical and bioactivity data to interrogate such multi-informative datasets.

The proof of concept and the exploration of such data in combination with results of various bioassays (anti-infective, anticancer, antiparasitic activities) will be exemplified. The potential benefits and challenges of using these approaches to transform the field of pharmacognosy in the current era of omics and digital science will be discussed.

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[5] Queiroz EF, Alfattani A, Afzan A, et al.. J. of Chromatography A; 1598: 85–91 (2019**)**

Synthesizing new molecules in the condensed phase using fragment Ions from mass spectrometers

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Leibniz-Institut für Oberflächenmodifizierung e.V., , Leipzig, Germany

Fragment ions observed in mass spectrometers are known to chemists usually as reactive species relevant to analytics and fundamental gas phase ion chemistry studies. Meanwhile, their potential as building blocks for condensed phase chemical synthesis remained largely unexplored. ESI-coupled ion soft-landing has recently developed into a method for small-scale chemical synthesis using gaseous ions.[1] The preparation of new compounds and surface layers using mass selected fragment ions[2] from the gas phase and the analysis of the new substances with conventional analytical methods, including infrared spectroscopy and nuclear magnetic resonance spectroscopy is shown. The new possibilities for chemical synthesis are demonstrated by binding of inorganic fragment ions generated in the gas phase to (bio-)molecules on surfaces, covalent binding of cluster ions of same polarity at surfaces[3] and binding of unreactive molecules like N2 to reactive fragment ions,[4] which are subsequently "taken out" of the mass spectrometer.

References:

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Schematic visualization of the synthesis of new products using gaseous fragment ion.

Breaking the "ppq(uint.)-barrier" – Offline ExC and automated online ExC-IC-ICP-MS for the determination of technetium-99 below ultra-trace levels

M. Horstmann[1], C. D. Quarles Jr.[2], S. Happel[3], M. Sperling[4], A. Faust[5], K. Rahbar[6,7], D. Clases[8], U. Karst[1]

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Only being discovered half through the last century, the role of technetium as an anthropogenic radiocontaminant has constantly increased. Being virtually monoisotopic, its primary isotope ^{99}Tc with a half-life of over 211,000 years, accounts for the entire natural and much higher anthropogenic occurrence. Today, due to its unique decaying pattern, the metastable state ^{99m}Tc is used as a radiotracer for scintigraphy in medical diagnostic techniques such as single photon emission computed tomography (SPECT). Nowadays, ^{99m}Tc is applied in an estimated 40 million procedures per year and makes up for about 85% of all diagnostic scans performed in nuclear medicine worldwide. Besides the resulting discharge through medical facilities, multiple other pathways, such as the reprocessing of nuclear fuels, contribute to environmental concentrations of the radionuclide at or even below ultra-trace levels and consequently require powerful monitoring methods.

As most samples contain expectedly low concentrations coinciding with difficult matrices such as wastewater, blood or urine, monitoring of ^{99}Tc is proving difficult. In addition, due to its radioactivity, elemental standards of ^{99}Tc are not easily available, therefore expensive and not easy to handle. To overcome these challenges, we developed a novel quantification method for ^{99}Tc using an automated single platform system for total metal and speciation analysis. In an online approach, extraction chromatography (ExC) is followed by anion-exchange chromatography (IC) to eliminate potential isobaric interferences of ^{99}Ru as well as potential matrix effects and to focus the wide peak shapes of the previous ExC preconcentration. The individual method provides detection limits reaching down to 6.3 ± 0.1 fg/kg for ^{99}Tc. To lower detection limits even further we added an integrated filter disk-based offline ExC purification and preconcentration step increasing the total sample volume to 1 L. With an additional 9-fold preconcentration factor, overall detection limits could be further decreased below 1 fg/kg.

After eliminating elemental interferences from the sample, the method allows internal quantification of ^{99}Tc with a strategy called isobaric dilution analysis (IBDA).[1] Exploiting the isobaric interference of naturally occurring ^{99}Ru, ^{99}Tc can be quantified online with a conventional elemental Ru standard. To compensate for any difference in elemental sensitivity within the ICP-MS, for the first time, an isotopic standard of ^{99}Tc was prepared from decayed medical technetium-99m generator eluate by means of ExC and counter-quantified using total reflection X-ray fluorescence analysis (TXRF). The combined method enabled online quantification of ^{99}Tc in a sample of clinical wastewater at a concentration of 89 ± 4 fg/kg.

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ABSTRACTS ORAL PRESENTATIONS

Session 1: Proteomics
Oral Presentation

Proteomics reveals that ribosome frequently changes reading frame during translation

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During protein translation, "slippery" sequences in the mRNA can cause the ribosome to change into a new reading frame. As a consequence of slipping, different codons are read and a different protein sequence is produced from the point of shifting. This process is known as ribosomal frameshifting. It may occur as a random error, but since this mechanism allows the production of several proteins from a single mRNA sequence, frameshifting is used by viruses, prokaryotes and eukaryotes to regulate protein expression and adopt an mRNA sequence to produce a new protein with a new function. To test and quantify frameshifting caused by short sequences, we developed an assay based on a reporter protein containing the putative frameshift site. In this construct, production of a set of peptides and a fluorescent protein is dependent on frameshifting (see fig. 1). By quantifying the peptides relative to peptides encoded in the 0-frame, and by relating the fluorescence to a non-shifting control, we can measure the probability of frameshifting by two independent readouts. Guided by predictions from a probabilistic model of translation developed in our lab, we tested several hundred short sequences for their ability to undergo frameshifting.

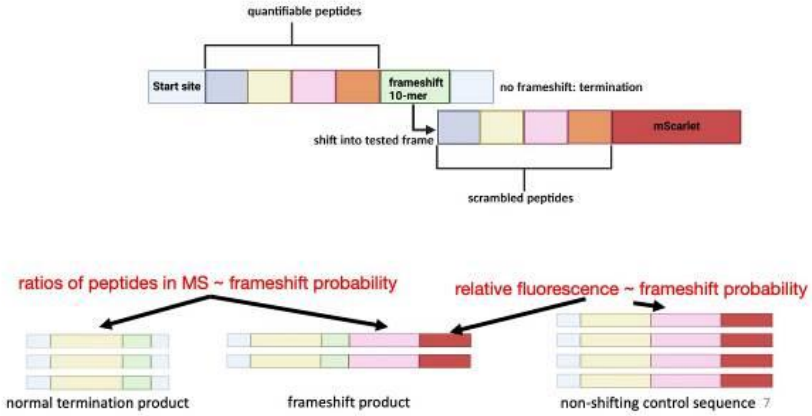
We identified dozens of new slippery sequences that cause frameshifting with probabilities of more than 10%, with some sites leading to more than 20% frameshifting. The majority of these sequences does not match known frameshifting patterns, and many occur often in genomic coding sequences. Based on the tested sequence library, our model can predict frameshifting with more than 80% precision.

Using targeted MS, we are able to identify the amino acid sequence at the site of frameshifting, and could show that frameshifting into a certain frame on a single sequence can occur by two distinct mechanisms.

These results indicate that ribosomal frameshifting is a phenomenon that organisms frequently have to contend with, by tolerating, avoiding, or co-opting it. This has implications for both the regulation of protein expression, and the evolution of new functions.

Figure 1. Concept of the protein construct for quantifying frameshifting. After translating the first set of peptides, at the tested 10-mer the ribosome will either continue in 0-frame or shift into one of the two alternative frames. Only shifting into the correct frame (that is being tested) will result in production of the second peptide set and the fluorescent protein. Relating the amounts of the two peptide sets by MS or the fluorescence relative to a non-shifting control provides estimates of the frameshifting probability.

Fig. 1


quantifiable peptides
Start site
frameshift 10-mer
no frameshift: termination
shift into tested frame
mScarlet
scrambled peptides
ratios of peptides in MS ~ frameshift probability
relative fluorescence ~ frameshift probability
normal termination product
frameshift product
non-shifting control sequence
7

Session 1: Proteomics
Oral Presentation

A region-resolved proteomic map of the human brain enabled by high-throughput proteomics

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Over the last decade, technical progress in the area of liquid chromatography, mass spectrometry and data analysis, advanced the sensitivity and reproducibility of LC-MS measurements. However, a sensitive, robust and fast LC-MS setup for use in everyday clinical diagnostics is still lacking. Therefore, we coupled a micro-flow LC (fast, robust) to a timsTOF-HT (fast, sensitive) in order to establish a system that can deliver speed while maintaining deep proteome coverage. To reveal the power of this combination, we determined the proteome of thirteen post-mortem human brain regions using formalin-fixed paraffin embedded (FFPE) material.

Aiming for deep proteome coverage, we developed a proteomic workflow for formalin fixed brain tissue including lipid depletion. High efficient protein extraction and de-crosslinking was achieved by boiling the tissue in 4% SDS, Tris buffer (500 mM, pH 9) and sonication using the Covaris® R230. Proteins were digested using the SP3 approach and desalted peptides were fractionated using basic reversed phase liquid chromatography. Samples were analyzed on the novel micro-flow timsTOF-HT setup.

First, we optimized a micro-flow LC timsTOF-HT setup including e.g. reducing the inner diameter of the emitter to 50 µm. This powerful setup convinces by combining speed and sensitivity. For example, we identify >6,500 protein groups in single-shot HeLa runs using a 15 min gradient. Having this fast and robust setup at hand, we analyzed 48 fractions of thirteen postmortem human brain regions. This resulted in >2000 injections of FFPE material which underlines the robustness of the system. We identified > 9,500 protein groups in each brain region, which is very close to what we can see in fresh frozen material (<5%). Bioinformatic analysis uncovered profiles distinguishing brain regions and highlighted candidates of regional driver proteins. While the core proteome was shared between the brain regions, we also identified unique finger prints pointing towards specific functions and cellular composition of each region.

In the scope of this project, we not only established for the first time the power of combining micro-flow LC with a timsTOF-HT which turned out to be a fast, robust and sensitive LC-MS setup, but also elucidated the proteomic profile of thirteen human brain regions in a deep manner.

Session 1: Proteomics
Oral Presentation

Miniaturized quantitative low-input top-down proteomics reveals infomation about proteoforms not accesible via bottom-up proteomics

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While most nanoproteomics approaches for the analysis of low-input samples are based on bottom-up proteomics workflows, top-down approaches enabling proteoform characterization are still underrepresented.

Using mammalian cell proteomes, we established a facile one-pot sample preparation protocol based on protein aggregation on magnetic beads and intact proteoform elution.

The method was then adapted for top-down proteomics sample preparation on a digital microfluidics (DMF) device, which is a tool enabling the manipulation (i.e. splitting, merging, mixing) of small droplets of liquids with volumes between ca. 0.5 to 2 µL on a chip by application of electric fields.

Single intact *Caenorhabditis elegans* nematodes, which consist of an invariant number of 959 cells, were lysed (with the additional challenge to crack the cuticule surrounding the organism), and the intact proteoforms were isolated and desalted on-chip, providing LC-MS ready proteome samples [1]. In combination with a recently developed multi-compensation voltage (cv) LC-FAIMS MS setup [2], the number of proteoform identifications compared to in-tube sample preparation was increased by 46% [3]. Label-free quantification of single nematodes grown under different conditions allowed us to identify changes in abundance of proteoforms not distinguishable by bottom-up proteomics [3, 4].

The presented workflow will facilitate proteoform-directed analysis on samples of limited availability

Novel Aspect

Development of a novel sample preparation platform for multiplexed low-input top-down proteomics.

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Advances in plasma-based mass spectrometry for the detection of single nanoparticles and imaging of microplastics

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In this Presentation, recent advances in plasma-based mass spectrometry for the detection of single nanoparticles and imaging of microplastics will be reviewed and some contributions from our laboratory to this field will be presented.

In the first part, recent developments in inductively coupled plasma mass spectrometry (ICP-MS) instrumentation for nanomaterials characterization in complex mixtures will be discussed. The current state-of-the-art in single-particle (sp) ICP-MS instrumentation for the detection and characterization of single nanoparticles (NP) as well as remaining challenges will be highlighted [1]. While millisecond dwell times were used in the advent of spICP-MS, the use of microsecond dwell times helped to improve nanoparticle data quality and particle size detection limits. Further to this development, we could show that a custom-built high-speed data acquisition unit with microsecond time resolution (µsDAQ) can be used to successfully address issues of split-particle events and particle coincidence, to study the temporal profile of individual ion clouds, and to extend the linear dynamic range by compensating for dead time related count losses [2]. Our next generation DAQ for spICP-MS features nanosecond time resolution. First results of a proof-of-concept study will be discussed [3]. For example, our new in-house built nsDAQ was coupled to a commercial quadrupole ICP-MS and gold nanoparticles (AuNP) as small as 7.5 nm were successfully detected. Due to the high data acquisition frequency, a statistically significant number particles can be characterized in under 60 s.

In the second part, we turn to a cooler plasma source, which proved useful in ambient desorption/ionization mass spectrometry (ADI-MS). Specifically, the use of molecular mass spectrometry with a home-built flowing atmospheric-pressure afterglow (FAPA) source (which was first developed in the Hieftje laboratory) for the direct analysis of microplastics will be discussed. Microplastics (MPs) are typically characterized by Raman spectroscopy and pyrolysis/thermal desorption coupled to gas chromatography, respectively. In this work, the FAPA source is coupled to a high-resolution mass spectrometer (HR-MS) and used to probe selected microplastics directly on a sample target without a preceding separation step. Characteristic mass spectra from MPs were obtained and multivariate statistical data analysis tools were used to process the raw data. FAPA-HRMS analysis in combination with principal component analysis is considered an interesting tool for microplastics analysis.

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MALDI mass microscopy for high throughput mass spectrometry imaging

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Introduction: Matrix-assisted laser desorption/ionization (MALDI) mass spectrometry imaging (MSI) maps the spatial distributions of thousands of molecules in tissue. However, its application as a standard imaging method, for instance in hospitals, is impeded by the low throughput of commercial MALDI MSI instruments, which is often less than 50 pixels s^{-1}. This low speed is due to each pixel being acquired sequentially, termed microprobe-mode. Consequently, ever-smaller pixel sizes in microprobe-mode are accompanied by quadratic increases in acquisition time, leading to impractically long measurements. Still, high throughput MSI at simultaneously high spatial resolution can be achieved with mass microscopy, in which a >100 μm^2 large area is ionized, preserved during time-of-flight analysis and magnified onto a fast spatially sensitive detector. Previously, we showed that mass microscopy combined with a Timepix3 detector allows acquiring images at >600,000 pixels s^{-1} and submicron resolution.[1] However, we conducted this study using secondary ion mass spectrometry (SIMS), a technique less suited than MALDI for the analysis of large molecules. Furthermore, the use of a slow, dim phosphor screen limited mass resolution and sensitivity. Here, we report the construction of a novel laser interface and the implementation of a fast scintillator. Using these modifications, we demonstrate MALDI mass microscopy at several thousand pixels s^{-1}.

Methods: A TRIFT II mass spectrometer (Physical Electronics, Chanhassen, USA) equipped with a C_{60} ion gun (Ionoptika, Chandler"s Ford, UK) and a TPX3CAM (Amsterdam Scientific Instruments, Amsterdam, The Netherlands) was modified by adding an improved optical setup with a homogenized laser beam (Explorer One, Spectra-Physics, Stahnsdorf, Germany) and a Cry 60 scintillator (Crytur, Turnov, Czech Republic). For characterization purposes, TEM grids (Agar Scientific, Stansted, UK) were put onto dried crystal violet, and onto sprayed 2,5-Dihydroxybenzoic acid (DHB) films containing cetrimonium chloride. Data processing was performed with custom software.

Preliminary results: To characterize the new laser interface, we imaged TEM grids at speeds of 2,000 pixels s^{-1}, and at a spatial resolving power of at least 6 µm independent of matrix crystal size. The use of the novel scintillator improved sensitivity and time resolution from ~290 to ~50 ns with further improvements being possible by enhanced data processing. In future, we expect to perform MALDI of tissues at higher throughput than currently shown by operating the laser at 5 instead of 1.2 kHz, by firing fewer than 1,000 laser shots at each position, and by the use of faster computing capabilities to tackle increasing amounts of data.

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Increasing Ion yield for time-of-flight secondary Ion mass spectrometry imaging by using MALDI-Type matrices and cluster primary Ions

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Time-of-Flight Secondary Ion Mass Spectrometry (ToF-SIMS) is capable of 2D and 3D imaging with a lateral resolution below 100 nm and a depth resolution of a few nanometers. For many samples, however, the achievable spatial resolution is limited by the ion yield rather than the instrumentation. Low molecular ion yields in SIMS have been attributed to both poor ionization efficiency and fragmentation of molecules. Matrix enhancement and cluster primary ions are two promising approaches for improving molecular ion yields. In this work, these two approaches have been combined to better understand the mechanisms important in the matrix enhancement effect. The influences of cinnamic acid matrices and cluster ion beam size and species on the molecular ion yield of biomolecules were investigated in model systems and mouse brain tissue. A series of cinnamic acid-based matrices varying in acidity was employed to systematically investigate the influence of matrix acidity on molecular ion formation. Model systems involved three biomolecules: a lipid, a peptide and an antibiotic. The most promising matrices were vapor-deposited onto brain tissue. The enhancement of lipid ion signals from different regions of the mouse brain tissue was investigated for a range of cluster primary ions. In the model systems, the positive ion signal for all three biomolecules showed a strong increase for more acidic matrices. Additionally, increasing cluster ion size led to increases in the ion yield. However, the relative ion yield enhancement by the matrices decreased for larger cluster primary ions. These cause less molecular fragmentation even in the absence of an enhancing matrix. The work demonstrates that proton exchange between the analyte and matrix plays an important role in formation of molecular ions. Additionally, the matrix can lead to "colder" desorption of the biomolecules, which further increases the number of molecular ions detected.

Comprehensive analysis of aroma stability in brewed coffee: Insights from molecular interactions and quantification of reaction products

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The enjoyment derived from a freshly brewed cup of coffee, attributed to its alluring aroma and pleasant taste, has been acknowledged by consumers worldwide. The characteristic flavor profile of percolated coffee is known to result from the generation of aroma- and taste-active molecules during the roasting process. While the volatile signatures of roasted coffee can be successfully reconstituted with 25–30 aroma-active compounds, this overall aroma of brewed coffee proves to be far from stable, particularly during storage in sealed containers.1,2

A holistic UPLC-TOF-MS-based approach was employed to screen for storage-induced reaction products between coffee's low molecular weight fraction and key coffee odorants, namely thiols, and aldehydes. Therefore, coffee beverages were spiked with odorants and stored under thermos flask conditions. Utilizing statistical data analysis, differences in the metabolite spectrum of spiked and non-spiked samples could be visualized, and marker compounds were identified. Structure elucidation of these marker molecules was achieved through TOF-MS and 1D-/2D-NMR experiments.4,5

Then, targeted quantification of the interaction products was conducted after synthesizing suitable internal standards using UHPLC-MS/MS. The concentrations of reaction products were determined in freshly prepared coffee beverages of different roasting degrees, and additionally, the impact of storage time and temperature on the concentrations of these reaction products was evaluated. 4,5

Key aroma compounds were observed to form non-volatile reaction products with low molecular weight constituents of coffee brew. These key coffee odorants were removed from the aroma composition, rendering them undetectable to consumers.

The use of high-resolution mass spectrometry to assess the aroma binding affinity of coffee's nonvolatile fraction proves to be a highly useful technique. Its application is promising for the development of coffee products with higher sensory stability, which can minimize the staling effects observed during coffee storage.

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Session 3: Food and Flavour Analysis
Oral Presentation

Lipid Profiling by LC-TIMS-MS and GC-MS combined with MZmine data processing to distinguish milk samples from cow, goat, and sheep

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Lipids are one of the major constituents of most food groups. In addition to being an important energy source, lipids add to the nutritional value and sensory profile of the food. Due to characteristic differences between the lipid compositions of different animal or plant species, the lipid profiles can be used to discriminate these and determine food authenticity. The differentiation of food types based on lipid profiles is also a powerful approach in the domain of ancient lipid analysis, where food sources are identified through lipid residues preserved in sherds of ceramic cooking vessels. A big challenge is the discrimination of milk fats of different ruminant species such as cow, goat, and sheep.

This study focuses on the identification of marker lipids, specifically triacylglycerols, which can be used to distinguish dairy lipids from different species. In this context, we investigated the potential of untargeted lipid profiling methods for the identification of characteristic lipid signatures in order to differentiate milk derived from cow, goat, and sheep. For this reason, lipids from milk samples were extracted and analyzed by two different analytical platforms. A methodological comparison was conducted between gas chromatography-mass spectrometry (GC-MS) and liquid chromatography-trapped ion mobility spectrometry (LC-TIMS)-MS to evaluate their effectiveness in generating lipid profiles to distinguish the three animal species. The open-source software MZmine was used for comparative data analysis, enabling a detailed examination of the lipid profiles obtained from both LC-TIMS-MS and GC-MS.

Our results reveal distinct differences in the milk lipid profiles of the three animal species using both methods, highlighting potential marker compounds. These findings are not only significant for current food analysis but also hold potential for archaeological research. The identified lipid markers and their stability need further exploration through extended stability experiments to affirm their applicability in answering archaeological questions. This study contributes to the field of food analysis and archaeology and showcases the capabilities of advanced analytical techniques in combination with powerful analysis software in understanding complex biological matrices.

Protein composition of wheat kernel layers and the impact on bread-making quality

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Wheat is one of the most discussed staple foods due to its gluten proteins, which trigger wheat-related disorders such as celiac disease or wheat allergy. However, the gluten proteins are responsible for the exceptional ability to bake tasty bread. Before baking, wheat kernels are first milled into flour, and depending on the milling process, the flour contains more bran (wholemeal flour) or only the starchy endosperm (white flour). Wholemeal flour is rich in protein, minerals and dietary fiber and has a higher nutritional value than white flour. In wholemeal flour, the outer layers of the wheat kernel (i.e., the aleurone and sub-aleurone) are more enriched than in white flour. Although the outer layers of the wheat kernel have a higher protein content [1], little is known about the exact protein composition in different layers. The aim of our study [2] was to elucidate the protein composition of wheat kernel layers (inner endosperm, sub-aleurone and aleurone) and to provide insights into the functionality of these proteins in relation to bread-making quality.

Tissue from the inner endosperm, sub-aleurone and aleurone were obtained by microdissection after cryosectioning. This procedure guaranteed the highest possible purity of the tissues with no cross-contamination. The tissues, containing about 2 µg protein, were hydrolyzed with a mixture of trypsin and chymotrypsin and the peptides were analyzed by nanoLC-MS/MS. The raw data were evaluated using MaxQuant, and the protein distribution (in percentage) was calculated by the iBAQ-algorithm. Proteins were first grouped according to their function into albumins/globulins, gluten proteins and proteins with unknown function based on InterPro. The gluten proteins were further divided into α-, γ- and ω-gliadins and low- and high-molecular-weight glutenin subunits (LMW-GS and HMW-GS).

In total, 780 proteins were detected in the three tissues, with 593 proteins belonging to the albumins/globulins, 181 to the gluten proteins and 60 to the group of proteins with unknown function. 291 proteins were present in all three tissues and 234 proteins only in the aleurone layer. The proportion of gluten proteins was very low in the aleurone layer (3.4%) compared to that in the subaleurone layer (69.0%) and the inner endosperm (59.5%). The most abundant proteins in the aleurone layer were albumins/globulins (92.5%). Regarding the gluten proteins, the sub-aleurone layer had a slightly higher proportion of HMW-GS than the inner endosperm. The HMW-GS play a key role in gluten functionality and the formation of the gluten network. Combining this finding with the knowledge of a high protein content in the sub-aleurone layer [1] indicates an overlooked potential of the sub-aleurone layer for baking quality because this layer is mostly removed during milling and ends as animal feed.

Determining site-specific and clumped carbon isotopic compositions of bio- and geomolecules using high resolution mass spectrometry

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Biomolecules generally exist as mixtures of isotopologues that differ in number and position of rare-isotope substitutions. The proportions of different isotopologues of a biomolecule depend on multiple factors affecting its biosynthesis and metabolic and diagenetic transformations. Molecular isotopic structure can thus be a valuable tool to elucidate biochemical mechanisms as well as to reconstruct physiological, ecological and climatic processes.

Traditionally, site-specific isotopic measurements have been performed using NMR techniques, which require large amounts of pure material and long acquisition times. Recently, high resolution Orbitrap mass spectrometry (Orbitrap-MS) has been shown to be capable of distinguishing isotopologues and has been used to determine stable isotope ratios at particular sites within a molecule (Eiler et al., 2017). In contrast to NMR, Orbitrap-MS can work with trace amounts of analytes and allows the isotope ratio measurement of multiple elements in a single analysis. To date, Orbitrap-MS has been successfully applied to study the site-specific isotopic composition of small compounds such as methionine (Neubauer et al., 2018), amino acids (e.g., Chimiak et al., 2021), acetate (Mueller et al., 2022), and inorganic oxoanions (e.g., Hilkert et al., 2021).

In addition, Orbitrap-based isotope analysis is capable of quantifying the "clumping" of stable isotopes in molecules (i.e. multiply-substituted isotopologues containing two or more heavy isotopes; Csernica and Eiler, 2023). "Isotope clumping" refers to the phenomenon that heavier isotopes are often thermodynamically more likely to bind to other heavy isotopes than one would expect by random chance. The extent to which isotope clumping occurs primarily depends on the temperature during chemical reactions (Eiler, 2007).

Most published isotopic applications of Orbitrap-MS have focused on relatively small molecules. In our laboratory, we run a number of projects exploring the isotopic composition of larger molecules using Orbitrap-MS coupled with electrospray ionization, atmospheric pressure chemical and photoionization. Site-specific isotopic composition of fatty acids, paracetamol, and cholesterol from different sources or the abundance of multiply substituted carbon isotopologues in phenanthrene in geological samples that experienced different burial temperatures are explored. The Presentation will give an overview of first results and also addresses problems and limitations of this new approach.

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Development of a new nebulization system for LC-MS coupling employing an inverse low temperature plasma ion source

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In liquid chromatography – mass spectrometry (LC-MS) couplings, the nebulization of the LC eluent and its transfer into gas phase are vital steps to ensure efficient ionization of the analyte molecules by e.g. common atmospheric pressure chemical ionization (APCI) or the novel low temperature plasma (LTP)-based ion sources.

In its basic usage, an LTP ion source consists of an electric circuit with two electrodes, a high voltage and a grounded electrode. Between those, there is an insulating material, e.g. quartz glass, and a gap (cf. Figure). Voltage is applied, and the electrons travel to the high voltage electrode. Once the voltage is high enough, a discharge occurs, and the electrons can pass the insulating material and reach the gap. There, they collide with atmospheric molecules or a supplied discharge gas, usually helium or argon. This leads to a chain reaction and eventually to the ignition of the plasma that – in contrast to well-known inductively coupled plasmas – only possesses a temperature of roughly 30 °C. This plasma can now be used for the ionization of the analyte molecules. Being a soft ionization, leading to little fragmentation and the preservation of $[M]^{+\bullet}$ and/ or $[M+H]^{+}$ ions, LTP-based plasmas are able to reach better selectivity and a wide range of analytes.[1]

For efficient ionization, the LC eluent needs to be nebulized, meaning sprayed into fine droplets, evaporated and focused onto the ionizing plasma region. To improve this process, ultrasonic nebulization (USN) was to be employed which is thought to provide smaller droplets than a commercial APCI nebulizer. Thus, two commercial nebulizers – a medical inhalator and a room humidifier – were disassembled and reconfigured for employment in the LC-MS coupling. Based on these results, a new spraying system was developed. To enhance the evaporation and focusing of the analyte molecules, custom guidance cones were designed and 3D-printed from metal. After comparation, the best operating parameters were found via a design of experiments. For this, the temperature, at which the cone was heated, played an important role, as well as supplied auxiliary gas flows for further heating of the analyte molecules and cooling of a heat-sensitive construction piece. The novel spraying system was then compared to a common APCI nebulizer. Furthermore, it was tested in combination with two different types of LTP-based ion sources.

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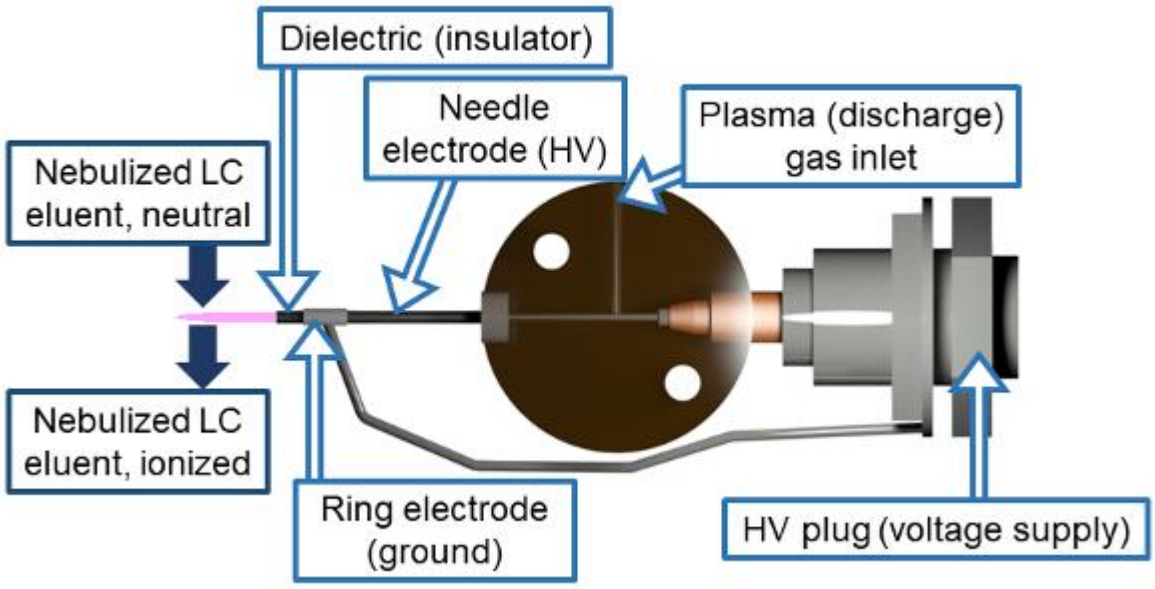
Fig. 1


Dielectric (insulator)
Needle electrode (HV)
Plasma (discharge) gas inlet
Nebulized LC eluent, neutral
Nebulized LC eluent, ionized
Ring electrode (ground)
HV plug (voltage supply)

How can we obtain structural information of very stable Fullerenes: High-Resolution mass spectrometry meets density functional theory

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The complexity of natural mixtures requires specialized equipment like high-resolution mass spectrometers (HRMS) to extract information on a molecular level. Unfortunately, the complexity often kind of sabotages the analyses and especially structural information is hard to find. Recent studies revealed the existence of fullerenes in crude oil and provides the material to study smaller fullerenes for which no synthetic material was available before.[1] Fullerenes are very stable molecules and doing *collision induced dissociation* studies on them is a paramount application for sector field MS. Here, we combine HRMS using *higher-energy dissociation* (HCD) in an Orbitrap MS in combination with density functional theory (DFT) calculations to understand structural details and the chemical and mass spectrometric behavior of smaller fullerenes.

The asphaltene fraction of a heavy crude oil was investigated. All mass spectrometric analyses were performed on a research-type Orbitrap Elite MS (Thermo Scientific, Bremen, Germany). *Collision induced dissociation* (CID) was performed in the HCD cell for higher collision energy dissociation (HCD) using Nitrogen as collision gas. The mass resolution was set to 480,000 at *m/z* 400, corresponding to a transient of 1.5 s. For atmospheric pressure chemical ionization (APCI) a corona discharge needle was used with voltages around 5 kV. Geometry optimization and frequency analysis were performed with the ORCA 5.0.3 package. The B3-LYP functional along with D3 dispersion correction as well as Becke-Johnson (BJ) damping was used for all calculations. The employed basis sets were Def2-SVP, Def2/J and Def2/JK.

This study focuses on understanding experimental details about structure of small fullerenes (C_{32} to C_{44}). To our knowledge these are the first experimental results of non-IPR (isolated pentagon rule) fullerenes at all. Their results of CID experiments show multiple C_2 losses at collision energies above 200 eV indicating a typical fragmentation pattern as reported before for C_{60}. However, their stability in comparison to C_{60} is considerably lower as it was predicted in the literature before. It could also be shown that fullerenes with "magic numbers" (C_n; n=32, 36) turn out be more stable than their congeners. Furthermore, the fragmentation pattern varies with decreasing size from dominating C_2-losses to bigger fragments with even and odd carbon numbers (C_{14}, C_{16}, C_{11}, C_{15}) indicating complete structural disintegration. The results of the DFT calculations indicated preferred structural motifs for the C_2 leaving groups during fragmentation. These data also indicate a Stone-Wales transformation as rearrangement reaction during fragmentation into a more stable configuration. DFT calculations support experimental findings and allow the interpretation of a fragmentation mechanism.

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Session 5: Environmental Analysis
Oral Presentation

On-line single aerosol particle laser mass spectrometer with double resonance ionisation: A new perspective on particle-bound transition metals and polycyclic aromatic hydrocarbons in combustion-emissions and ambient air

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Besides their climate effects, airborne particulate matter (PM) represents the largest environmental risk to human health (Lelieveld et al., 2015). Toxicological studies show that the most health-relevant PM compounds are soot, metals and polycyclic aromatic hydrocarbons (PAHs). Single-particle laser mass spectrometry (SPMS) can detect them in real time (Pratt & Prather, 2012), allowing the determination of the mixing state (Riemer & West, 2013). Particles are introduced as particle beam into the vacuum by an aerodynamic lens. The individual particle"s size and MS ion source arrival time is determined by laser velocimetry. An intense UV laser pulse is triggered to hit individual particles there, producing ions via laser desorption/ionization (LDI). Ions of both polarities are detected in a bipolar TOF mass spectrometer. Recording of individual particle"s chemical profiles is opening avenues to study transport, distribution and mixing state of aerosol compounds. However, matrix effects and fragmentation are limiting the achievable chemical speciation in conventional SPMS. We address these challenges by developing new laser resonance ionization approaches, addressing, e.g., atomic transitions of metals during LDI (Passig et al., 2020). Resonant LDI (RELDI) enhances detection efficiencies for relevant metals such as Fe and we could show that important atmospheric Fe-carriers are not detected by conventional SPMS but by SPMS with RELDI. We furthermore combine Resonance-Enhanced Multiphoton Ionization (REMPI) of aromatic molecules with LDI of refractory compounds (Schade et al., 2019) using spatial and temporal shaped IR and UV laser pulses. This technology produces detailed single aerosol particle PAH mass spectra combined with the inorganic composition (LDI), providing novel insight into the sources, distribution and ageing of atmospheric PM. We are addressing hereby both resonance techniques (RELDI/REMPI) for enhancing PAH- & Fe-signals (key compounds for PM-related health effects). To demonstrate the resonance enhanced SPMS method"s potential for risk assessment and source apportionment, we present number fractions and PAH profiles of long-range transported particles in ambient air measurements as well as PAHs in individual ship plumes (Passig et al.2022). Beyond that, we present results on safety-relevant dusts (explosives & narcotic drugs).

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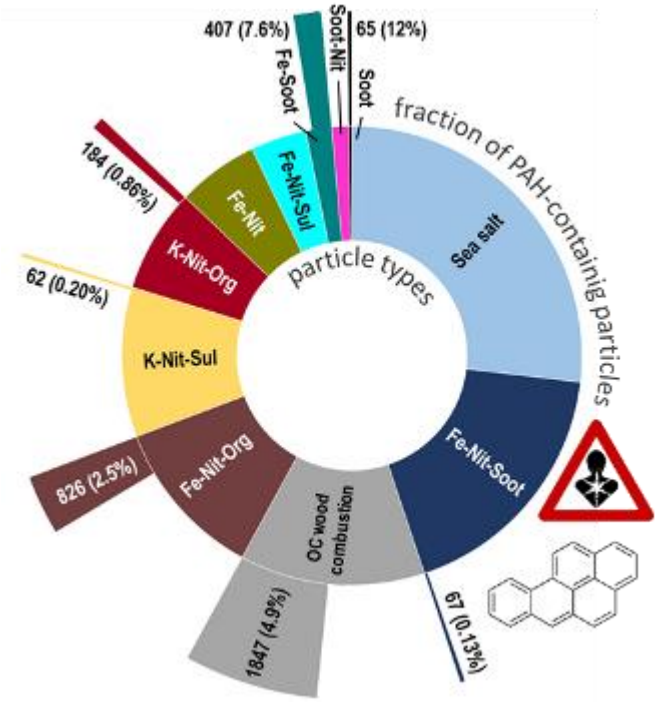
Figure: Result from analysis of 300000 particles for PAHs in a remote Swedish area. Inner ring: Relative abundance of particle classes. Outer ring: Fractions of PAH-containing particles (10-fold enlarged). Note that the PAH burden is delivered by only a small fraction of the particles.

Fig. 1


fraction of PAH-containig particles
particle types
Sea salt
Fe-Nit-Soot
67 (0.13%)
1847 (4.9%)
OC wood combustion
Fe-Nit-Org
826 (2.5%)
K-Nit-Sul
62 (0.20%)
K-Nit-Org
184 (0.86%)
Fe-Nit
Fe-Nit-Sul
Fe-Soot
407 (7.6%)
Soot-Nit
Soot
65 (12%)

Session 5: Environmental Analysis
Oral Presentation

Chemical and structural characterization of complex organic matter emitted from Boreal and Arctic peatland wildfires by 21T FT-ICR MS

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Peatlands are formed through incomplete plant decomposition and accumulation under waterlogged conditions. Globally, they are estimated to store one-third of terrestrial organic carbon. Boreal and Arctic peatlands cover the largest area of peatlands worldwide. Climate change, the higher frequency of extreme weather events, and thawing of permafrost grounds in the Arctic increase the number and scale of peatland fires in the Northern Hemisphere. However, the chemical composition of the emitted particulate matter (PM) is still vastly unknown.

Peat samples from four locations (Boreal: 2x Finland; Arctic: Norway, Russia) were burned in lab experiments simulating real-world peatland wildfires. The PM was collected on filters and extracted by MeOH/DCM for characterization by 21T Fourier-Transform Ion Cyclotron Resonance Mass Spectrometry (FT-ICR MS). Electrospray ionization (ESI+/-) was applied to address polar organic species and atmospheric pressure photoionization (APPI) to address non/-low polar constituents. Structural information was obtained by IRMPD fragmentation of isolated mass ranges in APPI 21T FT-ICR MS, in addition to thermal analysis of the original peat under inert or oxygen-depleted conditions hyphenated to 7T APPI FT-ICR MS.

FT-ICR MS chemical characterization of peat combustion emissions revealed an extremely complex mixture of organic aerosol compounds. Ultra-high field 21T FT-ICR MS enabled the assignment of up to 45,000 monoisotopic elemental compositions. The combustion type was predominately smoldering, with some variance between the samples, highlighting the influence of the local vegetation and microbial activity on the peat composition and its combustion. Compared to Boreal peat, Arctic peat generated a less oxidized and more nitrogen- and sulfur-containing organic aerosol that can easily be differentiated. An unprecedentedly high number of CHOS and CHNOS compounds was detected by ESI(-), and compounds were verified by their isotopic fine structure. The high abundance of sulfur-containing compounds is a result of the high amounts of organic sulfur species in Sphagnum moss derived peat, compared to other types of biomasses. Boreal and Arctic peat smoldering combustion therefore needs to be considered as a primary source of organic sulfur compounds to the atmosphere.

Three main structure motifs were characterized for each compound class in all peat samples: single-core aromatic ring systems with high aromaticity and minor alkylation (island-type, combustion-derived), multicore ring systems with medium to low aromaticity and a high degree of alkylation (archipelago-type, lignin decomposition derived) as well as aliphatic-like structures without an aromatic ring. Complementary thermal analysis (TA) highlighted the importance of the combustion conditions on the formation of OM in peatland fires, as TA with pure nitrogen-atmosphere (smoldering) generated more elemental compositions in common with the OM from lab combustion.

A UHPLC-HRMS method to record cyanotoxins and related secondary metabolites: A case study in Lake Stechlin (Ger)

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Introduction

Water is essential for life on earth. Besides the use as drinking water, clean rivers and lakes are of great importance for humans e.g., as a source for nutrition and recreational activities. Climate change and increased nutrient inputs may promote increased development such as algae. Among algae blooms in freshwater, particular attention is paid to the cyanobacteria, photosynthetically active microorganisms that are known to produce a large number of secondary metabolites. Some cyanobacteria genera can produce so-called cyanotoxins that can be harmful for humans and animals. Most frequently observed cyanotoxins are microcystins, cyclic heptapeptides that can lead to acute poisoning.[1,2]

Methods

For a more precise characterization of these toxins liquid chromatography coupled with mass spectrometry was used as it allows both qualitative and quantitative determinations. For analysis of cyanotoxins from Lake Stechlin in northern Brandenburg (Germany), the particulate material of the water was collected, extracted, and analysed using an UHPLC/HRMS method.

Results

By investigating additional cyanotoxins besides the standards available, two more microcystins, three cyanopeptolins, four anabaenopeptins, one aeruginosin as well as three additional secondary metabolites could be identified. Moreover, one unknown microviridin was detected and parts of its structure could be elucidated. In addition to these molecules six unknown metabolites were detected and can be linked to *Planktothrix rubescens* as native in Lake Stechlin. The application of this method yielded insights on cyanotoxin prevalence in the region of Northern German lowland Lake Stechlin and enabled for further studies in lakes nearby to elucidate additional ecological coherences by correlating the recovered data with additional bio-geo-ecological data. Furthermore, the method can deliver important information about the quality and toxicity of water samples.

Innovative aspects

- Improved (U)HPLC-(HR)MS method for cyanobacterial toxin analysis
- HRMS based identification of 14 additional toxins next to 7 microcystins
- 5 first HRMS based Annotated not jet described secondary metabolites

Acknowledgements

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Towards therapeutic drug monitoring and precision medicine – Mass spectrometric ITEM-ONE and ITEM-TWO analyses confirm and refine an assembled epitope of an anti-pertuzumab affimer

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Intact Transition Epitope Mapping - One-step Non-covalent force Exploitation (ITEM-ONE) analysis by nano electrospray mass spectrometry reveals that the assembled epitope on the surface of Pertuzumab which is recognized by the anti-Pertuzumab affimer 00557_709097 encompasses amino acid residues NSGGSIYNQRFKGR which are part of Pertuzumab"s heavy chain´s CDR2 as well as amino acid residues FTLSVDR which are also located on the variable region of Pertuzumab´s heavy chain. Despite not being part of Pertuzumab"s CDR2, the partial amino acid sequence FTLSVDR marks a unique proteotypic Pertuzumab peptide. The epitope-constituting amino acid residues from both partial peptides form a surface area of 1381.46 $Å^2$.

Binding between intact Pertuzumab and the anti-Pertuzumab affimer 00557_709097 was investigated using the Intact Transition Epitope Mapping - Thermodynamic Weak-force Order (ITEM-TWO) approach and quantitative analysis of the complex dissociation reaction in the gas phase afforded a quasi-equilibrium constant ($K_D{}^{\#}{}_{m0g}$) of 3.07 x 10^{-12}. The experimentally determined apparent enthalpy ($\Delta H^{\#}{}_{m0g}$) and apparent free energy ($\Delta G^{\#}{}_{m0g}$) of the complex dissociation reaction indicate that the opposite reaction, complex formation, is spontaneous and exothermic at room temperature.

Contrary to many other mass spectrometry-coupled epitope mapping procedures, such as HDX MS, FPOP, or cross-linking, there is no need for chemical labeling of the antigen.

Contrary to many methods which detect the presence of a protein complex, with ITEM-TWO there is no need for the use of additional antibodies, as is the case e.g. with conventional immuno-analytical methods like Western blot, FACS, and ELISA. Instead, ITEM-TWO makes use of the strength of native mass spectrometry in this respect.

Contrary to surface plasmon resonance or related methods for determining affinities, ITEM-TWO requests no chemical immobilization of one of the binding partners.

Performing ITEM-ONE and ITEM-TWO is fast and typically a series of measurements including blanks and negative controls is done within a few hours. Also, compared to all other above mentioned methods, ITEM-ONE and ITEM-TWO need very little material. Typically, a few micrograms (femto to pico moles) of antigen and binder macro-molecule are consumed.

Due to strong binding to Pertuzumab and because of recognizing Pertuzumab"s unique partial amino acid sequences the anti-Pertuzumab affimer 00557_709097 is considered excellently suitable for being implemented in Pertuzumab quantitation assays as well as for accurate therapeutic drug monitoring of Pertuzumab in biological fluids.

Characterization of Proteoforms of mAbs by CE-MS – What are the respective contributions of CE and MS?

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Introduction

The detailed characterization of monoclonal antibodies (mAbs) is crucial for their safe and efficient use as biopharmaceutical. Various post-translational modifications (PTMs) arise either in the producing cells or during subsequent processing steps, resulting in a large number of possible proteoforms. In contrast to the widespread analysis on the peptide level, the characterization on the intact level enables the characterization of proteoforms without "puzzling" and with less artefacts from sample preparation. Mass spectrometry allows for the analysis of many PTMs on the protein level, however, positional isomers or proteoforms of small mass difference are difficult to characterize. Here, separation comes into play. Capillary electrophoresis is one of the most powerful separation tools for charge or size variants. Various new CE methods will be presented and discussed in the context of characterization of proteoforms by online coupled mass spectrometry.

Methods Various mAbs were analysed by CE-MS using an Agilent CE coupled via the nanoCEasy interface [1] to an Orbitrap Fusion Lumos or an trapped ion mobility MS (timsTOF SCP). The separation was performed in a 60cm capillary using 10-30kV separation voltage applying various coatings, including neutral coatings based on polyethylene oxide (PEO) or hydroxy propyl methyl cellulose (HPMC) and cationic coating in a successive multiple ionic-polymer layers (SMIL) approach using diethylaminoethyl-dextran (DEAED). Acidic background electrolytes (BGEs) using 4M acetic acid as well as neutral non-denaturating conditions using ammonium acetate have been applied.

Results

The DEAED-based coating leads to a relatively low reversed electroosmotic flow with an absolute mobility slightly higher than the one of antibodies, enabling the separation of variants with slightly different mobility. Thus, it is possible to separate the C-terminal lysine variants from the main form as well as several acidic variants and monoglycosylated mAb forms. As "acidic" variants also proteoforms with a single reduced disulfide bridge are observed, concluded from both a mass shift of 2 Da as well as a charge distribution shifted towards higher charge states. Applying native separation and electrospray conditions, an even better separation of the basic lysine variants is observed when using the HPMC coating. However, fewer acidic variants are observed. Forms with reduced disulphide bridge seem not to be separated from the main peak.

Overall, the combination of new separation methods in CE with high performance MS analysis reveals detailed inside in various proteoforms, including difficult to detect variants of small mass differences. The results are presented in the context of different mAbs and will be discussed in the view of additional technologies including ion mobility MS.

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Session 6: Pharmaceutical Applications
Oral Presentation

Targeting non-biological complex drugs: Complementary high-resolution mass spectrometric techniques for a detailed chemical description of complex active pharmaceutical ingredients

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Question

For centuries, complex drugs have been used, and their pharmaceutical effects have been observed, investigated, and proven by numerous pharmaceutical studies and patient reports. 2014, the pharmaceutical class of non-biological complex drugs (NBCDs) was introduced [1]. These drugs consist of active pharmaceutical ingredients (APIs) that are neither derived from living organisms nor sufficiently accessible through analytical techniques. As a result, the chemical composition is often only partially discovered, and the API is defined as the entire matrix and controlled by the production process. The insufficient chemical description hinders the evaluation of pharmaceutical studies, the distinction from copycat products, and the optimization of the production processes.

Methods

This work focuses on bituminosulfonates, which are pharmaceutical products tentatively assigned to NBCDs. These complex APIs have been analyzed via comprehensive two-dimensional gas chromatography coupled to electron ionization high-resolution time-of-flight mass spectrometer (GC×GC EI-HR-ToF-MS) and direct infusion ultrahigh-resolution mass spectrometry (DI FT-ICR MS). For the FT-ICR MS, we used different atmospheric pressure-based ionization techniques (ESI, APPI). An online derivatization method was developed to enable the applicability of gas phase analysis for the highly polar APIs [2].

Results

While the DI ESI(-) FT-ICR MS selective and sensitive addressed the sulfonates of the APIs without a minimum extent of sample treatment, with the online derivatization and GC×GC EI-HR-ToF-MS detected non-sulfonated residuals and showed the core structural motives of the calculated sum formula. For the combination of both techniques, the data was unified. Although the orthogonality of the methods was visible, the similarity in their results was the basis for addressing the differences. Therefore, DI APPI FT-ICR MS was used to combine and verify the molecular information of both techniques. The complementary use of the three ionization techniques (EI, ESI, APPI), the ultra-high mass resolution of the FT-ICR MS, and the high chromatographic resolution of GC×GC allows the in-depth chemical description of APIs that are defined by their analytical inaccessibility.

Conclusions

APIs of complex drugs are still some of the most challenging matrices. Not only are their isomeric and isobaric hard to access, but also, the regulatory environment demands high reliability of the results. Here, the orthogonality of both high-resolution techniques covers a broad chemical space and allows the identification of unknown molecules via the fragmentation pattern and molecular ions.

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Exposure to doping substances through intimate contact: Multi-analyte LC-(HR)-MS/MS methods for the analysis of drug residues in human seminal fluid and case-related application

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[2]*University Hospital Muenster, Münster, Germany*
[3]*Science & Research / U.S. Anti-Doping Agency, Colorado Springs, CO, United States*
[4]*European Monitoring Center for Emerging Doping Agents, Bonn, Germany*

Potential scenarios as to the origin of minute amounts of banned substances detected in doping control samples have been a much-discussed problem in anti-doping case managements. One such debated scenario has been the contamination of female athletes" urine with seminal fluid containing doping agents and/or their metabolites. In this context, assays for the detection of seminal fluid in urine have been reported [1], contamination of seminal fluid with urine has been described [2], and the first comparison of metabolite profiles of anabolic steroids in seminal fluid and blood plasma using an animal model was presented [3]. Yet, there is a lack of comprehensive studies on substance concentrations and metabolite profiles in human seminal fluid.

The aim of this study was to analyse 480 seminal fluid samples from non-athletes for substances classified as doping agents. In a first step, a screening procedure was developed for 77 substances using liquid chromatography coupled to a triple quadrupole mass spectrometer. Subsequently, suspect samples (i.e. specimens indicating the presence of relevant compounds) were re-analysed using a high-resolution/high mass accuracy mass spectrometer, and possible candidate metabolites of the drugs were identified.

In about 17 % of the 480 seminal fluid samples, 93 observations of suspected substances were recorded by the screening method, and about 90 % of the suspected cases were eventually confirmed. Substances detected included salbutamol, anastrozole, hydrochlorothiazide, cocaine, methylphenidate, metoprolol and several antidepressants. Occasionally, also phase-I and -II metabolites were found in the seminal fluid.

Of particular interest was a seminal fluid sample in which the analyte GW1516 was detected, especially in consideration of a reported doping control case involving this substance, in which a contamination scenario through intimate contact was suspected. Subsequent investigations provided analytical evidence for the presence of seminal fluid in one of the athletes" doping control urine samples, and analysis of the seminal fluid sample provided the first data on an authentic concentration level of GW1516 and its metabolites in human seminal fluid. The combined facts support the possibility that the Adverse Analytical Finding was caused by unprotected sexual intercourse and the plausibility of the case-related arguments.

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Benchmarking sample preparation and data analysis for clinical proteomics of human biofluids

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Liquid biopsies like plasma, cerebrospinal fluid (CSF), or urine are a less invasive and inexpensive alternative to tissue biopsies. Additionally, these body fluids are known to contain a multitude of known and yet to be revealed biomarkers that can be detected using mass spectrometry (MS) based proteomics. There are several distinct advantages of this technology: (I) it gives unbiased access to the biofluid proteome, (II) it is species independent, (III) it can reveal information about isoforms and post-translational modifications and (IV) it does not require availability of antibodies. Furthermore, MS based proteomics has been rapidly evolving over the last years due to the development of new generations of MS instruments such as timsTOF or Thermo Astral, advancements in sample preparation like nanoparticle-based protein enrichment, as well as novel AI based tools for data processing. The continuous progress results in increased depth and throughput of clinical proteomics.

However, this highly dynamic nature of the field can also be a drawback, as applications of mass spectrometry-based proteomics for larger clinical trials is often hindered by a lack of standardization across laboratories and studies. To address this problem and to enhance the integration of MS-based proteomics into medical research the CLINPSECT-M consortium and its MS laboratories conducted a round robin study on clinical specimens such as human plasma and CSF [1].

Here, the data from the round robin study is compared to internal benchmark studies on human biofluids that were performed at Evotec. In addition, while the focus of the round-robin study was to harmonize workflows for undepleted plasma and CSF and track interlaboratory reproducibility, the Evotec study also measured longitudinal platform stability and shows how nanoparticle-based proteomics using the ProteographTM can provide deeper coverage of the plasma proteome and at the same time overcome some of the challenges associated with undepleted workflows.

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Pancreatic islets of patients with Type 2 Diabetes has consistent lipidomics signature

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Type 2 Diabetes is a metabolism disorder that globally perturbs lipid homeostasis. However, little is known on if and how it affects the lipid composition of human pancreas and, specifically, pancreatic islets. Proteomics and transcriptomics pinpointed changes in the expression levels of lipid-metabolism related genes [1]. However, they could not be unequivocally associated with any diabetes specific lipids patterns or translated into informative lipid markers in plasma.

We applied laser capture microdissection (LCM) to isolate pancreatic islets and surrounding exocrine tissue from native cryo-sections of biopsies from non- diabetes (ND, n=12), impaired glucose tolerance (IGT, n=12) and type 2 diabetes (T2D, n=10) patients. In each cryo-slice we collected the material from ca. 20 islets which was equivalent to the total surface area of 0.5 mm2; extracted lipids with MTBE/MeOH and quantified the absolute (molar) amount of 164 species from 15 lipid classes, including glycerolipids, glyco- and glycerophospholipids, sphingolipids and cholesterol esters by high resolution shotgun lipidomics (NanoMate Triversa/Q-Exactive).

Our findings demonstrated a clear separation of the T2D, IGT and ND sub-cohorts by both islets and exocrine lipid composition. Pancreatic islets of T2D patients were enriched in triacylglycerols. At the same time, changes in lipid plasma composition were not significant altered. We observed specific changes in the abundances of species, albeit with no apparent preference to lipid class, unsaturation and length of fatty acid moieties. Together with metabolomics data acquired from the same biopsies, the analyses lead to a better understanding of T2D perturbed metabolism in pancreas.

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Chemical profiling of fingerprints and illicit drug desorption using SICRIT Ion source: A rapid analysis approach

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Forensic toxicology, a multidisciplinary field, involves diverse analyses ranging from biological sample differentiation to identifying illicit and non-illicit analytes. This study focuses on efficient targeted and non-targeted analysis of analytes in fingerprints, utilizing novel instrumental and computational approaches. Recent advancements in ambient ionization mass spectrometry, specifically plasma-based dielectric barrier discharge ionization (DBDI) sources like the SICRIT Ion Source, cover a broad range of analytes. This, coupled with thermal desorption sampling, facilitates rapid analysis while minimizing sample preparation.

The study employed a custom-built device for thermal desorption of standards (Polar and Non-Polar Lipids, Fentanyl, Cocaine, Heroin) and fingerprints, followed by ionization with a SICRIT ionization source. Detection and quantification utilized a high-resolution LTQ Orbitrap XL mass spectrometer. Extracted ion chromatograms (EICs) were processed with Xcalibur for calibration curves and limit of detection (LOD) calculations. Lipidomic samples, standards, and fingerprints underwent processing using an R-script and Python machine learning pipeline.

The pipeline, incorporating dimension reduction methods, classifiers, and cross-validation grid search, identified optimal models for differentiating individuals based on their spectral data. This showcases potential applications in forensic analysis.

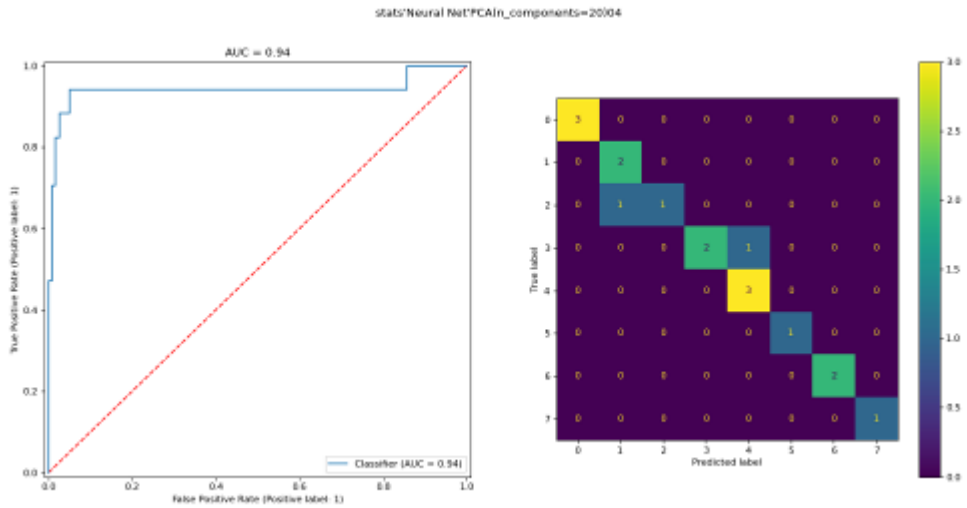
In a separate aspect, the study employed the high-resolution mass spectrometer to identify unknown compounds with exact mass measurement, focusing on Fentanyl, Heroin, and Cocaine in varying absolute amounts. Direct thermal desorption of samples in just 2 minutes revealed ionization of these compounds as protonated molecules. Despite manual sample introduction and instrument limitations leading to relatively high relative standard deviations (RSDs), sensitivity was deemed sufficient for qualitative or semi-quantitative measurements.

The technology's forensic potential expanded to differentiating individuals based on chemical fingerprint profiles, even for non-volatile compounds like lipids. Principal component analysis (PCA) and a machine learning pipeline demonstrated the ability to distinguish fingerprints from different individuals across multiple days with promising accuracy.

The study concludes that ambient ionization techniques hold potential forensic applications, offering rapid and effective differentiation of individuals based on chemical composition, particularly beneficial in cases with smeared fingerprints at crime scenes. Further investigation with diverse demographics and extended time frames is recommended to explore broader forensic use and comparisons with public databases. The ability to assign smeared fingerprints to potential suspects presents a valuable tool, showcasing the technique's potential impact in crime scene analysis.

Fig. 1: Confusion Matrix Classifier Example of Fingerprints

Fig. 1



MS1-base peptide identification and quantification by sparse encoding

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Peptide identification and quantification using Accurate Mass and Time (AMT) tags in liquid chromatography (LC) tandem mass spectrometry (MS/MS) data was proposed as an approach to partially or even completely obviate the need for subsequent MS/MS analysis. Recent developments in high-resolution mass spectrometers and deep learning offer higher accuracy of mass and retention time mapping, thus facilitating the further potential of AMT. Here we present a proof of concept experiment for joint peptide identification and quantification by expanding the AMT approach. We propose to de-convolute MS1 signals in a Scan-By-Scan (SBS) manner. For each MS1 scan, we construct a set of peptides from either an in silico digestion or prior deep proteome measurements, described by their accurate mass and predicted retention time. The joint identification and quantification is formulated as a spare encoding problem with LASSO loss, or a multi-variate regression problem focusing on inference of positive coefficients and sparsity regularization. We demonstrated that on the same dataset, the correlation between the quantification results from our method (MS1 only) and Maxquant reached a Pearson"s correlation of 0.948. In addition, our approach recovered and quantified 82.4% of the peptide sequences detected in the deep proteome measurement, while only 28.5% were identified by MaxQuant in the shallow measurement, highlighting the potential of MS1-based signal deconvolution. As a post-processing step to further select the elution peak in the case of multiple activation curves recovered by SBS, we trained a 1D-CNN true peak classifier that takes a single peak as input and predicts the score of the peak being a true elution peak, with an accuracy of 0.7.

Session 8: ComputationalMass Spectrometry
Oral Presentation

Streamlining MS data analysis and automatic spectral library generation in MZmine

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Liquid chromatography-high-resolution mass spectrometry (LC-HRMS) of complex samples rapidly generates gigabytes of data in a single study - of which only a fraction can be annotated, leaving a vast unknown chemical space to explore. This creates a need for flexible and scalable software tools for data processing. MZmine is a modular platform-independent software with a vibrant open-source community. Recent contributions have expanded its capabilities to support various MS platforms, leading up to integrative workflows that combine ion mobility spectrometry (IMS) data from LC-MS and MS imaging.1 Those workflows enable large-scale metabolomics and lipidomics research by spectral preprocessing, feature detection, and various options for compound annotation for thousands of samples in parallel. The modern graphical user interface and interactive charts facilitate data exploration and validation of results from every processing step. In addition, the MZmine Processing Wizard introduces easy-to-configure workflows for different MS platforms and research targets.

We will highlight the latest advances in the MZmine project, guiding large-scale untargeted MS data analysis as well as automatic reference data generation. The first part focuses on LC-MS and MS imaging data analysis and compound annotation by Ion Identity Molecular Networking2 to connect the known and unknown chemical space including the detection of a compound"s potential microbial origin.3 Furthermore, new data acquisition methods introduced for trapped-ion mobility spectrometry (TIMS)-MS imaging are presented, which increase confidence in compound annotation.4 The second part introduces a new open MS2/MSn spectral library that was generated with MZmine, leveraging a high-throughput MSn data acquisition strategy. A total of 15,000 bioactive compounds and natural products were analyzed within 10 days in both positive and negative ion modes (3 minutes for a mix of up to 10 compounds). Preliminary results gave an automatic annotation rate of over 80% of the compounds, yielding a mean of 44 MSn spectra per compound for various ion adducts and fragmentation energies. We expect this unique high-quality MSn tree library will accelerate the development of annotation tools and new machine learning-based tools.

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Comprehensive Plastic and Polymer Characterization by Combining DART and MALDI with High-Resolution Mass Spectrometry

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Plastic and polymers are widely in use in our society. To reduce the introduction of more material into nature, in particular as later microplastic, the EU started to set motivations and demands to recycle plastic material. For this purpose, a high level of information about pre-used material is needed to be able to recycle. Different analytical tools like FT-IR, GPC or pyroGC/MS are typically applied. Ionization techniques such as DART (Direct Analysis in Real Time) or MALDI allow for a direct and rapid fingerprinting of plastic material in combination with high-resolution mass spectrometers, for manufactured products as well as for used material or microplastic waste. DART was coupled with a thermal desorption/pyrolysis system to examine plastic and polymer compositions and compounds like additives and dyes in a direct way, based on their volatility in a temperature gradient. MALDI provides highly specific chemical information for polymer structural analysis, copolymer composition, complex polymer mixtures, and surface imaging, making it invaluable in various polymer analysis applications.

DART was coupled to a QTOF (both Bruker, Germany). While traditional GC/MS methods require sample preparation (10-20 min) and long analysis times (> 20 min), this new method allows for rapid and direct polymer analysis (< 5 min) without sample preparation. A small sample sliver is cut and placed in a copper pot of a thermal desorption system (IonRocket, Biochromato Inc., Japan) which delivers temperature gradients from ambient temperature to 600 ºC with a ramp rate of 150 ºC/min. When the thermal program is started, the QTOF collects MS spectra at 5Hz data acquisition speed for the entire run. MALDI was applied on an axial TOF/TOF MS (Bruker). Polytools (Bruker) and Polymerix (Sierra Analytics) software tools were used for detailed information retrieval down to the differentiation of structural isomers like polyethylene glycol methacrylate, methyl ether acrylate, and diglycidyl ether.

Depending on the temperature, different additives, the oligomeric basis as well as degradation products are released and detected at different time points with DART. Distinct m/z signals and general signal profiles of plastic materials were compared to characterize various plastic materials and to get a detailed overview of their complete composition. It was used in particular to track down the origin of microplastic material.

MALDI-MS/MS was used to characterize three types of polyethylene glycol structural isomers with equal oligomer masses. MS/MS has the advantage of being able to identify each end-group individually and is more specific than MS measurement alone.

Characterization of Beechwood Xylan using ultrahigh resolution mass spectrometry

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Xylans are the most abundant type of hemicelluloses in plant-based biomass [1], which is discussed as renewable alternative to fossil resources. For a useful application it is necessary to understand their properties and therefore characterize their exact structure. The identification of essential structural features of xylans can, for example, help to utilize xylan-rich wastes by improving the synthesis of commodity chemicals, such as furfural, from environmentally friendly sources [2]. One step to this structural elucidation is the development of new analytical methods to get the most information with the least possible effort. Therefore, a method to characterize the soluble and insoluble compounds of a beechwood xylan using graphite assisted laser desorption/ionization in combination with Fourier-transform ion cyclotron resonance mass spectrometry (GALDI-FT-ICR-MS) in the positive and negative ionization mode was developed in this study. To obtain more information about the beechwood xylan, also NMR and IR investigations were conducted. The analyses showed that the Xylan most likely is a partial acetylated glucuronoxylan which contains 4-O-methylglucuronic acid as substituent on the xylose main chain, which is typical for hardwood xylans [2]. Furthermore, it was possible to detect structurally different homologous series of xylan compounds using the HRMS analyses. The analytical methods developed here shall also be used in the future to characterize other xylans and biopolymers in order to establish biomass as alternative to fossil resources.

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100th anniversary and the expiration date has not been reached: aging patterns of Fischer-Tropsch-fuels (Kogasin)

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Significant reductions in carbon dioxide emissions are crucial to mitigate climate change. Transitioning to regenerative methods like heat pumps for domestic heat generation is time-consuming and costly. An efficient alternative is the use of synthetic carbon-neutral heating oils, requiring minimal adjustments to existing infrastructure. It has been a century since Franz Fischer and Hans Tropsch unveiled their groundbreaking Fischer-Tropsch synthesis in 1923, receiving a patent in 1925. This process enables the production of synthetic fuels (formerly known as Synthol) from diverse carbon sources[1]. Nowadays, coal (Coal-to-Liquid, CTL) no longer has to be used as a starting material; gas (GTL) or biomass (BTL) or, in future, CO2 extracted from industrial exhaust gases can also serve as a source and, thus, pave the way for the climate-neutral use of hydrocarbons, for example as heating oils. The use of regenerative heating oils, especially their joint storage with mineral residues in fuel tanks, remains largely unnoticed. Currently, precipitation from mineral heating oils is already a concern for operators and presents a challenge that must now be addressed and the question arises how stable are those Fischer-Tropsch fuels.

Two Kogasin samples from the Max-Planck-Institute were analyzed to investigate storage stability. The date of the samples can be pointed to the late 1950s, when the last Fischer-Tropsch synthesis experiments were carried out. A modern GTL was used as a reference sample, which, like the Kogasin samples, was stored in open containers, but only for six months. All mass spectrometric analyses were performed on a research-type Orbitrap Elite MS (Thermo Scientific, Bremen, Germany). The mass resolution was set to 480 k at *m/z* 400, corresponding to a transient of 1.5 s. For atmospheric pressure chemical ionization (APCI) a corona discharge needle was used with voltages around 5 kV. Electrospray was performed in positive and negative polarity. Spectral stitching in a mass range of 150-1000 amu was set to 30 amu windows with 5 amu overlap.

The results indicate that even paraffinic fuels such as Fischer-Tropsch liquids can age, albeit at a much slower rate that even natural fossil fuels or even other renewable fuels such as HVO and FAME. Here, we can show that fossil fuels and Fischer-Tropsch fuels all undergo an oxidative aging process, but at different time intervals. The samples are subjected to stepwise oxidation and potential oligomerization. While fuels typically undergo precipitation as part of the aging process, these examined fuels exhibit notable stability in this regard. This suggests that Fischer-Tropsch fuels serve as a viable substitute for non-electrifiable applications such as Kerosene or emergency power generators.

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High-resolution AP-SMALDI imaging of neurotransmitters in the parasitic flatworm *Schistosoma mansoni*

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The parasitic flatworm *Schistosoma mansoni* is causing schistosomiasis, a prevalent disease affecting hundreds of millions of people globally. Worm pairs reside in blood vessels where they generate approximatly 300 eggs daily, of which nearly half get trapped in host tissue, contributing to the pathology of schistosomiasis. The involvement of neurotransmitters is pivotal in the development and fertility of female worms. Therefore, exploring the occurrence and distribution of neurotransmitters in worms with varying mating status is a crucial area of investigation for identifying potential drug targets.

We studied the distribution of neurotransmitters in mature *S. mansoni* couples, immature worms and genetically altered worms. Before measurements, samples were derivatised with 2,4,6-trimethylpyrylium tetrafluroborate (TMP)[1] to enable neurotransmitter detection. On-tissue derivatisation and matrix application were performed on a high-resolution pneumatically assisted spraying device (SMALDIPrep, TransMIT GmbH, Giessen, Germany). Samples were analysed by atmospheric-pressure scanning microprobe MALDI mass spectrometry imaging (AP-SMALDI MSI). Sections were prepared using a cryotome. Imaging experiments were carried out using an AP-SMALDI5 AF ion source (TransMIT GmbH) coupled to a high-resolution orbital trapping mass spectrometer (Thermo Scientific Q Exactive HF, Thermo Fisher Scientific (Bremen) GmbH, Bremen, Germany).

Imaging neurotransmitters poses a significant challenge owing to their poor ionisability. On-tissue derivatisation using ionization-enhancing agents is imperative for MALDI MSI of neurotransmitters. In our investigation, we evaluated varying concentrations of TMP to optimize the derivatisation process for neurotransmitters. After TMP derivatisation, various neurotransmitters were detected in worm couples. The signal intensity of neurotransmitters was markedly higher in males compared to females, with no detectable neurotransmitters in immature females. These findings support the reported hypothesis of a male-dominated factor involved in female maturation.[2,3]

Preliminary MSI measurements of genetically modified worm couples, wherein the neurotransmitter-producing gene was silenced, revealed a signal at the *m/z* value corresponding to the targeted neurotransmitter. Intriguingly, no significant difference was observed between the silenced and control couples, indicating the presence of two isomeric forms of the neurotransmitter that remain indistinguished. We are exploring derivatisation reagents designed to generate distinguishable products in our ongoing investigations.

Acknowledgement
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3D mapping of molecules from a colon cancer spheroid model using MALDI MSI

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Matrix-assisted laser desorption/ionization mass spectrometry imaging (MALDI-MSI) is the go-to technique for describing label-free and *in situ* the molecular composition of biological samples [1]. Organoids and spheroids are very attractive 3D cell cultures due to their similarity with the *in vivo* physiological conditions, their human origin, and their contribution towards animal-free research [2]. Their analysis brings light on cell-cell and cell-microenvironment molecular composition and interactions, which creates opportunities in cancer research and drug screening. However, 3D molecular characterization is required for a complete understanding of the molecular composition and chemical changes of the system. Here, we developed a workflow that includes the generation of 3D molecular maps based on mass spectrometry imaging data of colon cancer spheroids composed of human HT-29 colon cancer cells and CCD-1137Sk fibroblasts [3,4].

Bi-culture spheroids were embedded using an in-house developed cryo-mold, flash frozen in liq.N_2 and sectioned at 20 µm with a CM 1950 cryostat (Leica). The sections were mounted on ITO slides, then sprayed with matrix using a TM-Sprayer M3 (HTX Technologies), and imaged at 20 µm lateral resolution and 40k spectral resolution using timsTOFflex MS (Bruker Daltonics) in tims ON mode, negative ionization mode. The acquired MS images were individually exported as imzML files using SCiLS Lab (Bruker) for automatic stacking and 3D visualization using in-house M2aia software [5]. Data analysis was done in SCiLS or using in-house developed scripts in R language.

We developed a MALDI-MSI workflow suitable for micron-scaled biological samples, such as spheroid cultures (Figure 1). We used multivariate analysis to select morphologically representative features which illustrated the layered distribution of the two cell lines used to build the spheroid, different distributions of cell-specific molecules, and intensity gradients of molecules present in both cell types. We then reconstructed spheroids in 3D by automatically registering ~45 adjacent tissue sections. Cell type and cell layer-specific metabolites were distinguished, suggesting the possibility to identify relevant markers for biological mechanisms typical to each cell type. Thus, cell-cell interactions, invasive cell migration, tumor progression, and other spatially relevant biological interactions can be monitored with this technology.

Figure 1. Full workflow developed for spheroid analysis, including sample preparation, MS image acquisition and 3D reconstruction of certain lipids.

Our high-throughput spatialomics method from micron-scale samples revealed cell-specific metabolic fingerprints.

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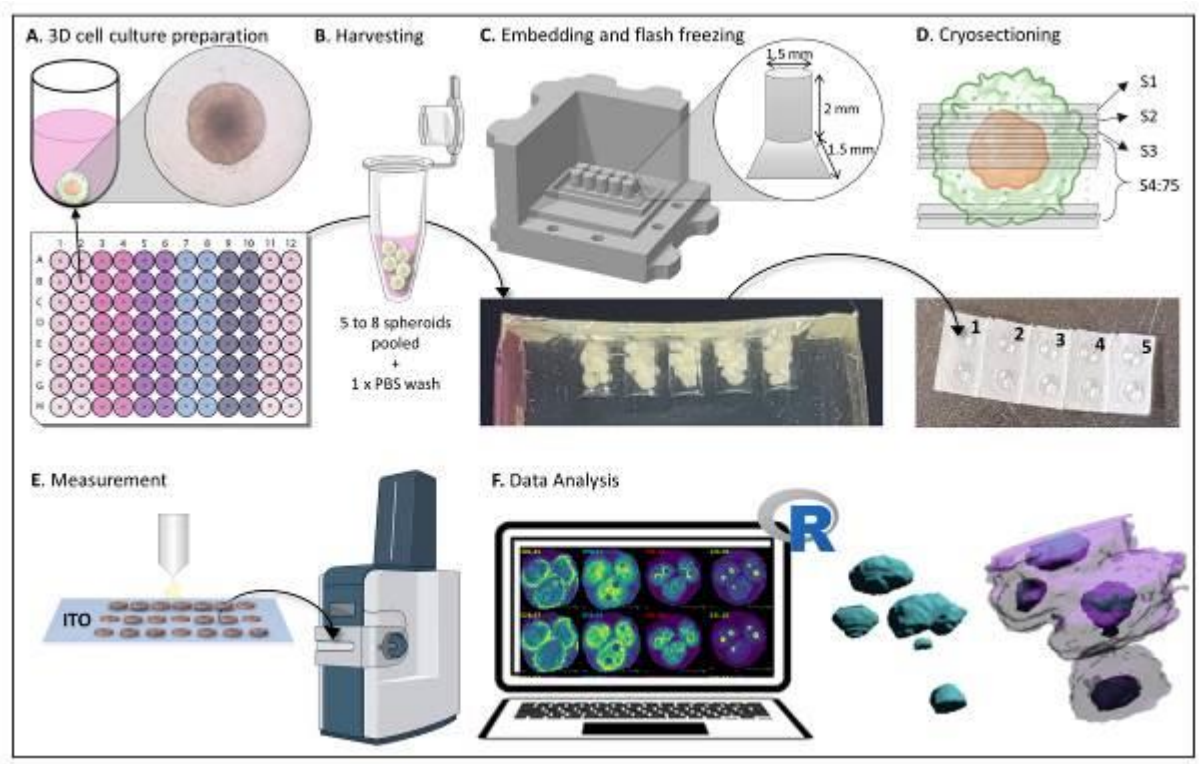
Fig. 1



Histology-guided single-cell MS imaging using a transmission-mode MALDI-2 ion source with integrated bright-field and fluorescence microscopy

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Introduction:

Transmission-mode MALDI combined with laser-induced postionization (t-MALDI-2) enables MS imaging (MSI) at single-cell resolution and pixel sizes down to about 1 µm x 1 µm. [Niehaus *et al., Nat. Methods,* 16 (2019)] Here we present the amendment of the method by on-line coupling with bright-field and fluorescence microscopy from the same sample slides including an inherent co-registration. We demonstrate the abilities of our correlative imaging method with selected tissues and cell cultures. A modified timsTOF fleX MALDI-2 instrument (Bruker) was utilized as mass analyzer.

Methods:

Tissue sections (7-10 µm thick) and cell cultures prepared on chamber slides were briefly fixed with formaldehyde and stained for fluorescence microscopy with small-molecule stains (e.g., Hoechst 33342) as well as fluorophores (e. g. Alexa Fluor 594) conjugated with specific antibodies. After being washed, dried, and scanned with a slide scanning microscope (VS200, Evident), samples were coated with MALDI matrix using optimized sublimation/recrystallization protocols. Inside the ion source, slide scanning bright field and fluorescence microscopy images were collected. Using the same piezo-actuated XYZ stage and objective, t-MALDI-2-MSI analysis was performed with pixel sizes of 1 x 1 µm². Overlays were generated using flexImaging (Bruker) and SCiLS Lab (SCiLS/Bruker).

Results:

Careful optimization allowed for the MALDI compatible staining of different tissues (murine brain and tumor) as well as cells cultured on a glass slide (THP-1 derived macrophages). This included unspecific stains of the cell bodies and nuclei as well as specific staining of lipid droplets and antibody based staining of neutrophils (anti-Ly6G) in a tumor as well as the Purkinje cell layer in the cerebellum (anti-calbindin). Subsequent t-MALDI-2-MSI was performed at 1 µm pixel size, yielding information-rich spectra, especially in the lipid mass range. Inherent co-registration with high fidelity between microscopy images and t-MALDI-2 allowed defining specific single cells as regions of interest (ROI) based on the high specificity and high resolving power of fluorescence microcopy. Together, this enabled the generation of single-cell mass spectra for specific cell types within the tissue allowing for a statistical analysis of their molecular make-up and its correlation to their exact location. Next to the definition of ROIs in data processing, pre-MALDI-MSI slide scanning also allowed for an accurate definition of measurements regions, effectively reducing measurement time. Next to tissue sections, we will demonstrate the analysis of cell cultures with sub-cellular resolution and direct co-registration of optical microscopy data. Here, a direct comparison of specific stains of cellular compartments (lipid droplets) and MSI data was used to characterize the molecular profile of THP-1 derived macrophages during maturation and differentiation on a cellular and subcellular level.

Comprehensive two-dimensional-LC-MS approach for profiling cardiolipin distribution in complex biological matrices

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Analyzing low-abundant phospholipids, particularly cardiolipins (CL), poses significant challenges. The class of CLs, found exclusively in the inner mitochondrial membrane, has gathered increased attention due to its association with mitochondrial dysfunction and various neurodegenerative diseases. It is imperative to understand the natural distribution of CLs, but their absolute quantification is challenging. To address this, we propose a referencing method using high-resolution mass spectrometry (HRMS) and charged aerosol detection (CAD) in a parallel setup following two-dimensional liquid chromatography (2D-LC) separation.

Phospholipids are categorized into subclasses based on their polar head groups. Utilizing an online 2D-LC heart-cut setup described by Helmer et al.[1], we achieve sensitive detection of CL species through HRMS by combining hydrophilic interaction liquid chromatography (HILIC) in the first dimension and reversed-phase (RP) separation in the second dimension. The analysis of biological samples faces challenges in comparing them, due to variations in sample amount and preparation. Hence, referencing is crucial for evaluating alterations in CL distribution. By incorporating CAD into the analytical setup, a quantitative approach for detecting high-abundance phospholipid classes, such as phosphatidylcholine (PC), is implemented post-HILIC separation in the first dimension.

HRMS is employed for the identification of CL distribution after species separation in the second dimension. Effective chromatographic separation of homologous CL species based on fatty acyl residues is achieved, and HRMS detection enables identification by accurate masses. This is confirmed through additional data-dependent MS/MS experiments. Characteristic fragments facilitate the identification of bound fatty acyl residues for most CL species. Kendrick mass plots, normalized on the CH2 Kendrick mass defect (KMD), are employed for data visualization. Despite complex sample matrices, our approach ensures reliable identification of CL species based on carbon chain length and degree of saturation in fatty acyl residues, considering the aforementioned detection and data processing techniques. The incorporated parallel CAD not only enables normalization of the total CL content for confident comparison between biological samples but also ensures online monitoring of the 2D-LC analysis in both separation dimensions.

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Lipids flux rate by full organism ^{15}N labeling and shotgun ultra-high resolution mass spectrometry

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Stable isotope tracers (e.g. ^{13}C, ^{2}H, ^{15}N) have been widely used in lipid flux analyses, and among ^{15}N labeling yields moderately simple isotopic profile and differentiates the lipid turnover from fatty acid synthesis. However, ^{15}N incorporation contributes minor shift in lipid masses, and is difficult to obtain an isotopic fine structure with conventional mass spectrometers.

Herein, we developed a workflow that combines *in vivo* ^{15}N metabolic labeling and shotgun ultra-high resolution mass spectrometry to systematically determine the absolute abundance and assess the turnover rates of lipids in body fluids and tissues of mice. Metabolic labeling was carried out by feeding young- and old-aged mice with a ^{15}N-labeled SILAM diet for five different lengths of time within 0 to 21 days. Lipids were extracted using methyl *tert*-butyl ether extraction and quantified by shotgun lipidomics. To achieve ultra-high resolution (~1.5M @ *m/z* 200), we coupled Q Exactive Orbitrap MS with an external data acquisition system (Booster X2) and accessed time-domain signals (transients). The transients were then processed using Peak-by-Peak software and lipids were identified and quantified by LipidXplorer software.

With the employed workflow, we resolved ^{13}C isotopes of unlabeled and monoisotopic peaks of ^{15}N labeled lipid species (Δm = 0.00633 Da). Further, concurrently we determined the molar abundance and turnover rates of over 120 nitrogen-containing species covering glycerophospholipids, sphingolipids, and glycosphingolipids in mouse plasma, whole blood, four distinct regions of brain and liver. Furthermore, a wide range of specimen-specific, lipid class and molecular species-characteristic turnover kinetics were observed across ages. Of note, lipids with ethanolamine (LPE, PE, PE O-) and serine (LPS, PS) head groups showed rapid turnover and relatively higher (ca. 2-fold) rate in comparison to the choline-containing (LPC, PC, SM) lipids. Further to mention, the kinetics of turnover strongly differed among diacyl glycerophospholipid species in contrast to lysolipids. Moreover, brain regions (cortex, cerebellum, striatum, and hippocampus) exhibited much delayed and lower (ca. 4-5 fold) lipid turnover rates and discrete kinetic profiles than body fluids and liver.

Taken together, for the first time we monitored lipidome-wide fluxes at the full organism level across ages, which might serve as a unique reference for a better understanding of the dynamics (metabolism and transport) of lipid species in mammals.

Dimethylglyoxal: A reactive pyruvate metabolite that mediates neurological complications of diabetes

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Complications of diabetes are often attributed to glucose and its reactive metabolites, such as methylglyoxal and glyoxal. Those α-dicarbonyls react with amino acids and modify proteins, known as advanced glycation end products (AGEs), thereby inducing oxidative stress and inflammation. In addition, acute hyperglycemia is associated with poor functional outcome after ischemic stroke. However, neurons and brain endothelial cells primarily utilize lactate, which is considered a safer route of energy consumption. Therefore, diabetes-related cognitive impairment may have a cause distinct from other diabetic complications.

α-Dicarbonyls were derivatized with deuterated *o*-phenylenediamine (d8-OPD) and analyzed by liquid chromatography-mass spectrometry (LC-MS/MS), using a C18 column and labeled methylglyoxal (d4-MG) as internal standard. Diabetic conditions in mice were induced by streptozotocin (STZ) injection. Long-term treatment was achieved via DMG in the drinking water. After 12 weeks, an object place recognition test (OPRT) was performed. Injection of glucose prior to a permanent middle cerebral artery occlusion (MCAO) was conducted to model hyperglycemic stroke.

Mice with chronic hyperglycemia displayed cognitive impairment in the OPRT, reflecting neurological complications of diabetes. Surprisingly, when analyzing α-dicarbonyls in plasma after derivatization with unlabeled OPD, we observed a higher signal for the internal standard 2,3-dimethylquinoxaline (2,3-DMQ) in diabetic mice. Assuming that hyperglycemia increases either 2,3-DMQ or dimethylglyoxal (DMG), the α-dicarbonyl that generates 2,3-DMQ upon derivatization, we replaced the derivatizing agent with d8-OPD and used d4-MG as internal standard. Indeed, increased levels of DMG in plasma and brain of diabetic mice were detected. Stable isotope-tracing in mouse brain endothelial cells showed that DMG is generated from glucose via two molecules of pyruvate with a pronounced production of under hypoxic conditions. *In vivo* results confirmed that cerebral ischemia increased DMG levels in both plasma and brain when accompanied by acute hyperglycemia. The knockout of Ilvbl, a gene homologous of acetolactate synthase that mediates DMG formation in bacteria, lowered DMG levels in the brain of diabetic mice and attenuated increased DMG brain levels after hyperglycemic stroke. Both *in vivo* and *in vitro*, DMG induced oxidative stress and inflammation. Long-term treatment with DMG led to signs of cognitive decline in the OPRT, resembling those observed in the STZ model. Therefore, treatment with DMG mimicked neurological complications of diabetes.

Our work reveals DMG as an unrecognized reactive glucose metabolite. The generation of DMG in the brain was mitigated by an *Ilvbl* knockout and DMG treatment led to cognitive impairment in mice. Therefore, the pathway leading to DMG formation is a potential pharmacological target to reduce neurological complications of diabetes and hyperglycemic stroke.

Analysis of Dried Blood Spots (DBS) in doping controls by means of mass spectrometry

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Apart from the ever-growing list of substances banned in sport and the resulting analytical challenges, sampling processes are also constantly being optimized. Therefore, in addition to the conventional analysis of already established sample matrices such as urine and blood (serum, plasma), the analysis of minimal-invasively collected dried blood spots as doping samples has recently been established. Numerous advantages in sample collection, transportation and storage are obvious for this matrix. However, automation of the sample preparation and measurement can also be a major advantage in sample processing, if the sample materials used are standardized. Here, cellulose-based DMPK cards and polymer-based TASSO systems are currently favored in doping controls, but further developments are expected soon. After extraction, target analytes are chromatographically separated and measured using HPLC coupled with mass spectrometry. High-resolution mass spectrometers are preferentially employed, allowing for comprehensive testing of a large number of analytes of various substance classes (anabolic agents, stimulants, corticoids, diuretics, peptide hormones, narcotics, etc.). The following contribution summarizes the existing MS-based methods, including their advantages and disadvantages. A special focus is placed on the current state of automation and the requirements for mass spectrometry.

Nanosecond infrared laser (NIRL) ablation for 3D tissue sampling: How we can apply spatially resolved proteomics on intact tissue specimens

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Several tissue types (e.g. intestine, skin) are structured in layers. To answer research questions related to processes in specific regions or the interactions between different cell layers, it is essential to take this tissue structure into account. Common approaches involve enzymatic, chemical, or mechanical methods for the separation of different tissue layers. However, important components of the tissue microenvironment (e.g., extracellular matrix) and spatial information in the tissue structure are lost. Moreover, artefacts are produced to the biomolecules prior analysis resulting in false results.

Therefore, a technique is needed for the layerwise sampling of tissues without causing changes in the sample composition.

A novel approach utilizes mid-infrared ultrashort laser pulses for the simultaneous homogenization and sampling of tissue. Water molecules within the cells are excited by irradiation with the IR laser. The absorbed energy is converted into vibrational motion of the OH stretching band causing an explosion of the water molecules. As this process is faster than the thermal relaxation time, the tissue is transformed into an aerosol by cold vaporization. During this soft homogenization process all biomolecules that were previously in the tissue are solubilized and remain intact. Furthermore, only the irradiated tissue area is removed without damaging the adjacent tissue.

Here, we utilized NIRL ablation for direct layer-by-layer sampling of embryonic mouse head to spatially resolve and analyze the tissue structures and forebrain regions by subsequent differential quantitative bottom-up proteomics.

NIRL-based 3D tissue sampling of embryonic mouse head (n=5) was performed using a wavelength of 2940 nm. The emerging sample aerosol was collected on a glass slide, tryptically digested and further analyzed by mass spectrometry-based bottom-up proteomics. Database search was performed with Proteome Discoverer. Data processing and statistical analysis was performed using R Studio. The HarmonizR framework was used for batch effect reduction.

In our study, we uncovered the proteome composition of embryonic skin and bone structures, meninges, and cortex lamination in situ with a spatial resolution of 40µm. Overall, we identified 5031 proteins including 3126 proteins with quantitative information. Our data showed unique proteome profiles for extra- and intracranial ablated layers comprising expected cell-specific proteins. Additionally, known cortex markers like SOX2, KI67, NESTIN, and MAP2 showed a layer-specific spatial protein abundance distribution from the scalp to the ventricular zone of embryonic mouse head. Furthermore, we identified MTA1 and NMRAL1 as potential new cortex marker proteins for future research and diagnostic purposes.

Our findings highlight the potential of NIRL-based 3D tissue sampling to specifically sample thin tissue layers in regions of interest like the scalp or meninges to address related research questions.

Session 13: Instrumentation and Automation
Oral Presentation

Improved target, suspect- and non-target analysis of environmental contaminants in wastewater using a GC-EI&CI-TOFMS

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Wastewater plants can release aqueous effluents containing harmful pollutants, posing risks to ecosystems and human health. Given the dynamic nature of wastewater composition, regular target and non-target analyses are frequently conducted. Due to its complimentary polarity range, gas chromatography mass spectrometry (GC-MS) is often employed alongside liquid chromatography (LC) MS to evaluate potential hazard profiles, to identify compounds and to gain a more complete overview of the potential hazards present. However, conventional GC-EI-MS is limited in its identification confidence for some compounds due to unspecific fragmentation pattern, absent molecular ion signals or by not being available in reference libraries, leading to a lack of compound identification. Additionally, with little to no prior knowledge of sample constituents, a broad extraction method is paramount.

For this study, 24h effluent samples were collected from an industrial plant over two months. Analytes were enriched by headspace solid phase microextraction (SPME) using a novel hydrophilic-lipophilic balanced (HLB) fiber coating (CTC, CH. An 7890A GC (Agilent, USA) and was coupled to a newly developed dual ionization source time-of-flight (TOF) MS (ecTOF, TOFWERK, CH) [1]. Here, a standard 70 eV electron ionization (EI) source and a medium pressure chemical ionization (CI) source are operated simultaneously on a single mass analyzer. Furthermore, the system allows for a simple selection of different CI reagents ions (e.g., [NH4]+, [N2H]+, [H3O]+) between chromatographic runs, which enables the adjustment of reactant selectivity and the degree of fragmentation [2].

This talk will outline different approaches for compound identification in GC-MS experiments by using the simultaneously acquired EI and CI mass spectra. Additional CI information on the detected compounds due to the selection of different CI reagents ions is shown to be highly valuable for the compound identification process. The available GC-EI&CI TOF-MS data is further used to adapt the widely used LC-based workflow to report confidence of identification for compounds found in the wastewater samples (Figure 1). Moreover, a novel HLB-SPME material was tested as a solvent-free headspace extraction of the wastewater constituents and shown to extract a broad range of analytes. In combination with the GC-EI&CI TOF-MS, this methodology can be used to complement commonly employed methods such as LC-ESI-HRMS used in industrial wastewater analysis.

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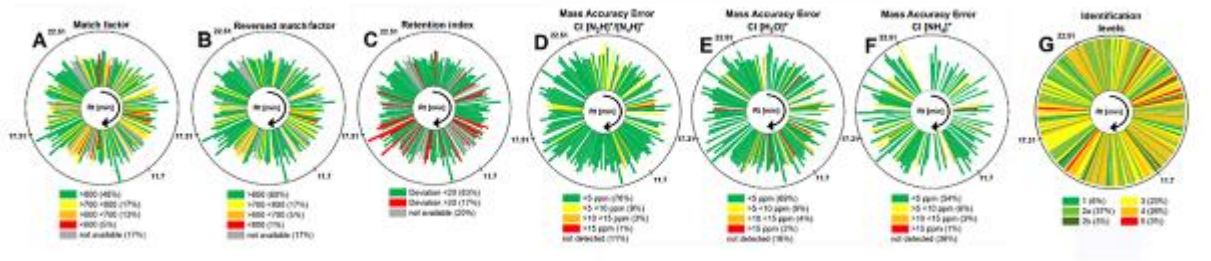
[2] Bräkling, S. et al. J. Rapid Commun. Mass Spectrom., 2023. 37:e9461, 3, 499–509.

Fig. 1



Bioanalytical forensic methods to prove poisoning by banned nerve agents: Current status of targets and techniques

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Nerve agents (NA) include organophosphorus (OP) poisons like sarin, VX and those of the Novichok group, that might cause death by respiratory paralysis.

OPNA react with acetylcholinesterase (AChE), an enzyme playing the essential role in the degradation of the neurotransmitter acetylcholine (ACh) in the synaptic cleft. Following this chemical reaction (phosphylation of the nucleophilic serine residue in the active center of the enzyme) AChE is inhibited, does not degrade ACh anymore and thus induces a cholinergic crisis resulting from the overstimulation of the effector cells.

Most well-characterized OPNA are excreted from a surviving organism within a few days after uptake thus hampering from their bioanalytical detection. Therefore, for forensic reasons (verification) biomarkers with a much longer half-life *in vivo* are required to prove poisoning even though some weeks might have passed between exposure and sample drawing.

Such beneficial biomarkers are generated from diverse endogenous proteins that had reacted with OPNA yielding phosphylated protein adducts.

This talk presents the current state of adduct analysis [1] focussing on i) reactive nucleophilic target side chains of amino acids and ii) mass spectrometry-based techniques for adduct detection and identification. Examples of real poisoning cases are presented obtained from the Middle East [2] and attempted suicides with related OP pesticides [3-5]. In addition, elaborated procedures applied to *in vitro* samples of Novichok agents will be discussed [6,7].

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The interplay of ubiquitination and phosphorylation in CaMKIIα function during synaptic transmission

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Communication between neurons takes place at the synapses through the action-potential-mediated release of neurotransmitters (NT). NTs are stored in synaptic vesicles (SV) at the presynaptic nerve terminal, where they undergo a trafficking cycle involving Ca2+-triggered SV exocytosis after depolarization of the plasma membrane, followed by SV endocytosis and regeneration. Protein phosphorylation is known to fine-tune these processes. Recent studies implicate protein ubiquitination as a further post-translational modification involved in NT release. Yet a comprehensive picture of the specific proteins and sites that undergo (de-)ubiquitination at the synapse is still lacking. Furthermore, there is no information on how specific ubiquitination sites of synaptic proteins change in response to depolarization and Ca2+ influx into synapses. Here, we performed a mass spectrometry (MS)-based, global quantification analysis of ubiquitinated proteins in isolated and functional active nerve terminals, termed synaptosomes. We identified more than 5000 ubiquitination sites in more than 2000 proteins. By using a TMT-based quantification analysis of ubiquitinated peptides we showed that more than 40 proteins undergo ubiquitination changes during Ca2+ influx. One of these is the key synaptic kinase Ca2+/calmodulin-dependent kinase II α (CaMKIIα), which was previously shown to undergo autophosphorylation at T286 in the presence of Ca2+ during activation of the kinase. We observed that CaMKIIα is simultaneously phosphorylated at T286 and ubiquitinated at K291 in the presence of Ca2+. As both modification sites lie within the same tryptic peptide, it was unclear whether the measured decrease in K291 ubiquitination reflects "true" deubiquitination or is due to the stark increase in T286 phosphorylation. To address this question, we quantified the absolute amounts of the CaMKIIα peptide singly ubiquitinated at K291 and doubly modified by phosphorylation and ubiquitination at T286 and K291, using parallel reaction monitoring (PRM) with standard peptides. PRM demonstrated that the observed deubiquitination at K291 upon Ca2+ influx is due partly to T286 autophosphorylation and partly to "genuine" deubiquitination. To monitor the effect of ubiquitination on CaMKIIα autophosphorylation at T286 and hence CaMKIIα activity we expressed the fluorescence resonance energy transfer (FRET) probe, Camui, along with a mutant form lacking the ubiquitination target lysine site K291 in HeLa cells. Using FRET measurements and PRM-MS we observed that the non-ubiquitinated variant exhibits dramatically higher levels of T286 autophosphorylation upon Ca2+ influx compared with the wild type (WT), suggesting that ubiquitination at K291 may inhibit CaMKIIα autophosphorylation at T286. Consistently with this, neurons expressing the non-ubiquitinated variant displayed greater synaptic activity than did the neurons expressing the WT Camui probe.

PTMNavigator: Interactive visualization of differentially regulated post-translational modifications in cellular signaling pathways

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Question

A central element of cellular signaling are protein post-translational modifications (PTMs). Upon a perturbation such as a drug treatment, many cellular proteins undergo a change on PTM level, which can have fundamental consequences for the phenotype. While PTM data nowadays is routinely acquired by proteomics labs, it is still hard to "connect the dots", i.e. to interpret the regulated PTMs in the context of signaling cascades. Here, we present PTMNavigator, a web-based software tool to explore PTM proteomics datasets from a pathway-centric viewpoint. We collected hundreds of manually curated pathways for 10 different species from publicly available databases. Our software is able to render these pathways in an interactive fashion and project experimental PTM data onto them. The tool is hosted on ProteomicsDB (https://www.proteomicsdb.org/analytics/ptmNavigator), and users can either browse through the data available there or upload their own experimental results.

Methods

KEGG and WikiPathways provide pathway definitions in the form of XML files that describe which proteins are part of a pathway and how they are connected. We first implemented a Python package that converts these files into a uniform internal rePresentation (https://github.com/kusterlab/pathway-importer). Then we developed a WebComponent that can combine these rePresentations with a list of modified peptides measured in a perturbation experiment and renders them together in an interactive fashion (www.github.com/kusterlab/biowc-pathwaygraph). Finally, we created PTMNavigator, which is a Vue.js-based user interface that is wrapped around biowc-pathwaygraph and is embedded into ProteomicsDB. Users can temporarily upload their data in order to visualize them with PTMNavigator. Our software is open source and we intend it be reused outside of ProteomicsDB as well.

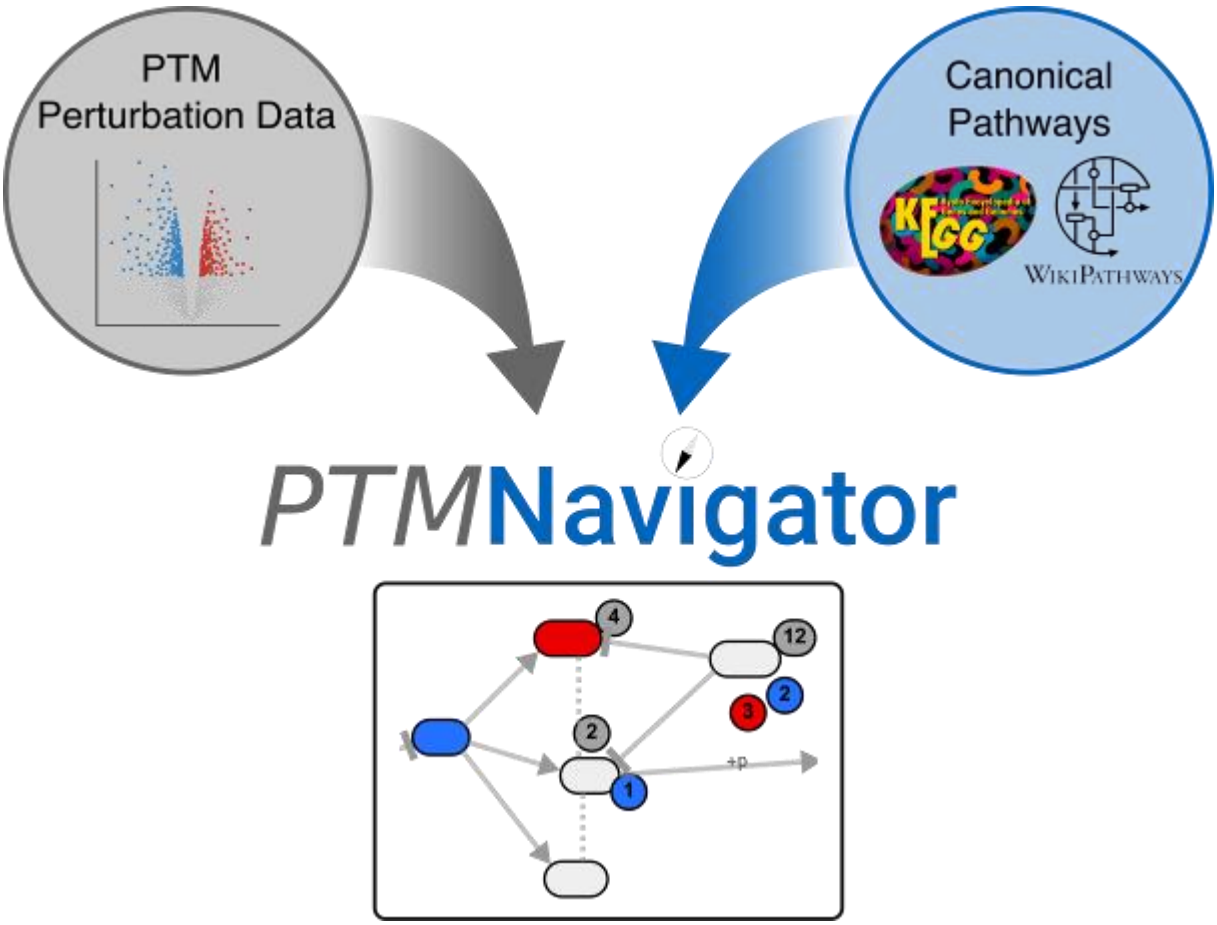
Results

Using a number of recently published datasets, we demonstrated some applications of our software.First, it enhanced pathway enrichment analysis by showing how regulated peptides are distributed in pathways with high enrichment scores, thereby helping the user to confirm or deny whether the suggested pathways are actually relevant. Second, it visualizedhow kinase inhibitors with three different targets perturb the PI3K/AKT/mTOR phospho-signaling cascade at different stages. Third, it aided in extending existing pathways by uncovering previously unknown relationships between PTMs and pathway components. Specifically, we were able to propose GRLF1 as an additional member of the signaling network affected by inhibition of MEK and uncovered a potential phosphorylation feedback loop from MAPK via p90RSK to SOS.

Conclusions

We present an easy-to-use web tool for the visualization of PTM signaling networks and the creation of publication-level figures. We envision that PTMNavigator will facilitate the study of PTM-pathway interactions and enhance our understanding of cellular signaling dynamics.

Fig. 1


PTM
Perturbation Data
Canonical
Pathways
KEGG
WIKIPATHWAYS
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4
12
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1

Characterization of metabolome alterations in barley (*Hordeum vulgare* L.) induced by *Bipolaris sorokiniana*

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Spot blotch caused by *Bipolaris sorokiniana* (teleomorph *Cochliobolus sativus)* is one of the most common foliar diseases of barley (*Hordeum vulgare* L.) worldwide and responsible for major losses of crop yield. Breeding resistant barley varieties has proven to be an effective countermeasure for protecting agricultural production. Plants react to pathogen attack by the upregulation or biosynthesis of secondary metabolites as one of their molecular defense strategies.

So far, quantitative trait loci (QTL) affecting resistance against spot blotch have been mapped, but the molecular understanding of the metabolic defense responses in the plant still needs to be included. In this project, molecular marker compounds for an infection of barley with *Bipolaris sorokiniana* are examined by means of untargeted UPLC-TOF-MS metabolomics and lipidomics techniques. By the analysis of quantitatively resistant and susceptible barley genotypes, metabolites are identified and quantified that activate resistance on a molecular level. Co-chromatography with reference substances, chemical synthesis and chromatographic isolation followed by UPLC-TOF-MS, LC-MS/MS and 1D/2D-NMR experiments revealed the chemical structures of key phytochemicals. Their localization in the infectious hotspots were further mapped using desorption electrospray ionization mass spectrometry imaging (DESI-MSI). We examined changes in the metabolism of fatty acids, fatty acid oxidation products as well as defense-related secondary metabolites, such as hordatines, phenolamides and flavone glucosides.

These metabolites can serve as biomarker molecules in screening plants for disease and resistance. The combination of known genetic and novel metabolic understanding of plant-pathogen interactions and resistance meachanisms is essential for purposeful breeding of resistant barley cultivars in the future.

LC-MS/MS detection of fossil metabolites in 45-million-year-old leaves

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Under favorable conditions, organic material of ancient angiosperm leaves may survive, at least to some extent, for millions of years [1]. However, little is known about the composition of individual fossil metabolites and degradation processes during fossilization. Some fossil leaves, such as those from the about 45-million-year-old brown coal from Geiseltal, Germany, are particularly well preserved, showing even preservation of green color. This exceptional color preservation is a phenomenon that has only been observed at very few localities worldwide. Early chemical studies [2, 3] suggested the presence of chlorophyll derivatives in green leaves from Geiseltal (see Figure) and motivated us to investigate such fossils using modern mass spectrometry techniques.

In order to extract as little as possible from the valuable fossil material, we applied highly sensitive and selective targeted LC-MS/MS for identification of individual metabolites. Extracts of fossil leaves were screened for more than 50 common plant metabolites and degradation products including polyphenolic compounds, carotenoids, and chlorophyll derivatives within a single LC-MS/MS run (negative and positive ion mode) using a Sciex QTRAP mass spectrometer with ESI source. As would be expected for fossil samples, for most of the targeted compounds, no significant signals above the background noise were observed. Remarkably, however, distinct multiple-reaction monitoring (MRM) transitions were obtained for two flavonoid pigments and several chlorophyll degradation products. The fossil compounds had the same retention times and MRM signal ratios of quantifiers to qualifiers as the standard compounds. To verify the identity of the analytes, a second elution gradient was applied, which again showed identical retention times and MRM ratios for fossil compounds and standards.

An even greater challenge was the identification of unknown fossil analytes. Non-targeted analysis of the extracts by HR-ESI-MS (Thermo Orbitrap) provided molecular formulae of a series of further compounds with very good agreement between experimental and calculated *m*/*z* values and isotope ratios. Based on additional fragmentation and UV/visible spectrophotometric data, one compound that could be assigned to the molecular formula $C_{33}H_{35}N_4O_2$ was preliminarily identified as a cyclic pheophorbide.

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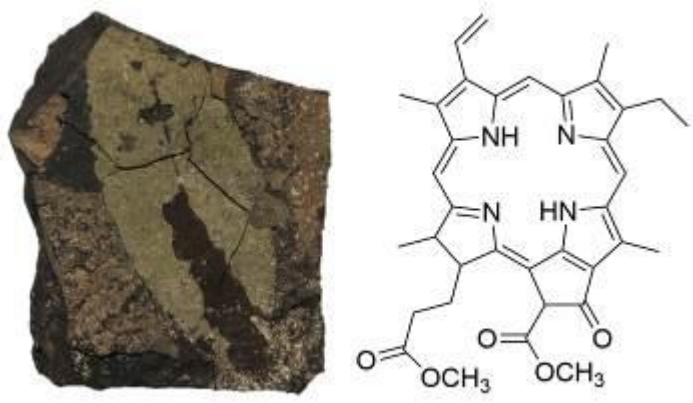
Figure legend

Fossil leaf with preservation of green color from Geiseltal, Germany, and chemical structure of a chlorophyll derivative previously described in reference [3].

Fig. 1



High throughput LC-MS based metabolomics study for the deep phenotyping of plasma sample from cohorts investigating heart failure

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The heterogenous clinical syndrome of Heart Failure (HF) affects millions of people worldwide and is characterized by significant reduction in quality of life and high morbidity. The increasing prevalence of HF and an unmet medical need require novel approaches to gain a deeper understanding of its molecular mechanisms in order develop improved diagnostic tools and future treatment options. The multidisciplinary research cluster "DIASyM" in Mainz aims to combine multi-OMICS, systems medicine and informatics, to perform a deep phenotyping of both the EmDIA study (N = 144), investigating empagliflozin treatment in patients with diastolic dysfunction and type 2 diabetes mellitus and the MyoVasc study on HF, comprising plasma samples from 3289 individuals across multiple time points.[1] Our research group will provide the highly automated, reproducible, and rapid DIA-LC-MS-based metabolomic profiling of the >10000 cohort plasma samples.

We initially evaluated different column chemistries to ensure optimal retention of a broad range of chemical compound classes within a short analysis time to ensure the throughput required to profile large clinical cohorts. The Kinetex F5 (2.1 mm × 150 mm, 2.6 µm, Phenomenex) as stationary phase provided excellent detection of a broad range of metabolites, from small hydrophilic compounds such as lactate to more hydrophobic molecules such as fatty acids, as well as chromatographic baseline separation of various isomeric molecules.

We subsequently focused on metabolite extraction from plasma, aiming to obtain a fast and reproducible protocol suitable for automated liquid handling. We compared solid phase extraction against liquid extraction with various organic solvents followed by protein removal at various centrifugation speeds. Liquid extraction with methanol resulted in the highest reproducibility with median CVs < 6%, regardless of the subsequent centrifugation speed. This provides us with a fast and highly parallelizable sample preparation protocol suitable for automation. In combination with a drastically shortened LC-MS gradient length, this allowed us to screen >500 samples of the emDIA cohort, achieving a throughput of 100 samples a day, thus providing an excellent platform to rapidly phenotype much larger cohorts such as the MyoVasc study in the future.

For improved metabolite annotation, we generated an in-house spectral library based on 612 metabolite standard compounds, fragmented with collision induced dissociation (CID) in both ion modes. These spectral libraries will now be implemented for targeted compound annotation from SWATH data and subsequent relative MS2-based quantification.

Once fully established, this novel high throughput plasma metabolomics protocol supported by liquid handling, will enable a highly reproducible and standardized analysis of plasma cohorts and improve our understanding of HF on a molecular level.

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ABSTRACTS POSTER

P 01

Deep proteome coverage at scale combined with reproducible quantitation on the timsTOF HT

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Introduction:

In the realm of proteomics, accurate and precise relative protein quantitation is the key to unravel the complex secrets of biological processes. Dia-PASEF is an advanced variant of DIA, capitalizing on the additional dimension of separation unlocked on the timsTOF platform by trapped ion mobility separation (TIMS). Demonstrating these capabilities resulted in optimizing a dia-PASEF acquisition method to analyze both single proteome samples and complex hybrid proteome mixtures for benchmarking.

Methods:

Tryptic digests of a HeLa, yeast and E. coli were either loaded directly on column or combined in defined ratios.

Samples were loaded on a 15cm C18 column (75µm, 1.6µm, Aurora, IonOpticks) using a nanoElute 2 nano HPLC (Bruker) coupled to a timsTOF HT (Bruker) via a CaptiveSpray 2 ionization source (Bruker) using a 15-min ACN gradient. For the dia-PASEF acquisition, a window placement scheme optimized via the py-diAID tool was used.

Data were processed in Spectronaut (v18, Biognosys) using directDIA+™.

Results:

In our study we were able to cover nearly the complete yeast proteome by identifying on average 4408 protein groups using a 15 min gradient. 95% of the protein groups were identified and quantified with CV values below 20% from triplicate injections.

When analyzing a human protein digest sample nearly 105,000 stripped peptide sequences from close to 8000 protein groups have been identified with excellent reproducibility.

For evaluation of the presented setup for quantitative proteomics we used samples mixed in defined ratios (HeLa, yeast, E.coli). Within a 15 min gradient we could identify and quantify on average 12,893 protein groups from 173,851 peptide precursors. The measured ratios obtained for human, yeast and E.coli were close to the expected ratios.

Conclusions:

dia-PASEF on the timsTOF HT enables high proteome coverage and accurate quantitation in short gradients of 15 minutes.

P 02

Determination of antimicrobial peptides in natural and recombinant microbial producers using LC-MS/MS

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The antimicrobial peptide GakC, an essential constituent of the trimeric peptide complex Garvicin KS, together with components GakA and GakB, is naturally synthesised in the human opportunistic pathogen *L. garvieae*. To bolster production efficiency in a non-pathogenic platform organism, GakC is subjected to heterologous expression within the recombinant producer *C. glutamicum*. This process entails the subsequent secretion of the peptide into the supernatant following its intracellular expression. Facilitated by the Sec pathway, this secretion is aided by the introduction of a small library of 24 different sec-dependent secretion signal peptides (SP). This secretion strategy is employed for its facilitation of more cost-effective downstream processing, attributed to the improved accessibility of the peptide compared to its intracellular counterpart. These SPs are fused N-terminally to the respective target peptide of interest. Upon recognition of the SP by the secretion machinery, peptide secretion is initiated. This approach not only augments production yields, but also streamlines handling procedures, presenting a promising methodology for the optimisation of both the synthesis and secretion of GakC in *C. glutamicum*. Within this study, GakC is subject to LC-MS assessment with the specific aim of pinpointing the most effective SP for enhanced production and secretion of GakC in the *C. glutamicum* system.

In the analysis of microcultivation samples, traditional assays like the pHluorin assay, which rely on measuring a peptides membrane-damaging activity, revealed limitations in providing comprehensive insights. The primary constraint stemmed from sensitivity restrictions, prompting the establishment of an alternative approach using LC-MS. For this purpose, a targeted multi-analyte tandem MS method in MRM mode was developed on an API4000 TripleQuad, focusing on the detection of tryptic fragments. Method optimisation on the LC side was a key consideration, particularly emphasising peak shape and time efficiency. This was critical due to the diverse nature of tryptic fragments characterised by varying lengths and isoelectric points, ranging from four to 17 amino acid residues and six to nine, respectively.

The successful development of a method for analysing microcultivation samples derived from a strain library, encompassing 24 SPs, marks a significant achievement. Beyond the primary analyte, GakC, a set of further peptides can be simultaneously investigated. Although the conventional pHluorin assay yielded negative results, failing to provide substantive insights, the adoption of LC-MS addressed the shortcomings of traditional assays, offering a more refined and comprehensive approach for assessing both peptide identification and quantitation in microcultivation sample analysis. This approach has facilitated the identification of a refined selection of top secretion producers, paving the way for subsequent process optimisation.

P 03

A new SPE Tips method based on an innovative sorbent for fast and efficient peptide fractionation in proteomic studies

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Introduction

Peptide fractionation plays an important role in bottom-up proteomic approaches, since it simplifies peptide pool for easier analysis by mass spectrometry, thus allowing more accurate interpretation. Indeed, full proteome characterization is often needed for complex biological matrices and, with growing interest in post-translational modifications, an extended protein sequence coverage is required. Most fractionation methods involve reversed-phase peptide fractionation at basic pH. This fractionation method is orthogonal to reversed-phase liquid chromatography, thus contributing to the simplification of the peptide separation and analysis, and does not require additional desalting step, contrary to ion-exchange fractionation approaches. However, efficient peptide fractionation is very challenging, especially in the case of complex samples containing peptides with a wide range of properties (charge, polarity and size). The objective of this study was to develop a simplified procedure for the efficient and fast fractionation of peptides, that can be adapted to different types of biological samples, both in terms of volume and quantity of peptides, in order to obtain a high-quality fractionation for a wide range of applications, from single-cell analysis to high throughput experiments.

Methods

A new reversed-phase sorbent, based on small particles tightly embedded in a monolithic membrane packed in SPE Tips, was used for the fractionation of peptides resulting from the enzymatic proteolysis of HEK293 cell lysate, and the results were compared to a reference commercial fractionation kit. Eight fractions were performed on both the commercial column and the SPE Tips, with an acetonitrile gradient, and each fraction was then evaporated to dryness before being resuspended in an appropriate solvent for nanoLC-MS/MS analysis.

Results

If the total number of proteins identified and the percentage of peptides eluting in only one fraction (50%) were similar for both sorbents, with a good distribution of peptides over the eight fractions, it appeared that the fractionation on the new sorbent presented several advantages compared to the reference kit since it can be stored dry at room temperature while the commercial columns have to be stored at 4°C in a storage buffer. Moreover, due to the SPE Tips format, the time required for the evaporation of each fraction is almost halved compared to the commercial columns.

Conclusion

The new reversed-phased sorbent developed in this study appears as a promising solution for the fractionation of complex samples or the generation of spectral libraries, since it leads to an increase of more than 25% in the number of proteins identified, compared to unfractionated samples. Finally, this new sorbent offers flexibility of format and capacity, since it is also available as SPE spin columns, for the fractionation of high amounts of peptides, or as 96 SPE well plates, for high throughput experiments.

P 04

Spatial proteomics with DISCO-MS: Applications and advances

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Spatial molecular profiling of complex tissues is essential to investigate cellular function in physiological and pathological conditions. However, methods for molecular analysis of large biological specimens imaged in three dimensions (3D) are lacking. Therefore, we developed DISCO-MS, a technology that combines whole-organ/whole-organism optical clearing and imaging, deep-learning-based image analysis, robotic aided tissue extraction, and ultrahigh-sensitivity mass spectrometry. DISCO-MS yielded proteome data indistinguishable from uncleared samples in both rodent and human tissues. We used DISCO-MS to investigate microglia activation along axonal tracts after brain injury and characterized early- and late-stage individual amyloid-beta plaques in a mouse model of Alzheimer"s disease. DISCO-bot robotic sample extraction enabled us to study the regional heterogeneity of immune cells in intact mouse bodies and aortic plaques in a complete human heart. Furthermore, DISCO-MS was applied to delineate the distribution of spike protein in SARS-CoV-2 mice models. Beyond previously documented regions, our investigation revealed a sustained buildup of Spike S1 protein in unexplored territories, particularly within the intricate network of the skull-meninges-brain axis. This observation suggests potential ramifications for neurological complications in the context of Long COVID. The applications of DISCO-MS have facilitated unbiased proteome analysis of preclinical and clinical tissues, following comprehensive 3D imaging of entire specimens, unveiling diagnostic and therapeutic prospects for intricate diseases.

P 05

Sample displacement batch chromatography (SDBC) for fractionation of proteoforms

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In proteoform research, the fractionation of proteoforms is currently one of the most challenging topics. The importance of studying proteoforms is not only high for the life sciences but especially for the development and production of therapeutic proteins (TPs) such as recombinant therapeutic antibodies (mAbs). Some proteoforms of TPs have significantly lower efficacy and some cause undesirable side effects. The identification and removal of proteoforms from the main form is challenging as the differences in composition are often very small and the concentration of proteoforms is usually very low compared to the main form.

In this study, we demonstrate that sample displacement batch chromatography (SDBC) is an easy-to-handle, economical, and efficient method for fractionating proteoforms. As a model sample a commercial ovalbumin fraction was used, containing many ovalbumin proteoforms. The most promising parameters for the SDBC were determined by a screening approach and applied for a 10-segment fractionation of ovalbumin with cation exchange chromatography resins. N-glycans of all ovalbumin proteoforms from the different SDBC fractions were released and analyzed separately. Mass spectrometry of intact proteoforms was used for characterizing the SDBC fractionation process. The relative quantities of the proteoforms were determined with UniDec using the signal intensities of the deconvoluted spectra. From the observed experimental masses, identification of ovalbumin proteoforms was performed by mass-matching.

Ovalbumin proteoforms were detected through mass spectrometry, revealing two clusters at 40 kDa (lower molecular weight/LMW) and 44 kDa (higher molecular weight/HMW). The study identified 43 ovalbumin proteoforms, considering post-translational modifications (PTMs) and N-glycan compositions. Sample Displacement Batch Chromatography (SDBC) effectively separated ovalbumin proteoforms into 10 fractions, demonstrating a significant enrichment of low-abundant proteoforms. Additionally, SDBC successfully separated closely related proteoforms, enriching them in early fractions and purified main ovalbumin proteoforms in later fractions. The technique effectively removed proteoforms not belonging to ovalbumin, demonstrated by bottom-up proteomics.

Despite challenges related to similar molecular weights, SDBC significantly increased the number of detectable proteoforms, providing a promising strategy for proteoform analysis and fractionation in proteomics. The study emphasized the economic benefits, scalability, and efficiency of SDBC, making it a valuable tool for accessing a larger number of proteoforms in the field of proteomics.

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P 06

Proteomic and metabolic adaptations in the kidney during pyelonephritis

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In the complex interplay of host-pathogen interactions, metabolites play a crucial role in shaping the immune response and affecting bacterial growth and survival. During urinary tract infection (UTI), i.e., adenosines, itaconate, and lactate are produced both by the pathogen and host cells and affect immune cell recruitment and activity. Label-free LC-MS/MS-based analysis of the human kidney proteome revealed downregulated oxidative phosphorylation and an upregulation of glycolytic factors, notably lactate dehydrogenase A (LDHA). Human kidney specimens were obtained from patients diagnosed with pyelonephritis (PN), an inflammatory disorder of the upper urinary tract. The measurements were performed on a quadrupole-ion-trap-orbitrap MS (Orbitrap Fusion, Thermo Fisher) coupled to a nano-UPLC (Dionex Ultimate 3000 UPLC system, Thermo Fisher). Positive correlation of metabolic factors, such as LDHA, with CD163 and other immune cell markers, indicated co-regulation of metabolic and immune processes. This co-regulation might be facilitated via metabolism-dependent post-translational modifications, such as lactylation.

In further experiments, we will characterize the kidney microenvironment through multiplex microscopy and MALDI-based spatial proteomics in tissue sections from PN and control patients. Moreover, a model of murine bone marrow-derived macrophages will be employed to study the polarizing effect of lactate on macrophages and identify targets of lactylation using flow cytometry and targeted proteomics. Investigating the interrelationship of metabolism and immunity will improve our understanding of the molecular factors that shape macrophage differentiation and guide progression and aggravation of PN.

P 07

Unraveling the ghost proteome (alternative proteome) by cross-linking MS and deep proteogenomic characterization

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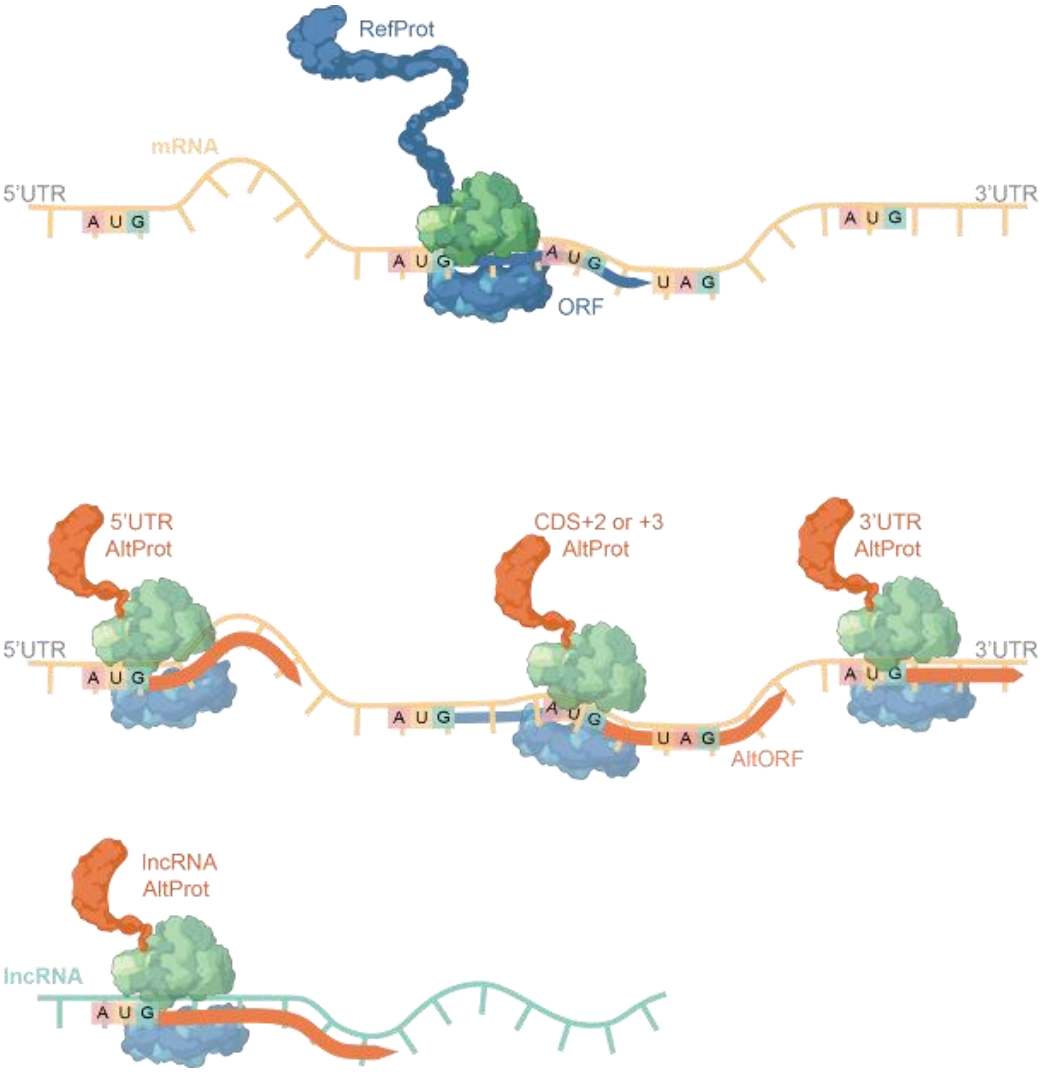
Ovarian cancer (OvCa) is often diagnosed late or misdiagnosed, resulting in high mortality rates. Treatment options include surgery or chemotherapy, but chemotherapy resistance is a major challenge. In this context the "non-referenced or ghost proteomes" is a potentially rich source of biomarkers. The latest contains proteins called "Alternative proteins" (AltProt). These proteins are translated from regions annotated as non-coding in transcripts, such as the 5"&3"-UTR regions, a shift in the reading frame, but also in ncRNAs. Obtaining functional protein information for AltProts requires complex and costly biomolecular studies. However, their functions can be inferred by profiling their interaction partners, known as "guilty by association" approaches. Indeed, assessing AltProts' protein-protein interactions (PPIs) with reference proteins (RefProts) can help identify their function and set them as research targets.

A methodology that combines proteogenomics, cross-linking mass spectrometry (XL-MS) and subcellular fractionation was developed. First, using RNA-seq data, customized protein databases for each cell line were generated employing OpenCustomDB. Then, we used a non-targeted protein-protein interaction (PPI) identification strategy: cross-linking-MS (XL-MS). To improve the detection of cross-linked peptides, we applied subcellular fractionation of the cells, allowing a de-complexification of the sample to improve peptide detection and assigning a subcellular localization for the identified AltProts. We highlight 13 PPIs between AltProt and RefProt. These interactions allow us to link AltProt to biological processes via Gene Ontology enrichment of the referenced interaction partners. To assess the validity of cross-linked interactions, we performed studies to measure the corresponding cross-linked distances.

A proteogenomic analysis was performed to investigate the proteomes of two ovarian cancer cell lines (PEO-4 and SKOV-3 cells) in comparison to a non-pathological ovarian epithelial cell line (T1074 cell). A total of 597 AltProts were identified. Among them were 41 cancers specific which included mutated AltProts. Differentially expressed proteins, including wild type (71) and two mutated AltProts, were identified between the cancer and normal cell lines. 11 AltProts were significantly regulated in more than one unique cellular compartment. This methodology also can be used to monitor changes under different cellular conditions. Finally, the expression of RefProts and their transcripts were associated with cancer-related pathways.

This work highlights the significant potential of proteogenomics in uncovering new aspects of ovarian cancer biology. It enables us to identify previously unknown proteins and variants that may have functional significance. The use of customized protein databases and the crosslinking approach have shed light on the "ghost proteome," an area that has remained unexplored until now.

Fig. 1


RefProt
mRNA
5'UTR
A U G
A U G
A U G
U A G
ORF
3'UTR
A U G
5'UTR
AltProt
5'UTR
A U G
A U G
CDS+2 or +3
AltProt
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AltProt
lncRNA
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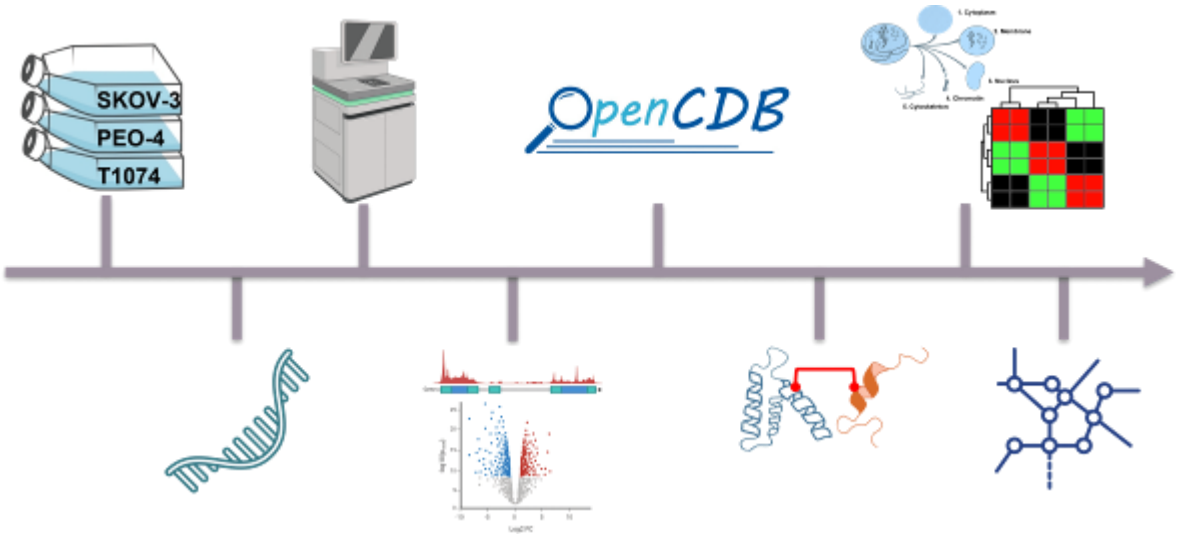
Fig. 2



P 08

Precise and accurate quantitation at 260 SPD with HT-DIA acquisition on Orbitrap Exploris 240 MS

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The ability to gain global proteome-wide information is key in the understanding of biological processes. To gain statistically significant quantitative information, study cohorts can comprise several hundreds of biological samples and replicates. A suitable workflow must accommodate for the need to process, measure, analyze a high number of samples with reproducible data and should maintain same performance over the duration of the whole study.

Here, we present a rugged workflow for LC-MS based high-throughput label-free quantitation using a Vanquish Neo LC system equipped with a 5.5 cm uPAC Neo High Throughput column, and an Orbitrap Exploris 240. We benchmark three different gradient lengths amounting to 100, 170 and 260 samples per day (SPD), showing high proteome coverage with reproducible quantitative results between replicates. We demonstrate the quantitative performance of these three methods by use of a three-species proteome mix, where we show accurate quantitation results for all three gradients. Additionally, we could show the robustness of the workflow with data from 1000 consecutive injections, as well as cross-site comparison of data collected at three different sites in Europe.

P 09

New CID cleavable cross-linkers suitable for IMAC enrichment and structure elucidation of proteins via MS

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Chemical cross-linking in combination with mass spectrometry (XL-MS) has developed into an important method for characterizing protein conformations and protein-protein interactions. [1] Especially the development of CID cleavable cross-linkers enhanced the effective detection of derivatized peptides and opened the avenue for automated MS data set analysis.[2] Nevertheless, it remains a challenging analytical task to differentiate cross-linked peptides from unmodified peptides in complex mixtures resulting from protein digestion.[3] To further improve the selective analysis of the cross-linking process tri-functional reagents for chemical cross-linking have been developed in the last years. These type of cross-linking reagents contain an additional moiety designed for affinity chromatography enrichment. We aim to expand the currently available group of trifunctional linkers by designing reagents that allow an enrichment of cross-linked species via immobilized metal affinity chromatography (IMAC). The symmetrical linkers for chemical cross-linking have the CID urea moiety in common, making them sensitive upon CID.[4] The projected trifunctional cross-linker containing a CID cleavable group in addition to an IMAC-enrichable function contains two Tyr moieties in the new linker reagent. The phenole side-chains should allow the characteristic complex formation with immobilized iron aimed to selectively retard cross-linked peptides via IMAC. This approach was inspired by the catechol based siderophores in terrestrial bacteria resulting in the idea of using the oxygen iron interaction for an IMAC purification.[5] This enrichment strategy has been established for a phosphonic acid containing cross-linker that allows the enrichment of cross-links based on IMAC. [6] The synthesis and initial proof-of-principle results with tandem MS of a NHS-Tyr-Urea-Tyr-NHS linker reagent are presented. We obtained the cross-linker from commercially available tyrosine over six steps with an overall yield of 20 %. Furthermore, we were able to react the cross-linker with thymopentin and BSA to prove its effectiveness as an amine reactive CID cleavable cross-linker. [1] L. Piersimoni, P. L. Kastritis, C. Arlt, A. Sinz, Chem. Rev. 2022, 122, 7500– 7531. [2] M. Q. Müller, F. Dreiocker, C. H. Ihling, M. Schäfer, A. Sinz, Anal. Chem. 2010, 82, 6958–6968. [3] D. M. Schulz, A. Sinz, BioSpektrum 2004, 10, 45–48. [4] F. Falvo, L. Fiebig, F. Dreiocker, R. Wang, P. B. Armentrout, M. Schäfer, Int. J. Mass Spectrom. 2012, 330–332, 124–133. [5] Y. Li, W. Jiang, R. Gao, Y. Cai, Z. Guan, X. Liao, 3 Biotech 2018, 8, 1–6. [6] B. Steigenberger, R. J. Pieters, A. J. R. Heck, R. A. Scheltema, ACS Cent. Sci. 2019, 5, 1514–1522

P 10

Development and optimization of a method for automated peptide desalting on the DigestPro MSi robot using AttractSPE® C18 tips

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Introduction

Bottom-up approaches are commonly used for proteomics analysis in LC-MS/MS. However, salts and digestion buffers can generate ion-suppression that limits peptide detection and greatly impacts protein identification. Therefore, peptide desalting and purification is an indispensable step prior to LC-MS/MS analysis, to ensure reliable and repeatable results.

Nevertheless, this desalting step can be very challenging since salts must be efficiently removed while peptides on the broadest polarity range must be retained to preserve all useful information about the sample, especially when working with low input material. To simplify and speed up the desalting procedure, an automated SPE tips-based method was developed and optimized.

Methods

The P3S proteomics facility is equipped with a nanoElute – timsTOF Pro LC-MS/MS system and has chosen to perform the desalting step prior to LC-MS/MS analysis with direct injection on the analytical column, to reduce sample handling and thus minimize potential sample loss.

With the aim to automate peptide desalting, P3S has asked CEM (previously Intavis) to create a custom needle to run home-made StageTips on the DigestPro MSi robot. The program was adapted from manual StageTips protocol and further optimized by playing with liquid aspirate and dispense speeds and volumes. Moreover, P3S has evaluated AttractSPE® C18 Tips (Affinisep) and compared them to home-made StageTips (with Empore C18 SPE disks) for the desalting of 2 µg and 100ng of protein digest.

Results

The AttractSPE® C18 Tips are packed with small sorbent beads embedded in a thin, uniform and mechanically stable membrane that combines high capacity and small dead volume, and are adapted for centrifugation or positive pressure assays. The comparison showed little difference between the two kinds of tips when working with 2µg of peptides but showed a 10% increase in the number of identified proteins with the AttractSPE® C18 Tips when working with 100ng of peptides. Moreover, the peptide intensities were higher with the AttractSPE® C18 Tips. In both cases, 2µg and 100ng starting material, the AttractSPE® C18 Tips performed better in the hydrophilic range. Indeed, AttractSPE® C18 Tips show better hydrophilic peptide retention as well as 9% increase in the number of identified proteins and 25% increase in peptide intensities.

Conclusions

In conclusion, the results show that AttractSPE® C18 sorbent offers a wider spectrum of interactions with a broadest range of peptides, from the most hydrophilic to the most hydrophobic ones, compared to home-made StageTips. In addition, the P3S team has optimized the desalting program on the DigestPro MSi robot to reduce the time from 40min down to 10min per sample. This shorter program did not lead to any loss in protein identification and quantification. On the contrary, a slight increase (11%) in peptide intensities was observed.

P 11

Enhancing treatment recommendations in precision oncology with proteomics and phosphoproteomics data

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Question

In precision oncology DNA and RNA sequencing are increasingly applied to profile rare and difficult tumours. However, the therapeutically important (phospho)proteome is rarely included. This can, in part, be attributed to the requirement of a deep, reproducible profiling as well as the lack of data analysis software taking care of all processing from raw data to patient-specific oncogenic activity.

We implemented an end-to-end clinical proteomics workflow into two existing molecular tumour board programs. Here, we present the automated analysis of (phospho)proteomic data enabling therapeutic suggestions within a turnaround time of two weeks.

Methods

Without healthy controls, our activity scoring relies on relative protein/p-site quantification compared to a pancancer background cohort. We are expanding our patient cohort for increased statistical significance and for broader coverage of rare cancer types and different oncogenic mechanisms.

2 batches/week (16 patients) are processed with a multiplexing strategy (TMT-11), allowing high throughput and high (phospho)proteome coverage. For most patients, expression data of >8,000 proteins and >20,000 phospho-sites are measured. After individual database searches per batch, the identified peptides and phospho-peptides datasets undergo a processing pipeline that combines data and makes it comparable. The data processing of the cohort includes steps that enhance data completeness, reduce batch effects and enable the identification of oncogenic signalling.

First, all peptides and phospho-peptides are normalised within batches after which they are normalised across batches by a novel approach turning them into quasi LFQ intensities. Protein quantification is done using the MaxLFQ method. We are able to show that most batch effects get successfully removed.

Single patient proteomes are then analysed at a protein abundance level and phospho activity level using z-scores calculated across patients to find aberrant activity. A novel strategy integrating different levels of signalling e.g. substrate phosphorylation, uses manually curated annotation and scores different cancer-related pathways to find best suggestions for targeted treatment.

Results

Currently, the cohort consists of >900 individual patient samples from various entities, predominantly sarcomas. Of these >500 prospective samples have been discussed in molecular tumour boards. Our results show that (phospho)proteomics can support genomic findings and provide new treatment suggestions.

Conclusions

Our automated analysis workflow has enabled proteomic addition to therapeutic suggestions in two molecular tumour board programs. It has shown to support original molecular profiling but also to give extra and new information where genomics and transcriptomics has shortcomings.

P 12

Pharmacoproteomic profiling identifies secreted markers for aberrant drug action

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Adverse drug reactions (ADRs) contribute significantly to late-stage attrition in drug discovery due to their unpredictability and enigmatic underlying mechanisms. Here we applied mass spectrometry-based proteomics to assess the effects of 46 drugs and 8 tool compounds with various levels of concerns for drug induced liver injury on the secretome of a hepatocyte model system. We observed distinct clusters of non-canonical secretion and intracellular thermal proteome profiling linked dysregulated mechanisms to extracellular markers. Functional follow-up confirmed lysosomal alterations by cationic-amphiphilic drugs, connected damage of the respiratory chain to Rab7-dependent secretion of mitochondrial proteins, and linked drug-induced endoplasmic reticulum stress to reduced basal secretion. Perturbation of sphingolipid biosynthesis pathways specifically induced secretion of the cargo sorting protein SDF4 whilst suppressing secretion of its cargo proteins. Thermal stability changes of clusters of membrane proteins in distinct subcellular compartments suggest local accumulation as important driver for unexpected drug effects through direct and indirect interactions.

P 13

Studying the interactions between SARS-CoV-2 nucleoprotein and G3BP1 and their influence on stress granule formation

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The nucleoprotein is one of the four structural proteins of SARS-CoV-2. It contains two structured domains and three intrinsically disordered regions (IDRs). The nucleoprotein plays an important role in RNA packaging, mediates viral replication and undergoes liquid-liquid phase separation in the presence of viral RNA. It is also involved in inhibiting the host-immune response as it binds to several host proteins involved in RNA processing and stress granule (SG) formation. SGs are formed in the course of cellular stress response induced by e.g. oxidative stress, heat shock or viral infections.

Ras-GTPase-activating protein-binding protein 1 (G3BP1) is one of the key factors in SG formation. G3BP1 consists of two structured domains, N-terminal NTF2-like domain (NTF2L), RNA recognition motif (RRM), and three IDRs. The IDRs comprise an acidic region, a proline-rich region connecting NTF2L with RRM, and an RG-rich region located at the C-terminus.

In response to cellular stress, translation initiation is arrested and polysomes start to disassemble. When free mRNA is released from the polysomes, it is bound to G3BP1 that forms clusters upon mRNA binding. Upon recruitment of other RNA-binding proteins these clusters assemble into mature SGs.

The SARS-CoV-2 nucleoprotein interacts with G3BP1, suggesting an influence of the nucleoprotein on SG formation. *In-vivo* studies have confirmed that SG formation is impaired in the presence of SARS-CoV-2 nucleoprotein.

The molecular details of the protein-protein interactions of SARS-CoV-2 nucleoprotein and G3BP1 are still elusive. It has been suggested that the N-terminal IDR of the nucleoprotein plays an important role in binding of G3BP1, while other studies indicate that the C-terminal region of the nucleoprotein might also contribute to G3BP1 binding.

Our aim is to gain detailed insights into the molecular mechanisms of SARS-CoV-2 nucleoprotein and G3BP1 interactions to understand the influence of a SARS-CoV-2 infection on SG assembly and disassembly. It is planned to use different methods of structural mass spectrometry for investigating these protein-protein interactions, such as cross-linking mass spectrometry (XL-MS), hydrogen/deuterium exchange mass spectrometry (HDX-MS) and native mass spectrometry (native MS). Those methods are perfectly suited to study the properties of IDRs. Our *in-vitro* studies will be complemented by *in-vivo* XL-MS studies in HEK293 cells to gain insights into the functional roles of SARS-CoV-2 nucleoprotein and G3BP1 in SG formation.

P 14

Improving depth of plasma proteomics using a timsTOF HT followed by benchmarking with CLINSPECT-M RingTrial data

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Blood serves as a vital reservoir of clinically relevant information, necessitating a comprehensive understanding of its protein composition. The exploration of plasma proteins is a highly competitive domain within the field of proteomics, while simultaneously facing challenges due to the high dynamic range spanning more than 10 orders of magnitude. Our objective was to enhance mass spectrometry-based plasma protein identifications using a timsTOF HT instrument (Bruker) connected to an Ultimate 3000 nano-RSLC (Thermo Fisher Scientific).

We compared two different sample preparation methods employing either the iST kit or the ENRICH-iST kit from PreOmics. Additionally, we evaluated various HPLC gradient lengths and data-independent acquisition mass spectrometry (DIA-MS) methods. Finally, three different software tools were used for data analysis, including a comparison of using a predicted spectral library versus a tailored library obtained through high pH fractionation.

Our results demonstrated a 45% increase in protein identification using the ENRICH-iST compared to the iST-kit. Gradient length analysis (30min, 70min, and 90min) revealed an approximate 20% increase between 30min and the two longer gradients, with negligible differences between 70min and 130min gradient length. Regarding the DIA-MS method, a tailored py_diAID method (Skowronek et al., 2022) exhibited around 10% more protein identifications compared to the standard DIA method provided by Bruker. Using the most successful method, we further evaluated software performance by comparing Spectronaut, DIA_NN, and MaxDIA with different spectral libraries. DIA_NN provided the highest number of protein identifications, reaching on average around 2000 IDs with a 75-minute gradient and a tailored py_diAID method.

Following the identification of the most effective combination, we used ring trail raw data from the CLINSPECT-M consortium originating from different laboratories across Munich to assess the scope of our own improved plasma proteomics. We reanalyzed DIA raw files from six different setups, each employing its best practices in plasma processing. DIA_NN was used for data analysis using a predicted human spectral library to enable comparative evaluations based on protein and peptide identifications, coefficient of variations, retention performance, and the overall overlap of identified proteins.

This study shows a systematic approach for the improvement of plasma proteomics using a timsTOF HT mass spectrometer. The implications of these findings extend to both the mass spectrometry community and clinical applications, particularly in the realm of biomarker discovery.

P 15

Ultra-high sensitive single cell proteomics on the timsTOF ultra

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For single cell proteome analysis, ultra-high sensitivity mass spectrometry is key to reach proteome coverages necessary for understanding the cellular heterogeneity on a cell-by-cell level. Latest enhancements in ion transfer with a larger transfer capillary, an additional higher-pressure segment for more effective ion collection, two orthogonal deflections, to maintain robustness, and high-capacity TIMS pushes the limits of detection to single cell level.

K562 cell digest (Promega) dilution series from 16 ng to 15 pg, were prepared. One, five, ten and twenty HeLa cells and FACS pre-sorted T-Cells (CD4+, CD8+) B-Cells (CD19+) and monocytes (CD14+) from peripheral blood mononuclear cells (PBMCs) were isolated and prepared with a cellenONE (Cellenion). Peptides were loaded onto an Aurora Elite column (IonOpticks) separated with a 22 min active gradient (32SPD) or an Aurora Rapid column (IonOpticks) with a 10 min active gradient (80SPD) using a nanoElute2, eluting peptide detection on a timsTOF Ultra in dia-PASEF, and analysis with Spectronaut 18.

Processing of K562 dilution series acquired in dia-PASEF mode identified >1,000 protein groups out of 15 pg, with 80SPD, and 32SPD and > 6,000 protein groups (80SPD) and >7,000 protein groups (32SPD) for 16 ng. Quantitative accuracy at 250 pg was around 8 - 9% and about 4 - 6% at loads of 4, 8 and 16 ng. Protein groups identified for peptide loads < 500 pg were comparable between 80SPD and 32SPD. Analysis of HeLa cells resulted in about 4,000 protein groups from single cells and up to 6,000 protein groups for 20 cells in 80SPD and 32SPD. For FACS pre-sorted PBMCs, the 80SPD workflow identified in total 1,713 protein groups, with distinct proteomic phenotypes for the four cell types.

Deep proteome coverage and high reproducibility using the timsTOF Ultra combined with automated single cell isolation and sample preparation on the cellenONE® platform.

P 16

BeatBox® and iST for streamlined FFPE-tissue processing: A xylene-free, robust, and high-throughput sample preparation for in-depth proteomic analysis

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Purpose

Access to formalin-fixed, paraffin-embedded (FFPE) tissue materials is relatively easy, as it is evaluated, preserved, and stored according to well-established procedures in routine clinical diagnostics. Extraction of information-relevant proteins from FFPE-tissues, however, is a process that requires expert knowledge. Using the BeatBox® tissue homogenizer and the iST kit for proteomic sample preparation, it is now possible to process FFPE-tissues easily, efficiently, and reproducibly without using toxic xylene-based deparaffinization. In combination with state-of-the-art LC-MS instrumentation, acquisition methods, and data-processing pipelines, biomarker discovery from FFPE-tissues reaches a completely new level in the field of proteomics.

Methods

Snap-frozen mouse organs (1-2 mg pieces) and matching FFPE samples (10 µm curls) were processed in 96-well format using BeatBox® homogenization coupled to iST sample preparation. For FFPE samples, an optimized workflow was established: FFPE-curls were homogenized in the BeatBox® (high power, 10 minutes), followed by an one-hour incubation at 80-95 °C, to de-crosslink, extract, reduce and alkylate proteins in one step. Applying the iST sample preparation protocol, tryptic digestion was followed by an optimized peptide clean-up with an additional washing step to remove last traces of paraffin. Peptides are analyzed using nano-LC coupled to a timsTOF mass spectrometer in DIA mode, and Bruker ProteoScape was used for data processing.

Results

In this study, the performance of a xylene-free workflow designed for BeatBox® + iST was compared to a standard xylene-based deparaffinization procedure. Matching fresh frozen tissue was obtained to perform a comparative proteomic analysis to the two different methods for preserved tissue. More than 10,000 proteins were identified in both fresh frozen and FFPE-tissues, with a high overlap of up to 73% of common proteins and a similar dynamic range.

Conclusion

The BeatBox®-iST workflow for FFPE-tissues enables in-depth proteomic analyses, is easy-to-use, and eliminates the need for xylene-based deparaffinization, providing a great solution for large-scale retrospective studies.

P 17

Improved dia-PASEF isolation window schemes for low input proteomics measurements

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Data-Independent Acquisition (DIA) is widely used for proteomics as it promises reproducible and accurate protein identification and quantitation. dia-PASEF is both, more sensitive and more selective, than traditional DIA approaches combining the advantages of DIA with the inherent ion-usage efficiency of PASEF. Making use of the correlation of molecular weight and CCS coded information, dia-PASEF enables highly confident identification. Two-dimensional mass and mobility space enables method creation with extensively different window schemes. Here, a variety of fixed-width or more advanced window schemes were evaluated for speed, sensitivity, and selectivity.

Dilution series of tryptic digests from human cell lines (HeLa and K562) from 125pg up to 200ng were separated using different nanoLC gradients (7 to 60min, nanoElute, Bruker). Depending on gradient length columns of 25cm, 15cm and 8cm length with internal diameters of 0.075mm or 0.15mm (PepSep, Bruker) were chosen. Isolation windows of 12, 25 and 50Da width, combined with one and two quadrupole isolation switches during TIMS separation were compared to variable window widths based on precursor density (py_DIAid). Data were processed using Spectronaut.

We limited the dia-PASEF windows to the mass and mobility range of highest precursor density, i.e. 400 to 1200Da and 0.7 to 1.4Vs/cm²in mobility (1/K0) dimension. For sample amounts in the 10-50ng range identifications were remarkable similar among the different tested acquisition schemes showing a variation of less than 10%. Lower sample amounts benefited from a lower number of broader windows as each individual precursor is fragmented more frequently. For higher sample loads, acquisition schemes of more narrow isolation windows resulted in improved identifications.

TIMS enables efficient ion usage and selectivity due to pre-separation of precursor ions into condensed ion packages, resulting in 5,400 protein groups identified and quantified with >47,000 precursors from 0.250ng cell digest and 8,500 protein groups with >105,000 precursors from 100ng.

P 18

Exploring the impact of the expression of different HRAS variants on the proteome and phosphoproteome of human keratinocytes

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HRAS is an early player in different signalling cascades including the MAPK/ERK pathway. The small GTPase HRAS acts as a molecular switch cycling between an active GTP-bound and inactive GDP-bound state. Pathogenic variants in the *HRAS* gene results in the constitutive activation of HRAS and are common in dermatological malignancies. This includes the germline variant HRASGly12Ser, which results in the developmental disorder Costello syndrome (CS), the post-zygotic variant HRASGly13Arg leading to skin disorders Nevus sebaceous and Schimmelpenning syndrome, and the oncogenic variant HRASGly12Val, which occurs in 7% of all cancers. Up to date, the molecular basis of cutaneous manifestations are unknown. Here, we aim to characterize the impact of different HRAS mutations on the proteome and phosphoproteome in human keratinocytes using Liquid-Chromatography-Coupled Tandem-Mass Spectrometry (LC-MS) to unravel different pathogenesis mechanisms. Co-immunoprecipitated proteins of HRAS were investigated in the same manner.

Therefore, we generated permanent immortalized human keratinocyte (HaCaT) cells stably expressing the above mentioned HRAS mutations. To obtain single cell clones, cells were transfected with constructs harbouring HA-tagged HRAS variants by electroporation, selected with Geneticin, and single cell sorted by Fluorescence-Activated Cell Sorting (FACS). Single cell clones were further harvested and lysed. For Co-Immunoprecipitation, cell lysates were subjected to Thermo Scientific"s Pierce Anti-HA magnetic beads. The immunoprecipitate as well as the whole cell lysate were then subjected to tryptic digestion following the Single-Pot, Solid-Phase Enhanced Sample Preparation (SP3) protocol (Hughes et al. 2019). For phosphopeptide-enrichment, Thermo Scientific"s High Select TiO2 Phosphopeptide Enrichment Kit was used. Peptides and phosphopeptides were subjected to bottom-up LC-MS/MS analysis using a C18-Reversed Phase chromatography coupled to an Orbitrap-Quadrupole Hybrid mass spectrometer. Resulting raw spectra were searched by Proteome Discoverer (Version 3.0), including the CHIMERYS algorithm. Data normalization and statistical analysis were carried out in the R software environment and Perseus (Version 2.0.3.). Pathway analysis were performed using Ingenuity Pathway analysis (IPA 84978992) and Funrich (Version 3.1.3) respectively.

We found significant differences between the variants on all levels, but HRASGly13Arg (Nevus sebaceous/Schimmelpenning syndrome) appears to be the most distinct variant. In particular, proteins involved in the mesenchymal to epithelial transition (MET) pathway as well as related functions, such as cell motility, migration and cell structure were notably affected by the various HRAS mutations. We aim to further explore implications of these changes in relation to the diseases associated with alll HRAS variants in the future.

P 19

High sensitivity class I immunopeptidomics

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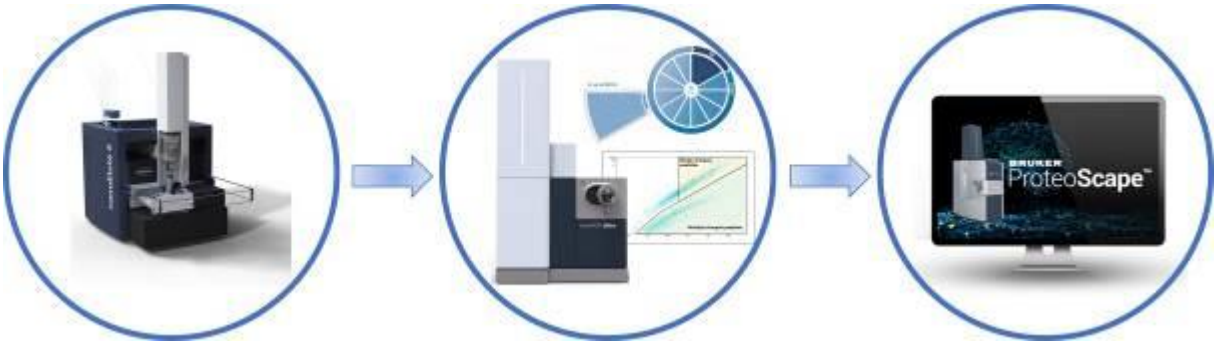
The identification of peptide antigens presented by the major histocompatibility complex (MHC) provides important insights for the understanding of cancer, infectious or autoimmune diseases and for the design and development of the corresponding immunotherapies. However, the low abundance of the peptides of interest, the diversity of their sequences and the complexity of the antigens make their analysis using conventional nano-LCMS a challenge.

Here, we present how the combination of sensitivity, selectivity, and speed of the timsTOF ULTRA makes it possible to overcome these challenges. The combined TIMS and TOF separation enables better discrimination of all peptides, even with the use of short gradients. Coeluting isobaric antigens can be separated and uniquely sequenced. In addition, singly charged MHC class 1 antigens can be analysed in parallel with doubly charged antigens, excluding singly charged background noise. All peptides are coded with their CCS (Collisional Cross Section) value, which increases confidence in identification or sequencing, especially for low intensity signals.

The result is an extremely sensitive setup for analysing very low sample amounts, both of immunopeptide-like standard samples and of true immunopeptidomes. This innovative approach increases the sensitivity of peptide antigen identification, enabling a more accurate and comprehensive understanding of the immunopeptidome landscape. The integration of TIMS with advanced mass spectrometry techniques such as Bruker's polygon filter on the timsTOF Ultra platform represents a significant advance in the field, in particular the identification of previously ignored singly charged antigens and provides new insights into immune responses and disease pathogenesis.

Fig. 1



P 20

Influence of software settings on peptide identification rate by microLC-IM-QTOF and reproducibility of identifications

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The influence of the data evaluation parameters for qualitative results of untargeted shotgun profiling of enzymatic and non-enzymatic post-translational modifications (PTMs) was investigated. For this purpose, the bovine whey protein α-lactalbumin was used as a model. First, the sequence coverage was investigated based on nine untreated samples. Individual adjustment of the protein database and enzyme settings of PEAKS studio software increased the sequence coverage from 93.5 % to 100 % and the number of identified unmodified peptides from 27 to 48. Second, the identification rate of peptides including PTMs was monitored for three untreated, three heated and three lactose-heated samples. Hereby, further adjustments of enzyme settings could increase the number of identified peptides from 322 to 535. In addition, the qualitative reproducibility was investigated based on 18 measurements of the same sample, that was heated with lactose, across three batches. A total of 570 peptides were detected, of which 89 peptides were identified in all measurements, but the largest share of 161 peptides were detected only once, and mostly based on non-indicative spectra. In summary, the study demonstrated that the number of identified peptides is significantly affected by the software settings. Furthermore, it was shown that the identification of peptides in multiple measurements was subject to considerable variation.

P 21

Single cell vs bulk proteomics on the timsTOF SCP

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Single cell proteomics is a rapidly evolving branch of proteomics that benefits from major technological breakthroughs significantly increasing the sensitivity of MS instrumentation and streamlining sample preparation processes. A variety of approaches including SCoPE and label free proteomics have been reported for single cell proteomics so far. We have established a streamlined workflow where cells are sorted in a FACS system directly into a multi-well plate format containing lysis buffer. After overnight the digestion, the plate is transferred to the autosampler of the mass spectrometer for proteomic analysis.

In this study we evaluate the new workflow by sorting a sample containing two different types of cells, namely HCT 116 and Jurkat 6.1, using the FACS machine. We investigated the depth of proteome coverage and the quantitative separation of the two cell types. Furthermore, we plan to investigate the gain in separation and loss of depth of proteome coverage of the two cell types in comparison to the bulk analysis.

Cells were sorted using a FACS Melody system into a 96 or 384 well plate system containing lysis buffer. After sorting, trypsin was added to digest the cells overnight. Samples were measured at a throughput of about 40 SPD (samples per day) by separating the peptides on an in-house packed column spraying directly in to the timsTOF SCP mass spectrometer. Raw data were processed using the directDIA workflow available in Spectronaut.

P 22

Mass spectrometric methods to study the biochemical effect of carbon-based nanomaterials on alveolar rat lung cell line NR8383

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Significance: Carbon-based nanomaterials (CBN) are of great interest in chemical science and are already present in our daily life inside various materials. Although they hold huge potential e.g., in medical use, they also bear great risks by interacting with proteins. Although some CBN such as carbon nanotubes are under the suspicion to be involved in tumour development and inflammation of the lung, little is known about the underlying processes causing these life-threatening diseases. Obtaining certainty regarding the effect of CBN is particularly important with respect to biomedical applications and worker protection. Of special importance in investigating signalling and interaction cascades is the change in the proteome, which can be analysed by LC-MS/MS. Unfortunately, nanomaterial can be a great threat when remaining in the samples due to their interaction with the chromatographic column. Therefore, it is highly important to find a protocol for studying biological effects of CBN with LC-MS/MS.

Methods: Carbon black (CB), as the easiest shaped and most accessible CBN, was selected as model particle. Considering that the lung is one of the major organs of exposure to CBN, the alveolar rat cell line NR8383 was chosen as the cell model. Due to the difficulties in separating highly hydrophobic carbon black particles, the behaviour of those was studied during different stages of the experiment from cell culture over cell lysis to protein digestion. Through this carbon black particle removal strategies were tested at cell level, protein level and peptide level.

Results: The first attempt of CB incubation with cells revealed the tendency of CB to agglomerate in the cell medium, making it unable to infiltrate the cells. This issue could be resolved by pre-coating CB with an excess of BSA. Thereby, the particles were not only able to enter the cells but also the agglomeration was decreased. Still the particles were not removable from the cell lysate after harvesting, which is why further purification techniques at protein and peptide level were investigated. As purification at protein level proved unsuccessful, three different attempts were made to remove CB at the peptide level.

Conclusions: The digestion protocol based on protein denaturation using sodium deoxycholate (SDC) has outperformed the other protocols and was the only one able to remove CB without yielding an insufficient amount of peptide for LC-MS/MS. Through this approach a biological difference between untreated cells and CB incubated cells could have been observed.

P 23

Deep proteomic screening and validation for systematic discovery of molecular glue compounds and novel degrader targets

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Introduction

Targeted protein degradation (TPD) with molecular glue compounds is a breakthrough therapeutic modality to eliminate disease-relevant proteins previously considered undruggable. Despite this enormous potential, systematic discovery of novel molecular glues and their cellular degradation targets has so far been challenging. Here, we present a deep proteomic screening and validation platform to advance TPD drug discovery programs at all stages and create broad pipelines of novel, high-value targets. Deep proteomic screening is a data-independent acquisition (DIA) based MS technology to screen compound libraries of (potential) molecular glues against cellular proteomes at unprecedented throughput, coverage, and sensitivity. It identifies and quantifies more than 11,000 proteins per sample from cell lines treated with molecular glues, enabling comprehensive proteomics-based drug and drug target discovery.

Methods

Treatment of cells with libraries of up to 10,000s of compounds and the subsequent sample preparation is performed fully automated in 96-well format. Samples are measured at enhanced throughput of 24 samples per day in single-shot mode on a highly optimized LC-MS platform on which data-independent acquisition is combined with ion mobility separation (DIA-PASEF). Raw data processing is performed using DIA-NN optimised for maximum coverage, precision, and data completeness. Data are further processed on an in-house custom-made analysis pipeline to maximize the yield of putative neosubstrates which can be mechanistically validated by various biochemical assays such as interactomics (to detect degrader-induced E3 ligase binding), ubiquitinomics (detection of up to 50,000 ubiquitination sites to demonstrate degrader-induced modifications), and others.

Results

We detect and precisely quantify up to 200,000 precursor ions per sample in a 50-minute LC-MS method (24 samples per day including overhead time), resulting in a coverage of up to 11,000 protein groups and a data completeness of more than 99% at protein level. This deep proteome coverage drastically increases the detection of low abundance proteins such as transcription factors. For example, among the 11,000 proteins detected in one sample more than half of all transcription factors encoded by the human genome can be detected. In a proof-of-concept study we treated a panel of cancer cell lines with a set of immunomodulatory imide drugs (IMiDs) or IMiD derivatives. Besides 18 known neosubstrates which we found significantly downregulated in at least one of the tested cell lines, we found additional likely neosubstrates for which we were able to detect treatment induced ubiquitination sites indicating that the respective proteins represent novel neosubstrates.

Conclusion

We have built a scalable, automated, and digitalized proteomics screening platform to detect novel degrader drugs and target candidates combined with their mechanistic validation.

P 24

Proteomic signatures of inflammation-induced early pancreatic carcinogenesis

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Pancreatic cancer still lacks suitable diagnostic biomarkers, especially for early diagnosis in (pre)invasive stages. Emerging evidence suggests that proteomic signatures, modulated by complex posttranslational modifications, may enhance diagnostic and predictive precision by focusing on entire protein profiles rather than individual proteins. To elucidate the initial stages of pancreatic intraepithelial neoplasia (PanIN), we are currently engaged in proteomics studies aimed at unraveling the molecular intricacies of early PanIN. Specifically, we are conducting quantitative comparisons between two cell lines, PanIN4994 and PanIN6585, which represent distinct stages of early PanIN. Additionally, we aim to illuminate the impact of inflammatory signals, such as interleukin 6 or interleukin 22 treatment, on the protein level in vitro.

Methods:

Three biological replicates of PanIN4994 and 6585 cells were cultured for 96 hours, harvested and lysed by sonication. For interleukin treatment experiment, the cells were cultivated to a confluence of 60% before Il6 or Il22 was added. Interleukin treated cells were cultured for further 24h before they were harvested and lyzed. Proteins were proteolyzed with trypsin before high-pH reversed phase fractionation or phosphopeptide enrichment. For quantitative proteomics all biological replicates were analyzed in three technical repetitions using either a label free data dependent analysis (DDA) approach or a reporter ion based quantification after TMT labelling. Samples were analyzed by LC-MS/MS on an orbitrap Q-Exactive Plus mass spectrometer coupled to an U3000 RSLC nano HPLC, linear 240 minute gradients were applied and the MS was operated with a top10 DDA method. All data were processed using MaxQuant and Perseus.

Preliminary results and perspectives:

The proteome analysis allowed the comparative quantification of about 4000 proteins in both cell lines. As anticipated, expression levels of the majority of the quantified proteins remain consistent during the early stages of PanIN, with significant regulatory changes observed in only 221 proteins. Notably, these variations in protein levels align well with previously obtained RNAseq transcriptome data.

For instance, the cellular tumor antigen p53 exhibited a fivefold downregulation in PanIN6585 cells. Other downregulated proteins, such as STAT1 and Nmi, known interactors of p53, are associated with the interferon-gamma mediated signaling pathway. Conversely, upregulated proteins like Sdhd, Aco2, and Dlat primarily participate in metabolic pathways, such as the tricarboxylic acid (TCA) cycle. These findings are consistent with a more advanced stage of cancer in PanIN6585 cells.

Moreover, we conducted an analysis of protein expression in response to interleukin stimuli in PanIN4994 cells. Based on this, we plan to extend our investigation to analyze protein expression in response to NFkB-mediated stimuli in both cell lines and eventually in pancreatic tissue.

P 25

Label-Free quantitation with high accuracy and precision on 250 Picogram to 500 Nanogram sample load scale with orbitrap astral mass spectrometer

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Introduction
Bottom-up proteomics has proven to be the most suitable technology for the analysis of very complex biological samples, such as cell lysates or blood. As the obtained data become more and more employed in biomedical research, the challenge of analyzing large numbers of samples in the shortest time remains. On the other hand, analysis of protein expression at the single-cell level, allowing to reveal cellular heterogeneity, gained increasing importance over the last 5 years and set even higher requirements for sensitivity and reproducibility of measurements.

An integrated workflow for label-free quantitative proteomic studies has been developed, to meet all these challenges. Orbitrap Astral mass spectrometer is very suitable for quantitative analysis of complex proteomics samples on a wide range of sample loads from single cell like sample loads to hundreds of nanograms or microgram sample loads, In this work, the performance of the system is demonstrated for label-free quantitation of a mixture of digests of 3 proteomes, on a level of 250 picograms to 500 nanograms per injection.

Methods
Digests of Human, Yeast and E. coli proteomes were mixed at different ratios and analyzed at total protein loads from 250 pg to 500 ng/injection. Peptides were separated on a 25 cm Aurora™ column (Ionopticks) in a direct injection workflow, using a Vanquish™ Neo UHPLC system under nanoflow conditions, at different gradient length. MS analysis was performed on the Thermo ScientificTM OrbitrapTM AstralTM mass spectrometer, using DIA methods with different settings, depending on the sample load per injection. MS1 scans were collected at 240,000 resolution using the Thermo ScientificTM OrbitrapTM analyzer in parallel with MS2 scans at a resolution of 100,000 using the Thermo ScientificTM AstralTM analyzer. Ultra-fast (up to 200Hz), narrow windows (2 Th) DIA experiments were used for high sample loads, whereas wide windows (up to 30 Th) and long ion accumulation times (up to 60 ms) were required for single-cell-level loads. FAIMS was used for loads from 250 pg to 10 ng. Data were analyzed using Spectronaut 18 software (Biognosys).

Results
As the result, more than 13,000 proteins were quantified with a median CV of less than 5% for 500 ng protein load, and more than 7,000 proteins were quantified with a median CV of less than 8% for a 250 pg protein load, with directDIA, library-free processing. Quantitation accuracy remained high throughout the entire range of sample loads, with the median numbers of data points from 3 to 6.

Figure 1. Spectronaut 18.2, LFQBench wiev for the 250pg load of a 3-proteome digest mixture. Method details: 20 min gradient, 25 cm Aurora column (Ionopticks), FAIMS on, Orbitrap 240K, cycle time 0.6s, IT 100ms, DIA 20Th window, Astral IT 40ms.

Figure 2. Spectronaut 18.2, CQEBench wiev for the 250pg load of a 3-proteome digest mixture.

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Poster Presentation

Fig. 1

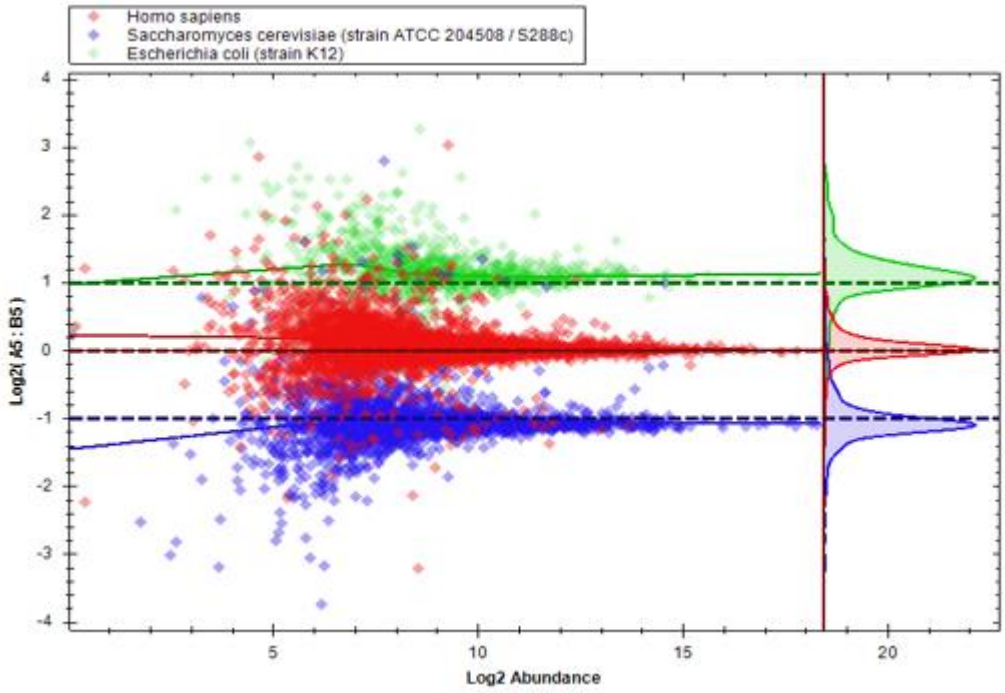
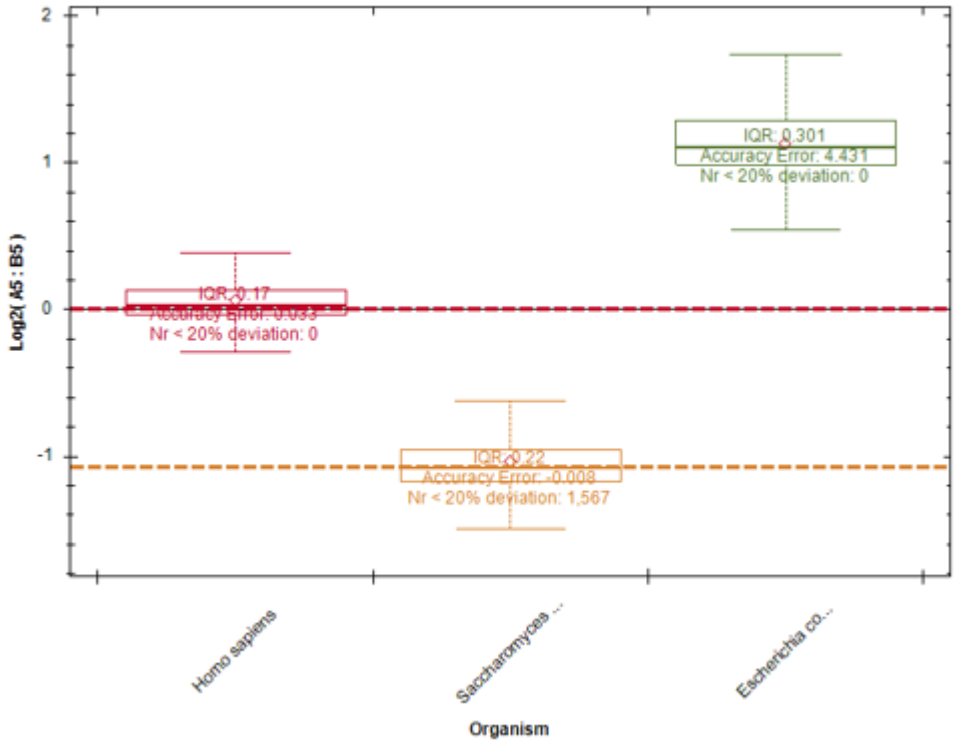


Fig. 2



P 26

Differential proteomics in the defense response of Zea mays to multitrophic biotic stresses

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Fusarium verticillioides is a phytopathogenic fungus that highly impacts crops of maize and other grasses, causing economic losses associated with stalk and ear rot. Furthermore, the production of mycotoxins present great health risk when the crops are consumed. Recent studies revealed the fungus" intimate association with the borer *Diatraea saccharalis*, an insect pest of great relevance to the same crops. Plants infected by *F. verticillioides* produce volatile organic compounds that increase *D. saccharalis* attraction. Also, *F. verticillioides* is capable of being transmitted vertically in the insect and to manipulate its behavior to increase dissemination. Knowledge of how these interactions happen at the protein level is scarce and poorly explored. As such, the aim of the current work was to explore the proteome changes of *Z. mays* B73 plants exposed to *F. verticillioides* infection (FV), *D. saccharalis* herbivory (DS), or the combination of both (FVDS), together with a mock treatment (MK). Through total proteome analyses using a TIMS-TOF device , 4122 protein groups were identified in all treatments after data processing in MaxQuant,. Differential expression analyses were carried out using R and the *limma*-package together with Perseus. KEGG pathway and GO enrichment analysis were carried out on differential abundant proteins (DAPs). Five comparisons were made to identify key proteins in multitrophic biotic interactions (DS/MK, FV/MK, FVDS/MK, FVDS/DS, FVDS/FV), which were further separated to identify unique and shared proteins between treatments. The upregulation of chitinase A1, germin-like protein 8-14, PRX12 and subtilisin-chymotrypsin inhibitor 1 seem to be a common response of *Z. mays* to these biotic stresses. In both DS and FVDS treatments, a strong induction of glutathione and phenylpropanoid metabolism was observed, together with many proteinase inhibitors and LOX metabolism-related proteins, as a common response against insects. Notably, LOX2 and LOX10 were only upregulated in DS, but not in FVDS, treatment. In the FV and FVDS treatments, a gibberellin receptor GID1L2 with no described function was upregulated, together with ASR2. Also, ASR5 and 6 were upregulated in FVDS, which could indicate a role of ABA in these interactions. In both FV and FVDS, many different 60s ribosomal proteins were also upregulated, a mechanism previously described to increase tolerance to plant pathogens. Most DAPs were unique to the FVDS treatment, and included a large set of known and unknown Pathogenesis Related (PR)-proteins, like OSM34, PRP3, CYP71Z18 and many others. Interestingly, BX11 and BX14 were only upregulated in the FVDS condition, although being known for the synthesis of the insecticidal compound benzoxazinone. Taken together, this exploratory research represents the firsts steps to identifying key proteins and pathways that are responsive to multitrophic interactions of agronomical importance in maize.

P 27

Proteomic differences in patients with high and low risk of endometrial cancer recurrence

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Endometrial cancer is the most prevalent gynecological cancer worldwide. Many patients with endometrial cancer have favorable prognosis. However, there is a substantial number of patients with recurrent disease and poor response to treatment (high-risk group). It is recommended to classify endometrial cancer patients into low- or high-risk groups based on various features including tumor grade, lymphovascular space invasion, myometrial involvement, and non-endometrioid histology [1]. Despite advancements in molecular profiling of endometrial cancer, a substantial group of patients are misclassified according to the current criteria. The aim of this research was to find proteins that may be used as biomarkers for grouping endometrial cancer patients into low- or high-risk groups. A total of 52 patients were classified as patients with a high risk of endometrial cancer recurrence, and 63 patients were classified as patients with a low risk of endometrial cancer recurrence. Serum samples from endometrial cancer patients were subjected to proteomic analysis using matrix-assisted laser desorption/ionization–time of flight mass spectrometry (MALDI-TOF MS). The data obtained were analyzed using chemometric algorithms to identify potential biomarkers. A total of nineteen precursor ions were recognized as fragments of eighteen proteins, comprising (1) connective tissue matrix proteins, (2) cytoskeletal proteins, (3) innate immune system molecules and stress proteins. Interestingly, 7 out of 19 of the discriminatory proteins in our study belong to cytoskeleton components as mulitple components of microfilaments are deregulated. This study utilizing MALDI-TOF MS combined with solid-phase extraction pretreatment to analyze plasma samples from patients with endometrial cancer highlighted the potential of proteomic analysis to identify biomarkers for risk stratification in endometrial cancer and improve treatment selection. Proposed potential biomarkers could potentially categorize patients into high- and low-risk groups, but their utility as biomarkers of high-risk endometrial cancer requires further investigation.

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P 28

Absolute quantification at proteome-wide scale using a generic internal standard

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Absolute (molar) quantification of proteins helps us to determine stoichiometric ratios within molecular assemblies and metabolic pathways. It also provides reference values for diagnostically important proteins and additionally, helps us to estimate the expression levels in cells and tissues. Here, we demonstrate that absolute quantification support the direct comparison of proteome composition of phylogenetically distant organisms that does not rely on the proteins sequence identity.

We have developed a workflow for proteome –wide absolute quantification by DDA with a family of FUGIS (Fully Unlabelled Generic Internal Standard) (1) proteins as the internal standard. A known amount of FUGIS chimeric protein is added along with protein test samples and they are all reduced, alkylated and digested together. The samples are analyzed by LC-MS/MS and the raw data files are processed with MSFragger (2). We have additionally developed a software- called GlobeQuant (1) and the output .tsv files from MSFragger is loaded into this software.The software calculates the molar amount of the FUGIS peptides and relates it to the median area of XIC peaks of FUGIS peptides peaks and further uses it as a single-point calibrant to determine the molar abundance of any codigested protein. The peptides of the codigested sample proteins are selected by the principle of BestNAverage, where the "BestN" is selected from the Top peptides based on a criteria of CV%.

This Proteome- Wide Scale absolute quantification is used to determine and compare the quantities of functionally related proteins in the whole cells of minimal JCVI-syn3A bacteria (3) and near minimal bacterium *Mesoplasma Florium* (4). We underscore that protein levels comparison could not be achieved by considering their genomes or (if available) transcriptomes.

These minimal organisms help us to understand the fundamental principles of life and the basic requirements for cellular function, including metabolism. Additionally, giving us the advantage of working with whole organism, allowing us to perform absolute quantification in an untargeted way to quantify the whole proteome across organisms hence quantifying and comparing the homologous proteins at the organism level.

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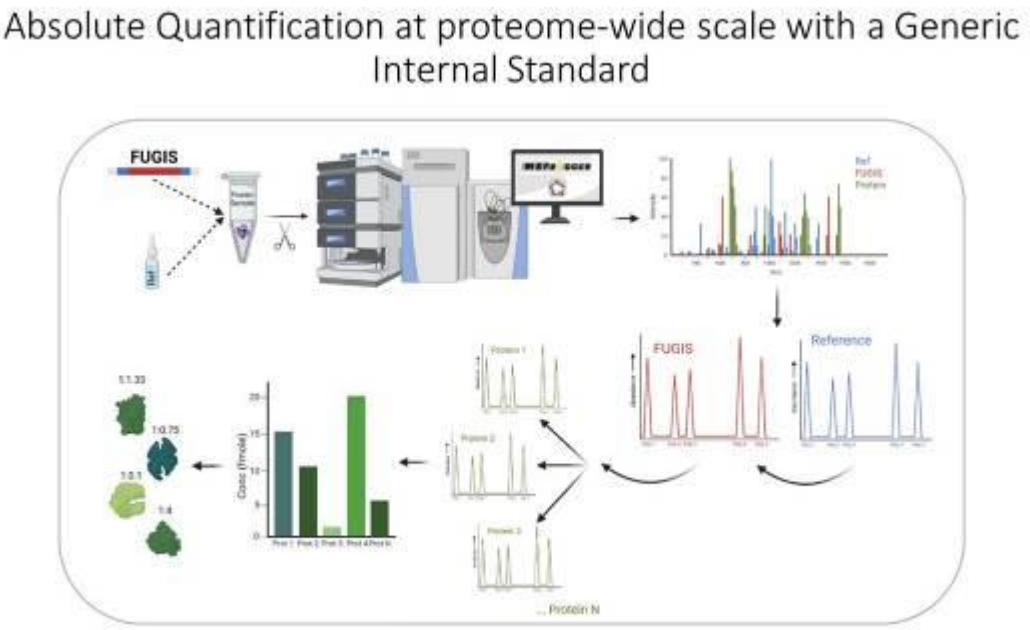
Poster Presentation

Fig. 1



P 29

A CCS-centric HLA-specific trained *de novo* module for precise and accurate real-time immunopeptide identification on the Bruker ProteoScape platform

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Introduction: Since its conception, Bruker ProteoScape (BPS) has transformed into a comprehensive proteomics data analysis platform that can integrate third-party tools while utilizing the concept of data streaming to realize fully customizable real-time processing workflows. To expand the capabilities of the BPS platform for immunopeptidomics, and other applications, we previously developed and integrated a timsTOF optimized *de novo* sequencing engine from Rapid Novor Inc., called BPS Novor. To further develop this tool and support the extremely rapidly growing field of timsTOF based immunopeptidomics, we have retrained this module with PSMs specifically generated from MHCI and MHC II samples.

Methods & Results: Novor was re-trained on a variety of timsTOF acquired data, where ground truth is taken from the Sage database search results filtered to 1% PSM FDR. Previously shown that BPS Novor is highly accurate and precise on a variety of datasets. On amino acid level, at 75% precision, BPS Novor achieved between 40-60%, whereas standard Novor achieved between 25-50%, and Peaks Studio achieved between 25-55% recall. We have also shown that BPS Novor is extremely fast by evaluating the processing speed across 5 datasets, with an average processing speed of 1338±226 spectra/second. Here we compare the re-trained BPS Novor against other tools across multiple immunopeptidomic datasets using standardized numbers of computing cores and input file formats to allow direct comparison between algorithms.

In this study, we both revaluated the performance of BPS Novor on previous datasets and confirmed similar precision, accuracy, and speed. We then focused on two recently published immunopeptidomics datasets and show the newly optimized BPS Novor module increases precision and accuracy over the previous versions for immunopeptidomic applications, while retaining the processing speed advantage over other algorithms.

Conclusion: We here introduce a purposefully optimized BPS Novor module, providing on-the-fly real-time de novo sequencing for timsTOF immunopeptidomics data.

Conflict of Interest Disclosure: R.Z., Q.L., M.X., B.M. are employees of Rapid Novor, Inc. Q.L., M.X., B.M. are co-founders of Rapid Novor, Inc. L.A., D.T., T.S., J.K., G.R. are employees of subsidiaries of Bruker Corp. Novor is a product of Rapid Novor, Inc. BPS Novor is a product of Rapid Novor, Inc. sold and distributed by subsidiaries of Bruker Corp.

P 30

Exposure of aged microplastic induces changes in the proteome of *Daphnia magna* – a comprehensive ecotoxicoproteomic study

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Microplastics are fragments of any type of plastic with particle sizes less than 5 mm. Microplastic pollution, pervades both marine and freshwater environments. In freshwater ecosystems, the cladocera *Daphnia magna* holds a pivotal role and is a well-established model organism in ecotoxicology. Furthermore, *D. magna* provides valuable insights into potential impact of microplastic ingestion [1][2]. Since morphological and chemical properties of plastic particles undergo significant changes as they age through mechanical stress and solar radiation, this study aims to explore the impacts of ingesting aged microplastics compared to their pristine counterparts. We therefore analyzed proteomes of *D. magna,* exposed to well-characterized pristine and artificially aged microplastics of multiple polymer types (PS, PP, and LDPE). Using a data-independent acquisition (DIA) method, DIA-NN analysis and a tailored bioinformatic workflow, we were able to identify around 4800 proteins, even though the *D. magna* proteome is not highly annotated. Strikingly, when compared to pristine PP, aged PP induced the smallest number of protein alterations, while we identified significantly more differentially abundant proteins for PS and LDPE microparticles. To further increase the analytic depth, we examined the proteome of dissected *D. magna* guts, where the exposure to MP is particularly pronounced [3]. Employing the same DIA approach, we were able to identify approximately 5600 proteins from individual daphnid gut samples. Similar to the analysis of entire daphnids, we found that aged PP caused the lowest number of altered proteins, compared to pristine PP, with PS and LDPE particles showing significantly more differentially abundant proteins.

In conclusion, we identified differentially abundant proteins when comparing aged and pristine microplastics as well as variations between different microplastic types. These findings underscore the impact of microplastic ingestion on *D. magna* proteomes and highlight the importance of considering both the aging process and polymer types in studies addressing the biological effects of microplastics. Overall, our dataset improves our understanding of the molecular mechanisms underlying the adverse effects of microplastics in freshwater ecosystems.

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P 31

Analysis of virus-host interactome from SARS-CoV-2 N-protein by structural mass spectrometry

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The multifunctional and highly abundant N-protein (Nucleocapsid) of SARS-CoV-2 is crucial for several processes during infection starting with protection and packaging of viral RNA but also replication, suppression of immune response or virus assembly. Although the mutation rate is lower compared to the spike protein, N also changes across variants indicating an adaption to the host under immune pressure. In this context, we want to identify host proteins that are involved in protein-protein-interactions (PPIs) with the N protein in a spatiotemporal manner and investigate possible changes in the N-interactome across the mutants. Here, distinct mutations may alter preferred binding partners and assembly states with an effect on the whole infection cycle.

Based on AP-MS from literature and computational predictions of possible N-specific host protein complexes using AlphaFold-Multimer, we selected candidates binding to mutation-prone regions of N or occupying common binding sites for expression and purification. Taking into account different proteoforms including post-translational modifications (PTMs), we purify proteins from mammalian cells and confirm complex formation by native mass spectrometry.

P 32

Duchenne muscular dystrophy carriers: Proteomic insights using a large animal model

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Duchenne muscular dystrophy (DMD) is a severe X-linked genetic disorder caused by frameshift mutations in the dystrophin gene (*DMD*), resulting in the complete absence of the encoded protein. DMD is characterized by progressive muscle degeneration and a substantial reduction in life expectancy. Although DMD primarily affects males, female carriers of the disease are also at a significantly increased risk of developing various cardiomyopathies as well as muscle weakness (Lim et al., 2020). However, studies addressing underlying molecular mechanisms are lacking. To fill this gap, both skeletal and cardiac muscle are of particular interest, of which human materials are not readily available due to ethical reasons. Tailored *DMDΔ52* porcine models of DMD which recapitulate important biochemical, histological, and functional features of the human disease have been previously developed (Stirm et al., 2021). At the same time, female carriers also serve as an excellent model for the effects of heterozygous *DMD* deficiency in women. The left ventricle and triceps muscle of six-month-old wildtype and carrier pigs were analyzed with data independent acquisition and DIA-NN. Using a peptide-level-based method (Ammar et al., 2019), we identified 32 significantly altered proteins in the left ventricle and 52 in the triceps. In both heart and skeletal muscle tissue, the carriers showed a slight decrease in dystrophin. Also, levels of dystroglycan and dystrobrevin, which are members of the dystrophin complex, were slightly reduced in the left ventricles of carriers. Various collagens and NADH dehydrogenases were altered in abundance in both tissues, with some of those proteins affected differently in the two tissues. In summary, our findings are giving first insights in molecular alterations with relevance for the observed increased incidence for muscle weakness and cardiomyopathies in female DMD carriers.

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P 33

Scalable mass spectrometry detergents identify a non-canonical lipid interaction in Gram-negative bacterial membranes

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Lipids surrounding proteins in the cell membrane of Gram-negative pathogens play roles in biological functions. The cell wall of Gram-negative bacteria contains two lipid bilayers known as outer membrane and inner membrane. Inner membranes contain phospholipids (PLs), which are important for membrane integrity and protein function. Lipopolysaccharide (LPS) is synthesized across the inner membrane and translocated to the outer membrane where it is displayed at the outer leaflet. The biological relevance of LPS binding has been widely studied for proteins involved in LPS synthesis and translocation, which delivered targets for antibiotics, such as polymyxins. However, LPS binding is also frequently obtained after purification of membrane proteins not associated with outer membrane biogenesis and function. The scientific question whether co-purified LPS binding to inner membrane proteins not assigned to outer membrane biogenesis represents a protein-lipid interaction existing in inner membranes needs to be clarified. The problem behind this scientific question is that current protein purification protocols inevitably involve the mixing of inner and outer membrane components which complicates our understanding and therefore assessment of critically important membrane lipids in the structural and functional regulation of inner membrane proteins in Gram-negative bacteria. To address this scientific question, a scalable mass spectrometry detergent technology is presented that was implemented into a purification method with a native mass spectrometry readout with several membrane protein analytes to decouple detergent-driven membrane solubilization from protein delipidation. Specifically, a new class of hybrid detergents with tuned fundamental detergent properties, i.e., molecular shape and polarity, is designed to gradually control the capability of detergent aggregates to retain or remove protein-lipid binding during membrane protein purification. The results obtained from this approach strengthen the hypothesis that lipopolysaccharide (LPS), an integral lipid in outer membranes of Gram-negative bacteria, can also bind proteins not assigned with outer membrane biogenesis. To exemplify, in the case of the vitamin B12 transporter BtuCD, a series of orthogonal techniques was used to decipher the biophysical properties that facilitate this apparent high-affinity and yet underappreciated protein-lipid interaction. Molecular dynamic simulations outline specific LPS binding patches on the BtuCD dimer surface and suggest a modulation of protein activity, which can be recapitulated using in vitro activity assays. Taken together, these results suggest redefined fundamental roles of LPS in membrane biology governing Gram-negative bacteria and spotlight a new membrane protein-lipid interaction that is generally interesting for the development of membrane-targeting antibiotics.

P 34

Deep proteomic characterization of more than 120 cell lines

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Biomedical research heavily relies on the indispensable utility of cell lines. Due to their ease of handling, widespread availability, and facilitation of straightforward investigations into diverse treatments, cell lines play a crucial role in exploring the mechanisms of action during drug development. To ensure the efficacy of such studies, a profound characterization of cell lines becomes imperative, aiding in the selection of the most appropriate cell line for a specific drug.

In our comprehensive study, we conducted a deep proteomic characterization encompassing over 120 distinct cell lines originating from six different organisms. Employing a semi-automated workflow, all samples were processed with a KingFisher Apex robot and subsequently subjected to 2-hour data-independent acquisition (DIA) runs utilizing cutting-edge LC-MS/MS systems, including the Vanquish Neo UHPLC System, Orbitrap Eclipse Tribrid Mass Spectrometer, and Orbitrap Exploris480 Mass Spectrometer from Thermo Fisher Scientific. Additionally, 12 cell lines underwent an extensive characterization involving six gas-phase fractions (GPF).

In human cell lines, single-shot DIA measurements yielded an average identification of more than 8,000 unique proteins. The implementation of GPF increased this number, resulting in the identification of over 10,000 unique protein groups. Mouse cell lines exhibited an average identification of 7,500 proteins, while rat cell lines displayed an identification of 8,100 proteins.

A key focus of our analysis was the expression profiling of different kinase groups within the characterized dataset. Human cell lines consistently exhibited the unique identification of 300 to 436 kinases, with 140 to 220 falling under the category of E3 ubiquitin ligases. Additionally, we identified 40 to 78 proteins belonging to the dark kinome. This rich dataset empowers researchers to make informed decisions regarding the selection of optimal cell lines tailored to specific research inquiries.

In summary, our extensive proteomic characterization of diverse cell lines provides a valuable resource for the scientific community, facilitating the identification of suitable cell lines for a myriad of research questions and enhancing the understanding of cellular responses to various treatments in the context of drug development.

P 35

Advancing bone proteomics: A novel extraction method applied to diabetic bone healing models

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Critical-sized bone fractures are characterized by a prolonged or even absent healing without surgical intervention. Individuals comorbid with the systemic metabolic disorder Type 2 diabetes mellitus (T2DM) even face an elevated risk for impaired healing. In order to optimize personalized treatment approaches using PCL-scaffolds, it is crucial to understand the molecular mechanisms at play.

For this, we performed a systematic comparison of various protein extraction methods from bone, including acid- or chaotropic-based buffers and enzymatic digestion of collagen. The goal was to optimize for lower collagen content in extracts, enabling deeper proteome analysis and a time-effective, reproducible protocol. We developed a rapid, two-step extraction method, enhancing reproducible protein quantification, reducing extraction time down to three days, and limiting analysed fractions per sample. The performance of the method in comparison to reference methods was evaluated using nano-LC-MS/MS in diaPASEF mode on a timsTOF Pro 2 instrument.

Utilizing this innovative extraction method, we explored PCL scaffold-guided bone regeneration in diabetic rats. Employing a diabetic rat model with a critical-size femur defect, scaffold-supported regenerated and contralateral tissue at 21 or 42 days post-implantation underwent LC-MS/MS-based proteomic analyses on a quadrupole-Orbitrap mass spectrometer (qExactive). This allowed for the quantification of over 4,000 proteins, providing a comprehensive view of bone healing under diabetic conditions. Functional analysis differentially abundant proteins confirmed an impairment of bone metabolism during diabetic bone healing, indicated by prolonged inflammatory protein expression and reduced structural protein levels necessary for soft callus formation. More striking, a protein cluster of an immune cell population was observed, that previously has been linked to impaired diabetic wound healing and has been discussed for their detrimental effect when overly active in later stages of bone healing. Taking together our metabolomic analyses confirming increased levels of their corresponding inflammatory mediators along with histological analyses proving an increased cell number, we identified a potential target population to address compromised diabetic bone healing.

Our findings offer vital insights into the disturbed molecular processes of diabetic bone regeneration. The study highlights the importance of deep proteomic profiling in understanding the adverse effects of T2DM on bone healing and lays the groundwork for scaffold optimization through biofunctionalization. This research marks a significant advancement in the field, potentially guiding future treatments to mitigate T2DM's impact on bone healing.

P 36

TwinScape: A digital twin-driven concept for improved timsTOF platform monitoring and data quality assurance

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Purpose: Resource and data quality management are key concerns for modern omics laboratories. The creation and use of virtual environments representing complex processes or systems, known as "digital-twins", has successfully enhanced system monitoring and decision making in a variety of fields including power utilities, railcar design and aircraft production. Here, the concept has been adapted for use in mass spectrometry-based omics research using automated information gathering and visualisation.

Methods: The instrument health and performance characteristics of advanced hyphenated proteomics mass spectrometry systems (typically comprising a nanoElute 2 liquid chromatograph and a timsTOF mass spectrometer) were monitored using Bruker TwinScape software. System performance was benchmarked using the Biognoysis iRT peptide standards mixture, and pre-processing of raw data was performed using Bruker ProteoScape.

Results: The use of a digital-twin approach (TwinScape) paired with standardised performance benchmarking (Biognoysis iRT peptide standards) enabled the comprehensive review of key data quality factors including total peptide and protein identification rates, as well as iRT peptide measurement parameters. Concurrent metadata and instrument health data acquisition allowed for interpretation of changes in performance metrics. Together, the approach provided assurance of instrument performance and enabled reproducible results for omics studies.

Conclusion: The digital-twin concept successfully facilitates bioanalytical platform monitoring and data quality assurance when combined with standardised benchmarking materials. When deployed across a modern omics laboratory, TwinScape enables "at a glance" monitoring of instrument health. In the future, the digital-twin concept may be further developed to deliver additional benefits including enhanced service support as a step towards enabling predictive maintenance.

P 37

Innovative high-throughput ENRICH-iST workflow facilitates fast and deep plasma and serum proteome profiling

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Introduction

Blood plasma/serum is one of the least invasive form of biopsy and a valuable sample for clinical research and patient health monitoring. However, the high dynamic range as well as sample heterogeneity and complexity pose significant challenges for LC-MS-based proteomics, thereby limiting access to the full proteome information and making in-depth profiling difficult at the rate required to study large cohorts. The ENRICH-iST workflow provides a robust and easy-to-use solution to the dynamic range challenge in plasma/serum and enables fully automatable high-throughput sample preparation for LC-MS-based plasma proteomics.

Methods

Starting with 20 µL plasma/serum, the novel ENRICH technology allows dynamic range compression by enriching low-abundance proteins onto non-functionalized paramagnetic microbeads. For subsequent LC-MS sample preparation, on-bead denaturation, reduction and alkylation, digestion and peptide clean-up were performed according to the iST-BCT protocol. Peptides were analyzed by nanoLC coupled to a timsTOF instrument (Bruker) using dia-PASEF® acquisition mode.

Results

Parallel processing of up to 96 samples using the ENRICH-iST workflow can be completed within 5 hours. Human plasma and serum samples processed with ENRICH-iST were compared to neat samples (iST-BCT) showing an increase in protein identifications by ≥2-fold for plasma and 1.5-fold for serum while demonstrating excellent repeatability with median CVs <10%. Mapping the identified plasma proteins to the Human Protein Atlas revealed a superior proteome coverage for the ENRICH-iST workflow over neat plasma and the classical depletion approaches. From the most abundant protein albumin (40 g/L) to proteins below 10 ng/L, ENRICH-iST covers a concentration range of 10 orders of magnitude.

Remarkably, as an ´antibody-free´ approach, the ENRICH technology is not specific to human samples and was successfully applied to mouse and rat plasma, demonstrating its compatibility with various mammalian species.

Conclusion

The ENRICH-iST kit is a cutting-edge, all-in-one workflow for low abundance protein enrichment in blood-derived biofluids coupled to bottom-up proteomic sample preparation. It allows deep proteome analysis of clinically relevant biofluids from various species at high throughput, in a robust, fast and easy-to-use manner.

P 382

Evaluation of the relative quantitative performance using tandem mass tags on a new high-resolution accurate mass platform

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Introduction

Isobaric tagging methods such as the Thermo Scientific Tandem Mass Tag (TMT) have emerged as the method of choice for relative quantitation of proteins across a high number of samples with good precision.

Mass resolving power exceeding 50,000 FWHM at low m/z is required to resolve TMT reporter ions and isobaric interferences. Orbitrap instruments are capable of these requirements however at the expense of lower scan speed.

A novel high-resolution accurate mass (HRAM) platform with mass resolving power up to 100,000 FWHM and scan speed up to 200 Hz in MS2 mode was evaluated for its quantitative performance using biological samples of different complexity.

Methods

A novel HRAM mass spectrometer was coupled to Vanquish Neo nano-LC.

Thermo Scientific™ Pierce™ TMT11plex yeast digest standard as well as a more complex TMTpro 16plex 3 proteome mix were used to optimize method parameters such as maximum injection time, dynamic exclusion, automatic gain control, FAIMS compensation voltage, as well as different acquisition schemes to achieve the highest number of quantified proteins with good accuracy and precision.

All data was processed using Proteome Discoverer software.

Results

The new high-resolution accurate mass platform has a top scan speed of up to 200 Hz in MS2 mode, limited by the maximum injection time.

Shorter maximum injection times results in higher protein identification (ID) numbers for individual samples but can limit the S/N of isobaric tag reporter ions resulting in fewer quantifiable proteins for multiplexed samples. This limitation can be overcome by increasing maximum injection times to provide sufficient reporter ion S/N for precise quantitation.

Increasing the maximum injection times for TMT-labeled samples results in speeds between 20 and 50 Hz for MS2 scans in DDA mode. Despite using slower scan speeds compared to label-free samples, the improved instrument speed and sensitivity for this new HRAM platform results in >3,000 quantifiable protein groups and >20,000 quantifiable peptides using a 50-minute gradient method for the TMT11plex yeast digest standard. This is a 1.8x increase in quantifiable protein IDs and 2x increase in quantifiable peptide IDs compared to results generated previously on Orbitrap hybrid instruments.

The optimized parameters and acquisition scheme from TMT11plex yeast digest standards were applied to the TMT-labelled plasma sample and used as starting point for assessing a 3 proteome mixture labeled with TMTpro 16plex reagents.

Preliminary data from the TMT16plex-labeled plasma sample mixture shows > 97% quantifiable protein groups in a 60 minute gradient. Preliminary data from the 3 proteome mixture shows >6,000 quantifiable protein groups in a 90 minute gradient.

Conclusion

The new high-resolution accurate mass platform provides the speed and sensitivity for accurate and precise relative quantitative proteomics.

P 39

Dynamic N-glycosylation pathway during mouse corpus callosum development by integration of N-glycomics and proteomics

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N-glycosylation is the most heterogeneous post-translational modification of proteins and more challenging to profile the whole landscape more than other modifications. By permethylation, 244 N-glycan compositions were quantified by label-free strategy across four postnatal stages of mouse corpus callosum and further verified by paired 12C/13C-permethylated N-glycans with isotopic labelling, showing precise N-glycan dynamics during the development of mouse corpus callosum by nanoLC-MS/MS in this study. We observed that N-glycome expression pattern gradually regulated in corpus callosum during mouse growth. Meanwhile, comparative proteomics was also performed by label-free quantification with a coverage of 3,686 proteins. The correlation between dynamic N-glycomics and proteomics involving into N-glycosylation pathway were further investigated, showing their high consistency and novel biomolecular features unique to development of mouse corpus callosum compared to other brain subregions. Focusing on the known N-glycosylation pathway, the accumulation of high-mannose N-glycans in early stages of mouse CC might demonstrate that N-glycan biosynthesis was faster and more glycoproteins were urgently required to promote the maturation of mouse CC, resulting into high-mannose glycoproteins escaped the further Golgi modification. That is the hindrance to understand their roles during the growth of mouse CC. As another molecular landscapes, functional proteomics provide direct phenotypes to understand the dynamic development of mouse CC. Overall, the proteomic profiling is consistent with previous study in spite of the usefulness of mouse CC in different time points. Gradually up-regulated proteins that are mainly involved into the generation of precursor metabolites and energy, ATP metabolic process and mitochondrion organization was found, which meant that higher energies were required to contribute to the maturation of mouse CC. Gradually down-regulated proteins were mainly involved into the step-down nutrient uptake, and biosynthesis and degradation of proteins. The proteomics landscapes meant that less proteins were required and degraded, and tend to be steady with the gradual maturation of mouse CC. The proteins involved in protein processing in ER were also demonstrated to be gradually decreased, showing consistency with the gradually decreased levels of high-mannose N-glycan that is attached on the nascent proteins in the lumen of ER. Thus, we first realized the correlation analysis between N-glycomics and proteomics in this study, and demonstrated their high consistency. In conclusion, we realized in-depth N-glycomics and proteomics using traces of mouse CC tissue. High coordination between N-glycomics and proteomics is firstly investigated in this study and showed comprehensive landscapes of dynamic N-glycosylation pathway during mouse CC development. The datasets are valuable resources for better understanding the CC development in human brain.

Fig. 1

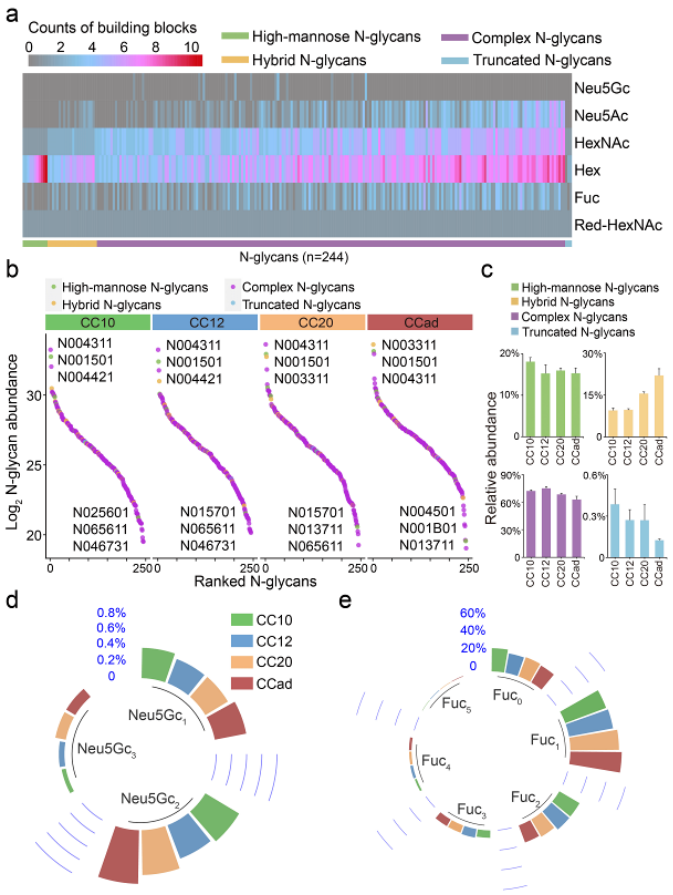

a
Counts of building blocks
0 2 4 6 8 10
High-mannose N-glycans
Hybrid N-glycans
Complex N-glycans
Truncated N-glycans
Neu5Gc
Neu5Ac
HexNAc
Hex
Fuc
Red-HexNAc
N-glycans (n=244)
b
High-mannose N-glycans
Hybrid N-glycans
Complex N-glycans
Truncated N-glycans
CC10
CC12
CC20
CCad
Log2 N-glycan abundance
Ranked N-glycans
c
Relative abundance
d
e

Fig. 2

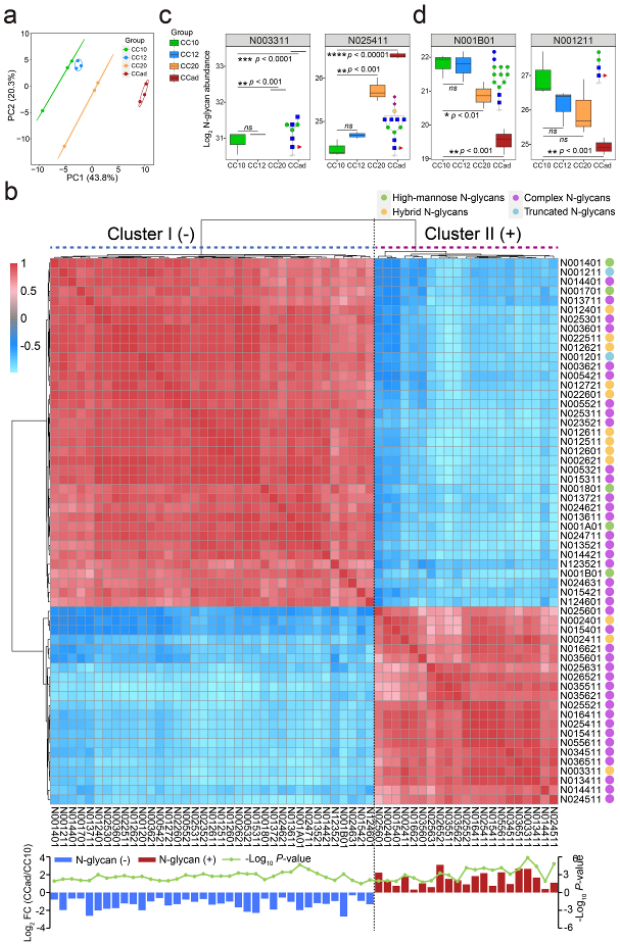
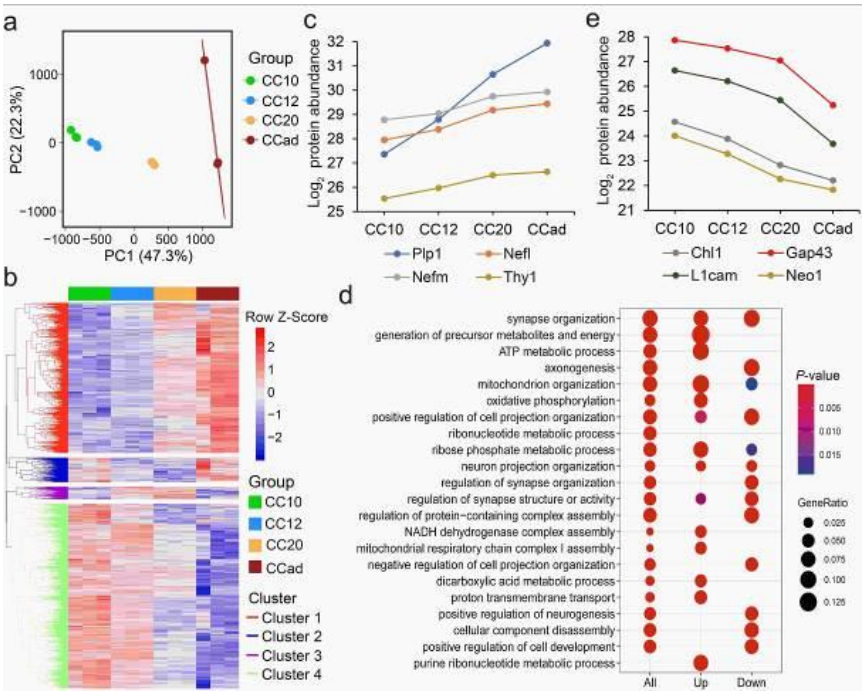

a
c
d
b
Group
CC10
CC12
CC20
CCad
PC1 (43.8%)
PC2 (20.3%)
N003311
N025411
N001B01
N001211
Log2 N-glycan abundance
High-mannose N-glycans
Hybrid N-glycans
Complex N-glycans
Truncated N-glycans
Cluster I (-)
Cluster II (+)
N-glycan (-)
N-glycan (+)
-Log10 P-value
Log2 FC (CCad/CC10)

Fig. 3


a
c
e
b
d
Group
CC10
CC12
CC20
CCad
PC1 (47.3%)
PC2 (22.3%)
Log2 protein abundance
Plp1
Nefl
Nefm
Thy1
Chl1
Gap43
L1cam
Neo1
Row Z-Score
Cluster
Cluster 1
Cluster 2
Cluster 3
Cluster 4
synapse organization
generation of precursor metabolites and energy
ATP metabolic process
axonogenesis
mitochondrion organization
oxidative phosphorylation
positive regulation of cell projection organization
ribonucleotide metabolic process
ribose phosphate metabolic process
neuron projection organization
regulation of synapse organization
regulation of synapse structure or activity
regulation of protein-containing complex assembly
NADH dehydrogenase complex assembly
mitochondrial respiratory chain complex I assembly
negative regulation of cell projection organization
dicarboxylic acid metabolic process
proton transmembrane transport
positive regulation of neurogenesis
cellular component disassembly
positive regulation of cell development
purine ribonucleotide metabolic process
All
Up
Down
P-value
GeneRatio

P 41

Visual proteomics of subregions in colorectal cancer FFPE samples

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Recent advances in mass spectrometry instrumentation have increased the sensitivity and thereby facilitated the analysis of single cells and other samples with low input material. Noteworthily, the advent of timsTOF SCP and Orbitrap Astral mass spectrometers have shown the feasibility of carrying out single cell studies to the depth of more than 3000 proteins from such low sample amounts. These recent technologies now ease a range of applications arising from low sample amounts both in a sensitive and high throughput manner.

Laser capture microdissection involves spatially selective analysis of cells, shapes or parts of tissues dissected from a biospecimen on a slide format under the microscope. Such analyses also profit from the latest sensitivity and throughput benefits of high sensitivity proteomics. Visual proteomics combined with high sensitivity sample preparation and analysis pipeline enable precise quantitative analysis of tumor microenvironment and normal regions of tissue biopsies.

Here, we combined our established high sensitivity proteomics pipeline together with Leica LMD7 laser capture microdissection instrument to build a visual proteomics workflow. We plan to investigate the proteomic profiles of colorectal cancer patient samples. The tissue biopsy was formalin fixed and sections were mounted on PEN glass membrane slides stained and examined under Leica microscope. Samples were dissected and collected into reaction vessels for lossless sample preparation and directly injected into the timsTOF SCP mass spectrometer. Raw data were processed in Spectronaut using the directDIA workflow.

P 42

A fast and novel workflow for screening smoke affected grapes and wine using SPMESH-DART-MS/MS

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Smokes from bushfires can seriously influence the quality of food and beverages in adjacent regions. E.g., in case of bushfire events near vineyards, potentially generated volatile phenols (VP) are monitored via chromatography-based analyses for quality control. Throughput limitations inherent to chromatography-based approaches can lead to analytical bottlenecks when a large sample number needs to be run in a short time period like during the harvest. We report a rapid screening method for routinely monitored VP using solid phase mesh enhanced sorption from headspace to a chromatography-free direct analysis mass spectrometer (SPMEsh-DART-MS) in a highly automated mode. This workflow provides enhanced data quality, faster results, and improved cost efficiency compared to traditional chromatographic screening approaches.

The screening workflow includes the volatile phenols 4-ethylphenol, 4-ethylguiacol, guaiacol, 4-methylguaiacol, and o-cresol. Sample preparation (~1.5 hours) was performed for 24 samples in parallel as described in [1]. Following sample preparation and extraction, the SPMEsh sheet was transferred to the automated positioning stage of an EVOQ DART-TQ+ triple quad mass spectrometer. MS/MS parameters were optimized (collision energies, collision cell pressure, and scan speed). Matrix matched calibration QC were analyzed, using d3-guaiacol as IS for all compounds. Regression curves were analyzed at 6 calibration levels in quadruplicate including matrix blanks. Accuracy was assessed using two QCs analyzed in quadruplicate.

The automated DART-MS/MS analysis of 24 samples was performed in just 12 minutes. Data processing was performed using a standard MS quantitation software with linear regressions of $R2 \geq 0.99$ and recoveries of 90 – 110% at 5 µg/L and 25 µg/L concentration levels. The total workflow time for 24 samples was < 1.5 h. Full workflow simplicity, sample throughput, and data quality meet or exceed the accepted metrics of conventional approaches.

P 43

High resolution mass spectrometry for the target and suspect screening of over 600 pesticides in different food stuffs: Adherence to SANTE requirements for identification

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UHPLC coupled with High Resolution Mass Spectrometry is increasingly being used in Target and Suspect screening for pesticides in food. With increasing numbers of pesticides required to be monitored and, increasingly, their metabolites too, conventional LC- triple quad (LTQ) methods are challenged to provide a single analytical method capable of monitoring > ca. 600 pesticides. Even with sub-ms dwell times, there is an eventual cycle-time limit on how many compounds (MRMs) can be monitored, in unit time, while still providing sufficient data points across the chromatographic peak to provide for sufficient reproducibility of peak areas. While LTQ acquisition frequently provides for the highest sensitivities, recent improvements in Q/TOF technologies provide sufficient sensitivity to address the MRLs associated with residue analysis in food. Additionally, since HRMS is essentially a non-target data acquisition, the number of pesticide residues which can be screen for is unlimited with no impact on cycle time by adding additional compounds; additionally, the data can be interrogated retrospectively.

While Target Screening and quantification routinely requires the use of a residue reference standard, Suspect Screening requires only that the residue"s retention time has been determined, using the screening method employed, and that qualifying fragments have been previously curated; any "suspect" hit should be confirmed by running the relevant reference standard. By employing data independent MSMS acquisition i.e. *All Ions MSMS*, pesticides can be detected and qualified, using fragment ion (s) data, as per the SANTE guidelines for compound ID. This present study shows the application of a generic UHPLC /QTOF method to the high compound-number screening of pesticides and metabolites in a variety of foodstuffs. Using a Target/Suspect screening method in MassHunter Quantitative software, >700 pesticides and metabolites were simultaneously monitored while complying with required SANTE identification requirements for accurate mass and overlay, of the detected ions and analyte peaks respectively. Additionally, supporting data i.e. retention time and isotope patterns were used in providing further confidence in compound identification. The method was used to evaluate its efficacy in detecting a suite of pesticides in different food matrices.

P 44

Unified flavor quantitation: Simultaneous determination of aroma- and taste-active compounds via UHPLC-MS/MS

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Regarding environmental sustainability and health-promoting aspects, many consumers prefer plant-based products or foods with reduced fat, sugar, or salt content. These changes in the food composition often result in an off-flavor in the final product. To develop products that have the desired favorable properties without having to compromise on aroma and taste, it is necessary to quantitatively assess the entire Sensometabolome consisting of taste- and aroma-active compounds. With the aim of simultaneous analysis of volatile and non-volatile compounds, the principle of "unified flavor quantitation" has been established recently, utilizing ultra-high-performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS). Usually, volatile odorants are analyzed using GC-MS. For this purpose, the odorants must be isolated from the food employing the SAFE technology, which is labor-intensive and time-consuming. Derivatization makes the highly volatile compounds, which are difficult to ionize by electron spray ionization (ESI), accessible for analysis with UHPLC-MS/MS. Combining only two derivatizations made it possible to determine 23 of the 27 key food odorants in a wide variety of food matrices. 3-NPH enables the detection of ketones, aldehydes, and carboxylic acids, whereas DGTA is used to analyze alcohols, phenols, and thiols (Bösl et al. 2023). Accordingly, the quantification of odorants can be integrated into the already established analysis of tastants. Sample preparation is significantly reduced, and a single measurement takes only a few minutes and covers the entire concentration range of aroma- and taste-active compounds in food. Among others, this combined methodology has already been applied to cocoa-based products (Hofstetter et al. 2023) and low-fat dairy products (Utz et al. 2021). The principle of "unified flavor quantitation" thus enables the simultaneous quantification of all key flavor compounds in food with minimal sample preparation.

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Hofstetter, Christoph Konrad, Andreas Dunkel, Ugo Bussy, Miriam Wimmer, Thomas Kauz, Timo D. Stark, John Didzbalis, Corinna Dawid, and Thomas Hofmann. 2023. 'Toward Unified Flavor Quantitation in Cocoa-Based Products', *Journal of Agricultural and Food Chemistry*, 71: 20243-50.

Utz, Florian, Johanna Kreissl, Timo D. Stark, Christian Schmid, Caren Tanger, Ulrich Kulozik, Thomas Hofmann, and Corinna Dawid. 2021. 'Sensomics-Assisted Flavor Decoding of Dairy Model Systems and Flavor Reconstitution Experiments', *Journal of Agricultural and Food Chemistry*, 69: 6588-600.

P 45

Uncovering the PFAS complexity: A powerful IMS-QTOF Workflow for biota analysis combining targeted and non-target approaches

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With their widespread presence in the environment and organisms, and their persistent, bioaccumulative, and toxic (PBT) properties, Per- and Polyfluoroalkyl Substances (PFAS) pose a significant challenge as organic micropollutants. The vast array of commercially available PFAS compounds and their transformation products necessitate tight monitoring within environmental compartments. This study aims to the development of a combined targeted and non-target workflow for the thorough characterization of PFAS in complex environmental matrices, particularly biota, by incorporating trapped ion mobility spectrometry (TIMS) into LC-HRMS.

Biota extracts were prepared by a generic protocol for the simultaneous extraction of 56 PFAS compounds from various sub-groups. RPLC was coupled to a novel heated electrospray source (HESI) for analyzing the extracts in negative ion mode on a timsTOF Pro 2 (Bruker). The targeted workflow utilized bbCID (broadband collision-induced dissociation) in data-independent acquisition (DIA), while the non-target workflow employed PASEF (parallel accumulation and serial fragmentation) in efficient data-dependent acquisition (DDA). The CCS-aware target analysis incorporated information for a list of 60 PFAS compounds, including elemental composition, retention time, MS1 and MS2 qualifier ions, and ion mobility-derived collision cross section (CCS) values.

The results of the targeted screening demonstrated improved sensitivity and lower PFAS detection limits due to HESI, along with higher quality for the full-scan MS and bbCID MS/MS spectra supported by the ion mobility filtering. Adding CCS values enhanced the identification confidence.

In the non-target workflow, raw data was transformed into a comprehensive feature table, and the detected features were filtered by Kendrick mass analysis. A PFAS suspect list with 5,000 compounds was employed for further annotation. *In-silico* prediction of MS/MS spectra and CCS prediction aided the identification of suspected compounds. Notable examples highlight the identification of suspected features as PFAS-related compounds, supported by the extensive MS2 coverage provided by PASEF.

This combined approach contributes to a better understanding of the chemical universe of PFAS in the environment and plays a crucial role in safeguarding the environment, wildlife, and human health in a One Health approach.

P 46

Characterisation of vitamin B6 glycosides in a model brewing process and in beer by a novel SIDA LC-MS/MS method

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As co-factor of numerous enzymes, vitamin B6 plays an indispensable role in human metabolism.(**1**) Worldwide, beer enjoys high popularity, thus substantially contributing to the B6-vitamin uptake. In plants, B6 mainly occurs in its glycosidic form (e.g. pyridoxin-5'-β-glucoside; PNG) but for beer, data on the full B6-vitamer spectrum including glycosides is still lacking.(**2**) To fill this gap, a novel SIDA LC-MS/MS method including the vitamers pyridoxine (PN), pyridoxal (PL), pyridoxamine (PM), PNG, PN-5'-β-maltoside (PN-malt) and PN-5'-β-cellobioside (PN-cell), as well as pyridoxic acid (PA), was developed.

After successful validation of the method the screening of 70 beers yielded B6-contents between 127 µg/L and 1110 µg/L, including PNG, PN-malt and PN-cell. Hereby, no significant difference was observed between alcohol-free and alcoholic beer of the same type. Wheat beer showed a significantly lower B6-content compared to lager beer.

Two different malt types served as raw material for the model brewing process, each of which was prepared in biological triplicates. Process monitoring of the vitamers confirmed a trend similar to literature (**3, 4**), but considerably higher overall B6 in each step. The vitamin B6 concentration reached its maximum during mashing, whereas a notable decrease was observed in the subsequent steps. While PNG represented the main vitamer in barley and malt, a rapid degradation to PN was observed after the addition of yeast. The newly identified PN-malt first emerged during mashing and was still present in the matured beer.

In conclusion, B6 concentrations in beer mostly seem to depend on the raw materials, the malt preparation and mashing. With the novel B6 method, a reclassification of the vitamer distribution and conversion, as well as the quantification of B6 in different beer types and during the brewing process was achieved.

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P 47

Comparison of different proteases in cereal analysis by LC-MS/MS

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Cereals are one of the most important staple foods in the world. One of the main uses of wheat and rye is to make bread, baked goods or pasta. Barley is mainly used for brewing beer. These cereals contain a complex mixture of various proteins, which are capable of triggering allergic reactions in some people. Gluten describes the proteins found in wheat, rye, and barley. With the help of proteases, these proteins can be hydrolyzed to peptides. The digestion of gluten proteins is mainly used for the identification of immunoactive peptides as well as for the analysis of protein distribution in cereal grains and is an important part of proteomics. The aim of our study was to compare different proteases (trypsin, chymotrypsin, thermolysin, pepsin and papain) in relation to the hydrolysis of cereal proteins (derived from wheat, rye and barley) for analysis with mass spectrometry. Additionally, different experimental conditions for different enzymes were also investigated.

Proteins from wheat, rye and barley flour, respectively, were extracted using the modified Osborne fractionation to obtain the gluten fractions prolamins and glutelins. After reduction and alkylation, the proteins were hydrolyzed with the different enzymes, respectively, and the peptides were analyzed by UHPLC-MS/MS. Finally, the raw data were evaluated using MaxQuant. In addition, a peptide identification experiment was carried out to show the differences between shaking and non-shaking during hydrolysis. Furthermore, an experiment was carried out on the time course of the hydrolysis of cereal proteins using the little used enzyme papain.

The variation in the number of peptide sequences identified int wheat, rye and barley fractions illustrates the different cleavage efficiencies and activities of the five enzymes used. In this case, these two factors depend, among other things, on the protein composition of the cereal. The results show that although most of the peptides could be identified with trypsin, only a small proportion of them could be assigned to gluten proteins. Additionally, no peptides of ω-gliadins (wheat) and of C-hordeins (barley) were identified using trypsin for hydrolysis. Overall, it was found that the different enzymes were able to identify peptides of the gluten proteins of the different cereals to varying degrees. Most gluten proteins in wheat could be identified by hydrolysis with chymotrypsin, in rye by hydrolysis with thermolysin and in barley by papain. There is no "perfect" enzyme for the gluten protein analysis of all three tested cereals using LC-MS. The choice of enzyme must therefore be evaluated depending on the research question.

P 48

Bitter compounds in potato protein hydrolysate

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To feed a growing world population, it is necessary to find new plant-based protein sources. Especially, protein-containing side streams must be made usable for human nutrition. One of these products is potato protein, which is a side-stream product of the starch production.1 In comparison to other plant proteins, potato protein has a high biological value of 90, showed a high content of the essential amino acid lysine, and no known allergens.2,3 However, potato protein has some techno-functional disadvantages, such as the often-occurring sandy consistency and the low solubility in water, which severely limits its applications and consumer acceptance. To eliminate these problems, enzymatic or acidic hydrolysis can be applied to the potato protein.4 As a result, the techno-functional properties such as solubility, emulsifiability, foam stabilization and gel formation can be improved and specifically adjusted by hydrolysis parameters and enzymes.4 However, hydrolyzed proteins have the disadvantage of a more pronounced bitter taste compared to the native protein. This can be attributed to the formation of bitter-tasting peptides and amino acids. To identify such bitter peptides, the sensoproteomics approach can be applied, which combines an activity-guided fractionation coupled with human sensory and proteomics analysis.5 As a result, the first bitter peptides in commercially available potato protein hydrolysates have already been identified. Furthermore, it was shown that the free amino acids contribute to the overall bitterness of the protein hydrolysate.

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P 49

Novel triple quad approaches for robust and reliable pesticide analysis with ultimate sensitivity

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Pesticides are by far the largest number of hazardous compounds analyzed in food and feed worldwide. The Rapid Alert System for Food and Feed (RASFF) network alone has reported nearly 1000 notifications of the presence of pesticides in fruits and vegetables during border control and on the marker. The challenge for current technology is the number of pesticide compounds that can be analyzed per single run. Screening techniques are becoming more popular especially for organic and pesticide-free products, while maintaining the ability to further quantify the positive samples in more detail.

We propose a methodology for the analysis of pesticides in food using a novel triple quadrupole mass spectrometer. The first calibration level for most of the compounds in the study was 0.2 ng/mL in plant matrix (QuEChERS extract) with a total run time of 10 minutes (2 uL injection). The scan time used for each compound was less than 5 ms, resulting in a method that can accommodate more than 1000 pesticides (positive and negative with fast polarity switching) with at least 2 transitions each in a single run.

Under these conditions, the calibration curve showed an R2 value greater than 0.99 for 95% of the compounds included in the study. Typical ion ratios for most compounds show less than 10% deviation. The RSD for 10 consecutive injections is less than 10% for most pesticides in a real matrix spiked at a concentration range of 2 ppb. All of these parameters indicate a robust and reliable method for testing up to 1000 pesticides in a single run.

P 50

Identification and quantification of several mycotoxins in plant-based food alternatives

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In today's dietary practices, the discernible trend toward alternative nutritional choices, notably vegan and vegetarian lifestyles, has gained considerable traction. Consequently, consumers are increasingly embracing plant-based substitute products ('alternative proteins') as viable replacements for traditional animal-derived fare such as fish, meat, and dairy products. These substitutes predominantly leverage plant-based proteins, encompassing sources like soy, pea, wheat, nuts, and oilseeds. Additional constituents of these products are derived from botanical origins.

However, the prevalence of mycotoxin contamination in plant-based raw materials, originating from molds, introduces a substantial challenge to the food industry. Robust control measures and mitigation strategies become imperative considering this issue. Current estimates underscore that nearly 80% of food and feed samples exhibit mycotoxin contamination.[1] Moreover, these toxins exhibit resilience through various food processing techniques, retaining their potency across manufacturing processes, thereby warranting attention even in processed foods. Prior investigations have indicated elevated levels of mycotoxin ochratoxin A in the blood serum of vegans in comparison to their non-vegan counterparts.[2]

In the ambit of our research, diverse vegan and vegetarian substitute products underwent comprehensive qualitative and quantitative analyses for mycotoxins. This involved the optimization of an LC-MS/MS multimethod tailored to the specific matrices, followed by detailed validation. Our approach facilitated an analysis of various commercial samples, encompassing the identification of 15 mycotoxins. These include deoxynivalenol (DON), DON-3-glucoside, 3-acetyl-DON, 15-acetyl-DON, HT2-toxin, T2-toxin, tenuazonic acid, alternariol, alternariol monomethyl ether, aflatoxin B1, aflatoxin B2, aflatoxin G1, aflatoxin G2, sterigmatocystin, and ochratoxin A.

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P 51

Targeted and untargeted LCMS analysis of prenylated flavonoids in mulberry and other plant samples

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Prenylated flavonoids (PFs) are a group of secondary plant metabolites that show a wide variety of biological activities and have great potential for the development of nutraceuticals. Morusin and mulberrin are two structurally related PFs that have frequently been studied in mulberry products, however so far only with HPLC.

The poster presents the development of a combined targeted and untargeted LCMS/MS method for the analysis of PFs in different plant samples. The samples include seven different food and medicinal plants (white and black mulberry, common snowball, raspberry, durian, jackfruit, common hawthorn) with up to three plant compartments (branches, leaves, berries) investigated. A fast and convenient workup procedure was developed to obtain PF-containing extracts of all plant materials. Untargeted analysis of PFs was carried out on a *Shimadzu* LCMS-9030 Q-TOF in data dependant acquisition mode. An *R*-based algorithm was used to filter the obtained data for hits with characteristic prenyl group losses. Data evaluation was carried out with the open source softwares MS-DIAL and MS-FINDER. Additionally, a targeted LCMS/MS method was developed on the same instrument, using a stable isotope dilution assay (SIDA) for two well-known PFs, morusin and mulberrin. The respective isotopically labelled standards were synthesized by microwave-assisted deuteration.

The untargeted screening method was used to filter 460000 MS2 spectra for characteristic prenyl group losses, resulting in a reduction to 17000 elements. Further filtering steps and peak alignment with MS-DIAL yielded 500 tentative hits for PFs. For about a third of these hits a PF structure had the highest score in MS-FINDER. According to the criteria of Schymanski et al.[3] the method, therefore, annotated over a hundred compounds as PFs with a confidence level of 3. Since a SIDA was carried out, as well, the analytes morusin and mulberrin could be annotated with a confidence level of 1. Both of their structures were the top hits in MS-FINDER. Relative quantification of morusin and mulberrin in MS-DIAL was in accordance to the SIDA results, with highest concentrations in white mulberry branch material.

Principal component analysis (PCA) was performed for all plant samples in general, as well as for the different plant compartments individually (all wood, all leaf, all fruit samples). Qualitative differences between mulberry and other wood samples were visible in several PCA score plots, further indicating the distinctiveness of mulberry as a source of PFs. SIDA results confirmed existing knowledge on morusin distribution in different plant compartments of mulberry. Further, common snowball was discovered to be a source of both morusin and mulberrin, making the respective fruit juice an interesting subject of future analysis.

P 53

Intrinsic native electron capture dissociation of oligomers of cytochrome c from different species

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Native mass spectrometry (MS), coupled with electron-based dissociation, has provided valuable insights into protein structure. Two decades ago, Breuker and McLafferty observed a distinctive dissociation behavior by electrospraying highly concentrated cytochrome c from equine heart. The fragment ion types they observed were typical for electron-based dissociation of proteins. However, no exogenous electrons were added, indicating that cytochrome c has an intrinsic ability to undergo this type of fragmentation. We will therefore refer to this mechanism as intrinsic native electron capture dissociation (iNECD). The electron transfer was proposed to happen during a highly asymmetric dissociation of a noncovalent cytochrome c dimer, which assembles during the ionization process of a highly concentrated sample. However, no dimeric states of cytochrome c were observed in the initial publications. The application of this mechanism to other proteins would be a valuable tool for structural analysis. To gain more insight into the iNECD process, we performed an in-depth study of equine cytochrome c, and also investigated whether cytochrome c samples from other species exhibit iNECD behavior.

We used a nanoESI-equipped quadrupole-ion mobility-time-of-flight (TOF) instrument (Waters Synapt XS), and cytochrome c samples were sourced from Merck. Data analysis was conducted using MassLynx 4.2 and CIUSuite 2.

In the initial experiments, we used a highly concentrated equine cytochrome c solution (37.5 µM). Following optimization of sample preparation, we successfully observed iNECD behavior in cytochrome c, even at a low concentration of 10 µM. The observed fragmentation patterns were consistent with prior reports. Notably, we detected cytochrome c dimers and trimers for the first time even at low concentration. Further tested solution conditions indicated the formation of the oligomers in bulk solution rather than during the ionization. We isolated and slightly activated individual charge states of cytochrome c dimers and trimers and detected iNECD fragments, supporting the hypothesis that dissociation of the cytochrome c oligomers is the starting point for the iNECD mechanism. Like we did for the equine cytochrome c, we prepared various solution conditions for cytochrome c from other species. We tested closely related species such as bovine cytochrome c with a very similar sequence and more distant species such as yeast cytochrome c for their iNECD behavior and were able to expand the mechanism to further sequences. This study represents the first in-depth investigation of iNECD exhibited by specific oligomeric states of equine cytochrome c and the extension of the mechanism to other cytochrome c species with the outlook of expanding this dissociation method to even further proteins.

P 54

Living libraries of metal clusters: exploring chemical complexity with LIFDI-MS

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Ligand stabilized metal clusters are useful study objects that can serve as models for the surface of complex heterogeneous catalyst systems.[1,2] Such studies usually require the prior isolation of pure clusters, which implies that the isolated species are sufficiently stable, i.e. have limited potential for chemical reactions.[3] We therefore developed a novel mass spectrometry based set-up and methodology for efficiently exploring highly air sensitive ligated *heterometallic* clusters in the form of "living libraries" (Fig. 1). Information is gained by directly dealing with chemical complexity without the separation of clusters, thus breaking with conventional research strategies involving targeted syntheses of singular species.

Each cluster library, undergoes characterization *via in situ* liquid-injection field desorption ionization mass spectrometry (LIFDI-MS), enabling the identification of all ionizable molecular species. However, two key challenges must be addressed: firstly, assigning an exact and unique sum formula to each pattern in the spectrum, and secondly, differentiating between molecular and fragment ions to define a distinct set of species characterizing the library (Fig. 1).

A double labeling strategy is employed to address this: Cp*Et (Δm/z = 14) and isotopically enriched 68Zn2Cp*2 (Δm/z = 2.62) are introduced to provide distinct signatures resulting in definite sum formulas [CuaZnb](Rk) (Rk = Cp*cMesdHh; c + d + h = ***k***) assigned to nearly every observed peak pattern. Furthermore, collision experiments in the higher-energy collisional dissociation cell (HCD) of the ORBITRAP mass spectrometer allow for discriminating between molecular ions and fragments.

This approach can also be applied to study the reactivity of clusters with reactive substrates. It thus reveals the potential for a paradigm shift in cluster science and supports the exploitation of special capacities of highly reactive mixed metal clusters to stimulate chemical reactions yet to be explored.

Figure 1: Set-up and workflow for the determination of the molecular species in cluster libraries.

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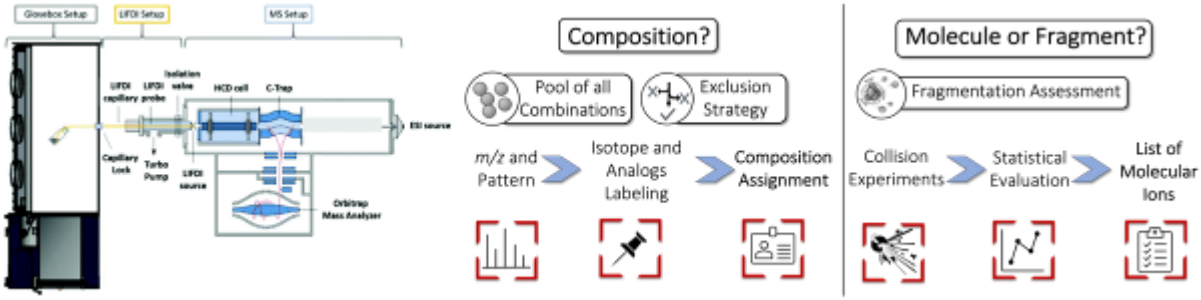
Fig. 1



P 55

Investigation of aspirated charged droplets from ESI in a Linear Ion Trap (LIT)

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Electrospray Ionization (ESI) is one of the most common ionization methods in mass spectrometry (MS). One of the biggest advantages of ESI compared to other ionization methods is the generation of multiple-charged droplets, which contains the analyte. But it has also some disadvantages, because these multiple-charged droplets can pass through the different vacuum stages of the mass spectrometer even to the mass analyzer region. This contamination by droplets can affect the analytical performance. For this reason it is important to understand which parameter influence the aspirated droplet ensemble. An ion trap is used to investigate these aspects, as it is particularly well suited to the study of droplets due to its ability to isolate and fragment different mass ranges.

Experimental studies are performed with a Thermo fisher linear ion Trap (LIT) Velos Pro. An isolation range of 800-2000 m/z was chosen and common ion source and trap parameters, e.g. capillary temperature, flowrate, isolation window or Helium gas pressure was changed systematically. The analytes used for these experiments are para-substituted benzylpyridinium ions (thermometer ions) and reserpine in different solvent systems. The solvents used was 1:1 mixture of ACN and water with 0.1% formic acid.

The experiments show signals in a broad mass range up to 2000 m/z. The isolation and fragmentation of different mass ranges above the m/z of the bare analyte, which should therefore not include the analyte signal, leads to significant intensities of analyte signals in the fragment spectra. Similarly to earlier results in different instruments, the observed analyte was obviously transported into the mass analyzer region in large charged aggregates, charged droplets or their large fragments. Besides the observed analyte signal with fragmentation of the isolated species, there are several signals with higher m/z values than the isolated mass range. A possible explanation is the charge loss of the multiple-charged droplets / droplet debris, resulting in higher m/z values. The variation of different source parameters affects the formation and the stability of these species. It was shown that the analyte signal depends strongly on the inlet capillary temperature, with a signal maximum at moderate capillary temperatures. Similar results were observed for other parameters, e.g. flow rate or Helium gas pressure. Besides the stability of the droplets / droplet fragments, the variation of the parameters also effects the signal intensity of analyte-dimer and -trimer peaks, which are commonly visible in the mass spectra.

We present an overview of the mass spectrometric observations and an analysis of possible reaction / fragmentation pathways of the aspirated droplets / droplet fragments in the LIT mass analyzer.

The measurements show possible existence of charged-droplets in a LIT and their dependency on different source parameters.

P 56

A novel Ion processor device for high-throughput analysis in a high-resolution mass analyzer

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Ion accumulation devices are routinely used to pre-process ion packets prior to pulsed extraction into an analyzer. Compared to conventional orthogonal extractors found in Q-ToF instruments, such devices benefit from a vastly higher duty cycle but require a compromise between buffer gas pressure, to capture and thermalize ions, and minimize fragmentation and scatter during extraction. A novel ion accumulation and pulsed extraction device containing two unique pressure regions for parallel accumulation/fragmentation and extraction is introduced [1]. It has a unified, phase-locked RF pseudopotential channel for seamless transfer of pre-cooled analyte ions. Axial accumulation and movement are performed via DC electrodes, allowing rapid processing at reduced pressures.

The design of the ion processor was optimized with simulation software (MASIM3D) and through experimentation. Special consideration was given to the operational speed so that an entire MS/MS cycle could be executed in under 5 ms. The ion processor was coupled to the Astral analyzer [2] for performance characterization. Direct infusion experiments with Pierce™ FlexMix™ calibration solution and proteins were carried out to investigate key characteristics including ion transfer times, shot-to-shot mass accuracy, fragmentation and extraction and space charge performance. In addition, proteomic LC-MS analyses were performed to assess the peptide identification and quantification performance in high-throughput applications.

Simulation results, design and experimental data of the ion processor will be presented. Emphasis will be given to the extraction region, where an optimized design is presented that increases the depth of the quasi-potential well during accumulation and improves uniformity of the acceleration field during extraction. Experimental MS and MS/MS data at repetition rates >200 Hz are shown and key characteristics such as the ion packet spatial and temporal distribution post-extraction will be highlighted. Accumulation capacities of several 10^5 charges per ion package and signal linearity of several 10^3 for single m/z have been achieved. These results show how the high transmission of the device makes it especially suitable for applications that require high resolution and accurate mass in the low ppm regime. This allows for peptide identification and quantitation at highest throughput for short LC gradients of ~5 min. Furthermore, the feasibility of the ion processor for use in intact protein analysis at high repetition rates is demonstrated for myoglobin and carbonic anhydrase. After initial implementation and testing, automatic calibration routines were developed and the extraction parameters were optimized without performance loss.

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P 57

Simulation of ion chemistry in traveling wave IMS with an open simulation framework (IDSimF)

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Traveling wave ion mobility spectrometry (TWIMS) is an analytical method used for the separation and identification of gas-phase ions. A TWIMS device consists of a gas-filled RF-only ion guide onto which a repeating waveform pattern is applied, resulting in a sequence of continuously propagating potential waves. Ions within the device can either be swept along by the wave or they can be overtaken by the wave in so-called roll-over events. The result of this is a separation of ions based on their electrical mobility, although the molecular dynamics are more complex compared to other IMS methods.

All featured simulations were performed using the Ion Dynamics Simulation Framework (IDSimF), an open-source software written in C++ containing various models and programs, which allow for the simulation of ion trajectories. Among its features are a number of different simulation applications, each representing a different experimental setup, e.g. IMS devices including TWIMS. In this application the electrode geometry and potentials were defined through SIMION potential array files. The potential wave is created by the combination of a waveform profile and a set of phase shifts which are applied to the electrode stack in a repeating pattern. This repeating potential pattern is then rapidly switched across the electrodes, leading to traveling potential waves.

Simulations of ion trajectories inside TWIMS devices allow for the study of ion-wave interactions and subsequently the trajectories and dynamics resulting from these interactions. The goal of this work is to examine the ion dynamics of a reactive system within a TWIMS unit, using a system of nine different water clusters in addition to nitrogen and water as an example. A cluster of a given size can react with water and nitrogen to increase its size via the reaction path:

$C_n + H_2O + N_2 \rightarrow C_{n+1} + N_2$

Consequently a water cluster can undergo a declustering reaction when it collides with a nitrogen molecule.

$C_{n+1} + N_2 \rightarrow C_n + H_2O + N_2$

The gas-phase ion chemistry within such an analyzer unit is not just dependent on the concentrations of the relevant species, but also on the surrounding electrical field. It was found that the temporally and spatially oscillating field in the TWIMS unit influences the reaction equilibrium and the concentrations of the cluster species. As the wave passes the clusters a regular pattern was observed, wherein a spike in the number of lower weight clusters coinciding with the wave passing underneath is followed by a gradual increase in the concentrations of higher weight clusters as they react with water in the time between two wave cycles.

The possibility of ion chemistry occurring in the analyzer stage is a commonly neglected topic and the ability to include chemical processes is limited in many trajectory simulation frameworks. As such we intend to present both the capabilities of IDSimF in this field and the influence of TWIMS field conditions on a reactive system.

P 58

Exploring the long-term stability of analyte signals in electrospray mass spectrometry through ion current measurement and optical spray monitoring

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Electrospray ionization (ESI) is a frequently used ionization technique in atmospheric pressure ionization (API), due to its ability to ionize large molecules without severe fragmentation. Essentially, a solution containing the analyte is sprayed within a strong electric field, resulting in the generation of charged droplets. These droplets undergo a series of shrinking and fission reactions, ultimately resulting in the release of bare ions. Despite its significance, the underlying mechanism of droplet generation and their course through ion source and MS vacuum system remains incompletely understood. While it is commonly assumed that all droplets evaporate within the ionization chamber, calculations and experiments indicate a lifetime of more than 1 ms, significantly exceeding the residence time within the ion source chamber.

Recent experiments provide evidence suggesting that a considerable fraction of the charged droplets penetrate the high vacuum region in typical ESI-MS systems. A commercial mass spectrometer (Bruker micrOTOF) allows the direct observation of droplet signals on an auxiliary ion detector (SEM) downstream of the acceleration stage of the TOF. Intense ion signal bursts, magnitudes higher than the ordinary ion current, were observed, attributed to fragmented, aspirated charged droplets. Furthermore, highly intensive single mass spectra were observed which we attribute to aspirated droplets as well. Previous experiments highlighted that essential parameters affecting spray generation, such as capillary voltage, liquid, and various gas flows significantly impact the frequency of droplet signature appearance in the pusher region.

This work investigates the long-term signal stability of ion signals in Bruker micrOTOF systems in the presence of droplet signatures under analytical conditions. The measurements reveal an intriguing phenomenon of a decreasing droplet occurrence frequency and analyte signal, respectively, within a few hours of measurement. Although the low droplet occurrence persists in immediately following measurements, it recovers during the instrument's standby time. After a few hours, the droplet occurrence experiences a rise and fully recovers after more than one day of standby time. Additional measurements show that changing the MS polarity for a few minutes leads to the same recovery phenomenon as a long standby time. To enhance experimental throughput, an automated method is established for systematically altering measurement conditions and controlling the LC pump for the direct injection of the analyte system in use.

It was noticed that there are some events leading to sudden changes, like a sudden drop in all signals or a slow decrease of signals after a distinct event. To correlate these events with spray conditions, the electrospray plume was monitored with a microscope camera searching for changes in the spray mode.

P 59

Investigation of neutral electrolysis products in an electrospray Ion source

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An electrospray (ES) ion source can be described as an electrochemical cell. In ESI, the application of an electric field between the electrodes leads to electrophoretic charge separation in the solution. As a result, ions of one polarity accumulate at the capillary tip, which leads to the formation of charged droplets. In order to maintain the formation of the charged droplets, charge balance takes place by electrochemical reactions at the electrodes. Often small quantities of electrolyte are added to the analyte solution to ensure charge seperation.

Electrochemical reactions that take place at the emitter electrode can change the analyte solution composition and therefore may influence the gas phase ions formed and analyzed by the mass spectrometer. A better understanding of the electrochemistry, along the various different parameters of an ES device, is expected to lead to an improvement of ES-performance.

To investigate the electrochemical processes the electrolysis products (CO_2 and H_2) of formic acid were measured in the gas phase. To accomplish this goal a costum experimental setup was designed. Therefore a Turbolon Spray Sonde of an Sciex6500 and a plate of stainless steel as a counterelectrode were used in a grounded-emitter system. The sprayed solution consisted of a mixture of water and acetonitrile with 0.1 % formic acid (HPLC grade). The mixing ratio of the electrolysis products CO_2 and H_2 in the gas phase were measured by different gassensors. Additionally the current at the emitter electrode was detected using an VC870 digital multimeter.

Using this custom experimental setup, the effects of different variable paramters of the ES device on the CO_2 mixing ratio were investigated. Initial results show an increase in both the CO_2 and the H_2 mixing ratio at the start of the ESI-process. Furthermore, it can be observed that a variation of the ion mode has no significant impact on the measured CO_2 mixing ratio. A comparison between measurements with an applied voltage of 3 kV and without applied voltage shows a clear difference in the measured CO_2 mixing ratio. A much higher CO_2 mixing ratio can be observed at the measurements without voltage. These results lead to the assumption that other effects besides the electrochemical processes impact the measured CO_2 mixing ratio. It is assumed that the results obtained are related to the solvent, particularly water, and the gas solubility of carbon dioxide in water. Futher experiments using only water, show a lower CO_2 mixing ratio for a measurement with degassed water than a measurement with non-degassed water.

P 60

Development of a new nebulization system for LC-MS coupling employing an inverse low temperature plasma ion source

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In liquid chromatography – mass spectrometry (LC-MS) couplings, the nebulization of the LC eluent and its transfer into gas phase are vital steps to ensure efficient ionization of the analyte molecules by e.g. common atmospheric pressure chemical ionization (APCI) or the novel low temperature plasma (LTP)-based ion sources.

In its basic usage, an LTP ion source consists of an electric circuit with two electrodes, a high voltage and a grounded electrode. Between those, there is an insulating material, e.g. quartz glass, and a gap (cf. Figure). Voltage is applied, and the electrons travel to the high voltage electrode. Once the voltage is high enough, a discharge occurs, and the electrons can pass the insulating material and reach the gap. There, they collide with atmospheric molecules or a supplied discharge gas, usually helium or argon. This leads to a chain reaction and eventually to the ignition of the plasma that – in contrast to well-known inductively coupled plasmas – only possesses a temperature of roughly 30 °C. This plasma can now be used for the ionization of the analyte molecules. Being a soft ionization, leading to little fragmentation and the preservation of $[M]^{+\bullet}$ and/ or $[M+H]^{+}$ ions, LTP-based plasmas are able to reach better selectivity and a wide range of analytes.[1]

For efficient ionization, the LC eluent needs to be nebulized, meaning sprayed into fine droplets, evaporated and focused onto the ionizing plasma region. To improve this process, ultrasonic nebulization (USN) was to be employed which is thought to provide smaller droplets than a commercial APCI nebulizer. Thus, two commercial nebulizers – a medical inhalator and a room humidifier – were disassembled and reconfigured for employment in the LC-MS coupling. Based on these results, a new spraying system was developed. To enhance the evaporation and focusing of the analyte molecules, custom guidance cones were designed and 3D-printed from metal. After comparation, the best operating parameters were found via a design of experiments. For this, the temperature, at which the cone was heated, played an important role, as well as supplied auxiliary gas flows for further heating of the analyte molecules and cooling of a heat-sensitive construction piece. The novel spraying system was then compared to a common APCI nebulizer. Furthermore, it was tested in combination with two different types of LTP-based ion sources.

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P 61

Simulation of the pressure dependent Dynamic Ion Acceptance Volume (DIAV) of an electrically biased external ion sampling stage

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One of the most important challenges encountered when sampling ions directly from a (moderately) pressurized vacuum recipient are geometric restrictions concerning the interface. In order to maintain the desired vacuum inside the ion transfer system of a mass spectrometric detector (in this case a TOF-MS), we employed an arrangement of electrodes best described as an einzel lens with a very short focal length, focusing the ions generated in a high-density plasma region through an electrically biased sampling orifice (gas flow restrictor, i.e., an electrode with a particularly narrow aperture, radius 0.25mm) - into the aforementioned transfer system. As expected, the necessary small diameter of this restrictor causes severe sampling inefficiencies of this system, with simulations suggesting significant loss of ions impacting on the electrode. Thus far the geometry and electrostatic configuration of the lens stack has been optimized with regard to ions moving towards the aperture at a rather acute angle, but recent reconsiderations of our experimental results obtained so far stoked interest in ions originating from the immediate region in front of the restrictor. We therefore calculated the "Dynamic Ion Acceptance Volume" (DIAV) of this configuration towards H_2^+ ions generated from hydrogen background gas molecules with a Maxwellian velocity distribution prevailing at 298 K by photoionization or photoelectron ionization. For the calculation of the DIAV, for which the boundary is defined as the 1/e iso-intensity surface relative to the maximum intensity with the volume, we investigated the trajectories resulting from this particular species in the electrostatic region of the interface using SIMION.
We furthermore extended this well-established method: Due to our experimental results that were obtained at different H_2 background pressures and surprising at first sight, we were also especially interested in a potential dependence of the sampling efficiency on pressure. Pressure dependent simulations were carried out with both SIMION and the Particle in Cell (PIC) and DSMC based plasma simulation software PICLas to explore one potential explanation of the peculiar experimental results described elsewhere. Our findings have implications for the future design of electrostatic sampling interfaces in dedicated plasma metrology tools based on mass spectrometry.

P 62

Development of a novel accumulation Ion source for instable metal compounds

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In research there are sometimes challenges that require tailored solutions instead of run-of-the-mill products. The analytical question in this case involves instable metal hydrides of about E-14mbar partial pressure in an atmosphere of few Pa hydrogen. In order to achieve analysis of the gas phase concentration a novel ion source including a fitting vacuum system and transfer into the MS was designed.

Since the gas phase concentration of the metal hydrides is below the LOD of the employed TOF-MS (Tofwerk AG, Thun, Switzerland) it is necessary to accumulate for a given timeframe on a sample probe with subsequent analysis of the surface of the sample. In order to generate ions electrons are accelerated onto the sample which lead to ionization on the surface and to volatile ionic species. The electrode stack of the ion source is built to collect the ions, achieve universal kinetic energy and guide the ions towards the MS. The potentials applied to the electrodes have to be altered between electron mode and ion mode because the electric fields needed for fitting electron trajectories towards the sample and ion trajectories towards the MS are mutually exclusive. An additional challenge of this analytical question are the spatial restrictions of the setup. The ion source has to be located 75cm away from the plasma region, where the SnH4 is located, and a sample must be taken without breaking the vacuum. For this reason the sample is mounted on the tip of a moving actuator that covers the distance and reaches through a gatevalve. The valve is needed to differentiate the pressure regimes from few Pa hydrogen in the plasma region and deep vacuum in the ion source region. As the actuator is retracted entirely the sample becomes a functioning part of the ion source including a heatbed and electrical contact.

Prior experiments from our lab inspired this method. When we exposed a commercial EI source to synthesized stannane we were able to achieve a full spectrum of resolved tin peaks even after removing the source of SnH4. This proves that the stannane molecules decomposed on the metal surfaces inside of the ion source and were either directly ionized by electron interaction on the surface or transfered into the gas phase with subsequent ionization. Additional experiments indicate that a heated metal surface yields more ions, which is why the sample holder of the novel ion source is going to be heatable as well.

Simulations are promising, showing a 75% sample coverage of electrons and 82% succesful transmission of cations into the MS however experiments are yet to be conducted. The ion source can also be used for conventional EI measurements for background spectra for example.

fig 1: Depiction of the ion source including all electrodes

fig 2: Trajectories of electrons towards the sample

fig 3: Trajectories of cations towards the MS

Fig. 1

Fig. 2

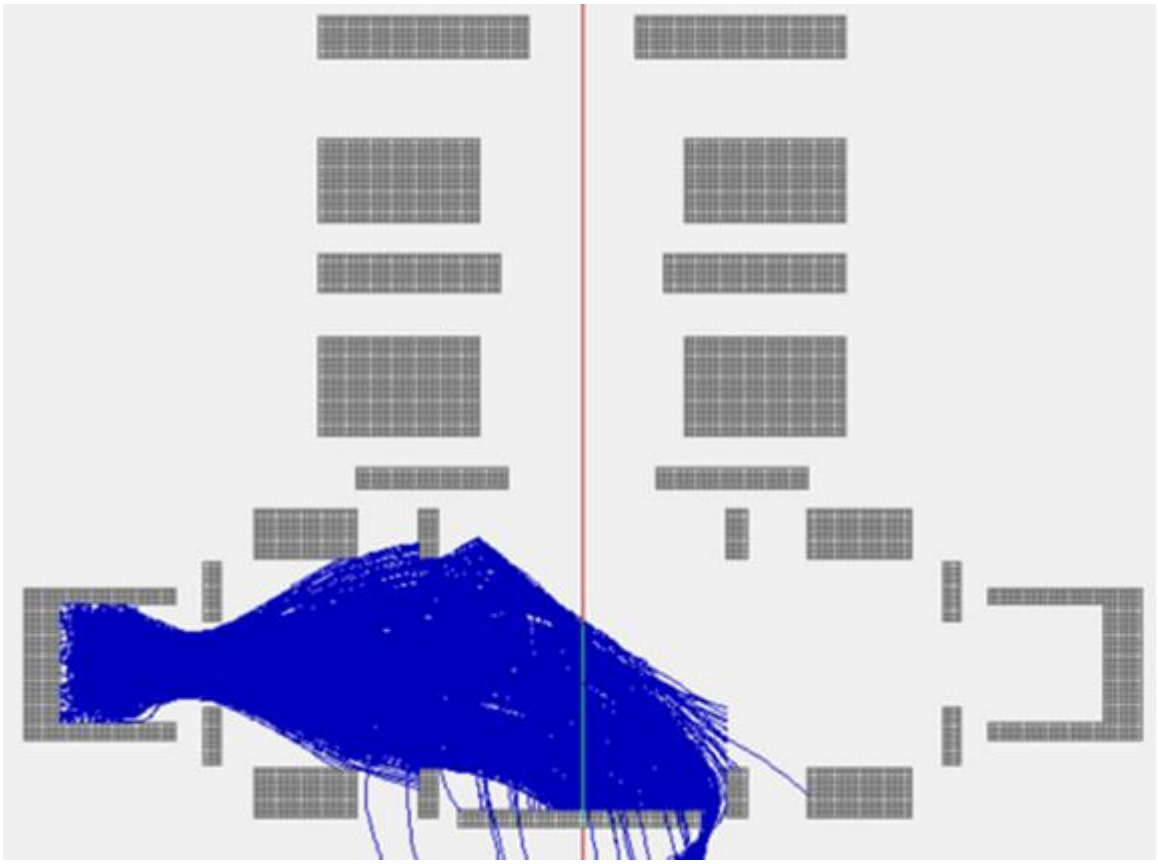
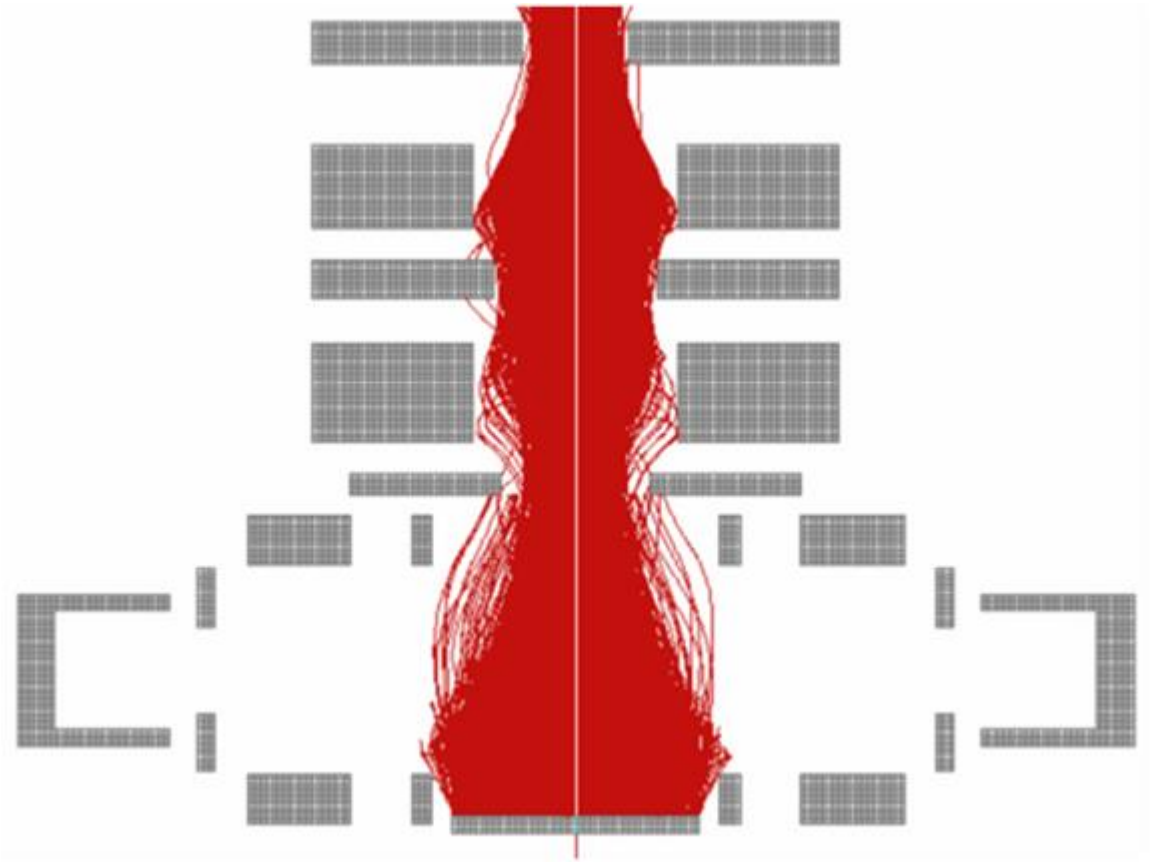


Fig. 3



P 63

Analysis of charged droplets from ESI in a vacuum system by displacement current measurements

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Electrospray ionization (ESI) stands as one of the most crucial ionization techniques in mass spectrometry (MS). An analyte solution is sprayed into an electric field, resulting in the formation of highly charged droplets containing the analyte. Experimental observations show that a significant portion of these charged droplets generated by ESI can pass through the MS inlet into the vacuum system of the instrument due to their long lifetime. The experimental observations led to the conclusion that the droplets not only impact analytical performance but also result in contamination of the mass spectrometer. This results in increasing expenses for maintaining the instrument. Experimental findings reveal that large charged aggregates can infiltrate into the mass analyzer region of ion trap instruments. Measurements of displacement current were conducted to directly determine the absolute charge of individual aspirated droplets. Charged droplets are created through electrospray ionization at atmospheric pressure and are subsequently transported into a vacuum chamber. This chamber is designed to replicate the conditions of a typical first vacuum stage in the mass spectrometer. A voltage of 4 kV is applied to the ESI needle, causing the generation of charged droplets. The sprayed analyte solution for these experiments are Reserpine in different solvents (1:1 mixtures of Acetonitrile or methanol and water with formic acid). These droplets enter the capillary, which is grounded, and are subsequently transported into the vacuum chamber through the flow of neutral gas. The charged droplets pass through a wire ring in the vacuum region, which serves as a detection electrode. The displacement current generated by the passing charged droplets on the measuring electrode is amplified using a sensitive amplifier before being transmitted to an oscilloscope. In all measurements in the described experimental setup, the predominant observation was the occurrence of strong signal pulses at highly regular intervals. The findings were interpreted, drawing on additional insights from numerical simulations using SIMION. These results offer an initial estimation of the actual charge carried by the measured particles. In the simulation, information regarding the speed, amount of charge, and amplitude of the simulated droplet can be derived. Combining information from both - the experiment and the simulation - allows an estimation of the absolute amount of charge carried by the experimentally observed droplets. The results indicate that the particles carry charge quantities ranging from $1x10^5$ to $1x10^6$ elementary charges while passing through the ring electrode.

P 64

Wavelength dependence in surface-assisted laser desorption/ionization mass spectrometry

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[2]*University of Liège, Mass Spectrometry Laboratory, Liège, Belgium*

Surface-assisted laser desorption/ionization (SALDI) mass spectrometry (MS) [1,2] relies on the use of (usually inorganic) nanostructured surfaces (*e.g.,* coatings of colloidal nanoparticles [3], nanostructured solid supports [4,5] or sputtered metal nanoclusters [6]), instead of organic matrices, as in the conventional matrix-assisted laser desorption/ionization (MALDI) MS technique. While the interplay between the laser wavelength and the absorption profile of the matrix has been widely studied and proved to be a crucial factor in MALDI-MS [7-10], very few, if any, fundamental studies have been carried out in SALDI-MS. Yet, the laser wavelength is a key parameter that needs to be tuned to correspond to the maximum of optical absorption of the assisting material, in order to provide optimal analytical results [7-10]. Indeed, the desorption in SALDI-MS has been proved to be mainly driven by thermal processes, resulting from the heating of the nanosubstrate surface upon absorption of the photon energy [1]. However, almost all SALDI-MS studies use standard laser wavelengths of 337 or 355 nm, even though the peak absorption of the SALDI nanosubstrate might completely differ from these values. Here we employed wavelength-tunable optical parametric oscillator (OPO) lasers to investigate the wavelength dependence in SALDI-MS, using citrate-coated gold nanoparticles (AuNPs) as SALDI nanosubstrates and model analytes (*e.g.,* benzylpyridinium thermometer ions, lipids). The uniform spray deposition of the AuNPs and analyte ions on Superfrost™ glass slides was performed using an ultrasonic spray coater. We then recorded gold and analyte ion signals as a function of the laser wavelength and laser fluence in the UV-visible range, knowing that the maximum absorption of the colloidal AuNPs is at 529 nm. The results of this study provide new insights into the SALDI desorption/ionization processes and could assist the improvement of the analytical performance of the SALDI-MS technique, through instrumental and methodological adjustments.

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P 65

A combined LIFDI-MS/Glovebox setup for *in situ* investigation of metallocene isomerization for *Iso*-selective polypropylene polymerization

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Group IV metallocene complexes are essential as catalysts in polymer chemistry. Since the meso isomers of such metallocene catalysts only lead to atactic polyprolpylene (aPP), which is to be avoided due to its poor physical properties, only the racemic isomer leads to the industrially relevant isotactic polypropylene (iPP). Beside the tedious separation of the isomers, the meso complex can be converted towards the racemic isomer by the use of trimethylaluminium (TMA).[1] To uncover the underlying principles necessary for the formation of the racemic metallocene and to find ways towards the direct formation of this isomer, elucidation of the isomerization mechanism is essential.

In this contribution, we will present the mechanistic investigation of the underlying reaction mechanism of the complex" isomerization using different analytical methods, focusing on the elucidation *via* mass spectrometry, leading to the proposal of a complete isomerization mechanism. Therefore, a LIFDI-MS setup was accessed, which was connected to an Orbitrap as mass analyzer on the one side, and a Glovebox system on the other side, for liquid sample injection under inert atmosphere.[2] This setup allowed the *in situ* investigation directly from the reaction liquid combined with a soft ionization for accessing short-lived, highly sensitive and not-isolable metallocene reaction intermediates, making it a useful tool for mechanistic elucidation of organometallic compounds.

Figure 1 Isomerization of a Zr metallocene *via* TMA.

Figure 2 Adduct of a metallocene with TMA as isomerization intermediate.

Figure 3 Coupled LIFDI-MS/glovebox setup (right).[2]

References

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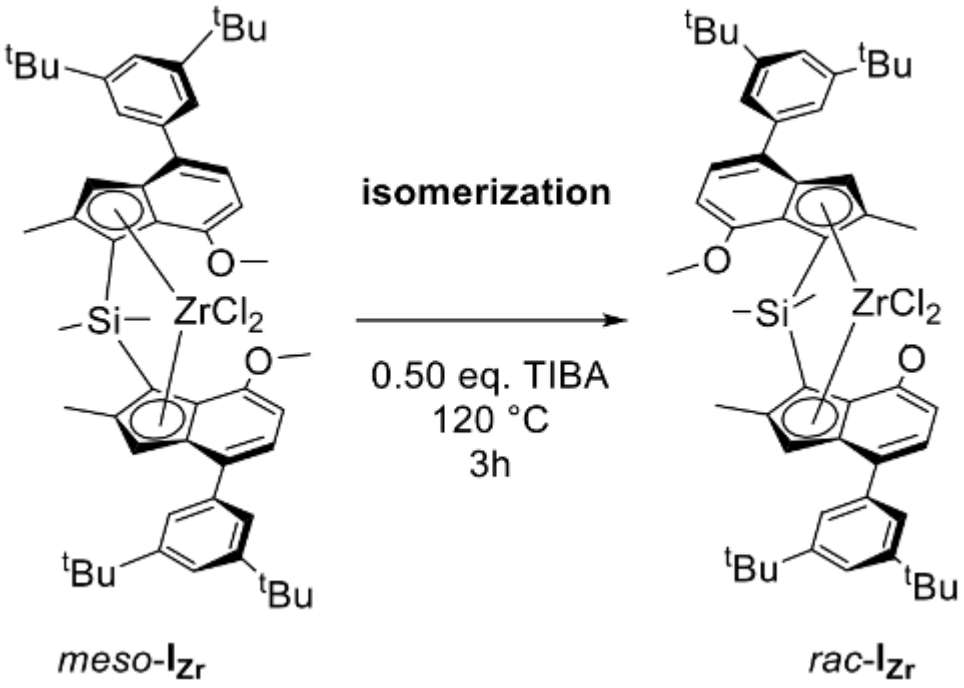
Fig. 1


tBu
tBu
O
Si
ZrCl2
O
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tBu
isomerization
0.50 eq. TIBA
120 °C
3h
tBu
tBu
O
Si
ZrCl2
O
tBu
tBu
meso-IZr
rac-IZr

Fig. 2

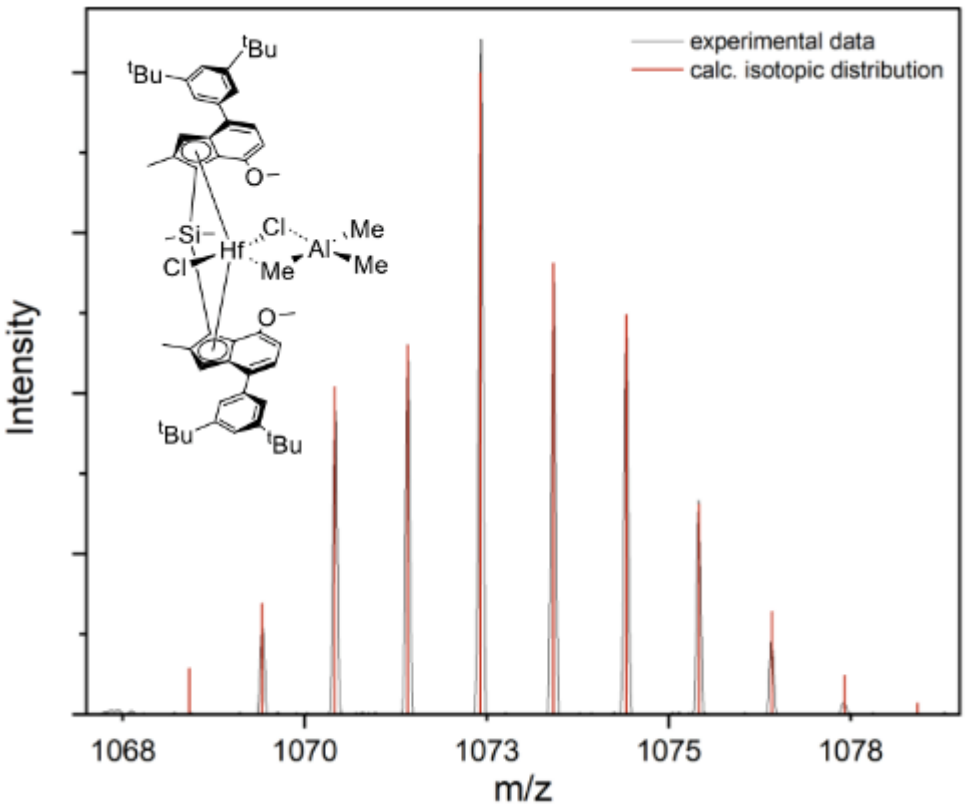
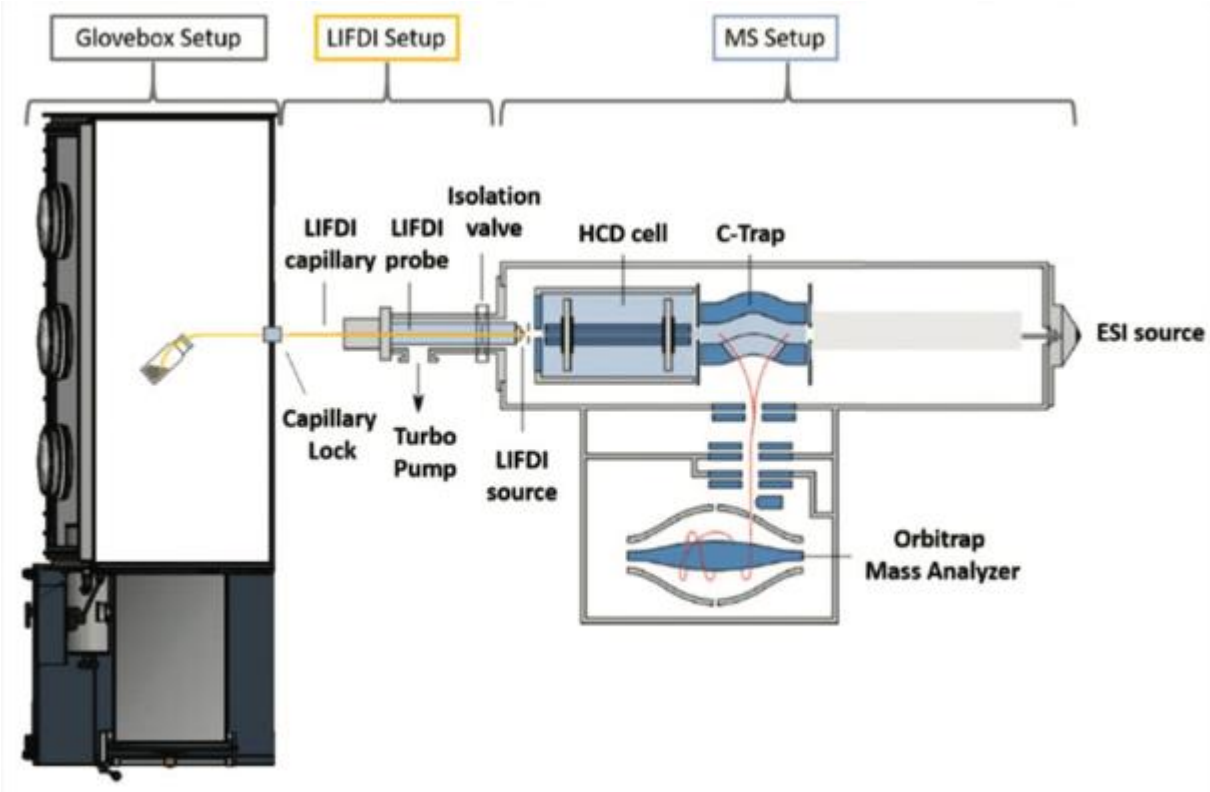

experimental data
calc. isotopic distribution
Intensity
1068
1070
1073
1075
1078
m/z
Si
Hf
Cl
Cl
Al
Me
Me
Me
O
tBu
tBu
tBu
tBu

Fig. 3


Glovebox Setup
LIFDI Setup
MS Setup
LIFDI capillary
LIFDI probe
Isolation valve
HCD cell
C-Trap
ESI source
Capillary Lock
Turbo Pump
LIFDI source
Orbitrap Mass Analyzer

P 66

Mass spectrometric studies on the fissociative electron attachment to thiazole and 2-Bromo-5-nitrothiazole

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[2]*University of Notre Dame, Radiation Laboratory, Notre Dame, IN, United States*
[3]*University of Notre Dame, Department of Physics and Astronomy, Notre Dame, IN, United States*

Radiosensitizers are used to increase the efficiency of radiation in the hypoxic conditions of the cancerous cells. Water ionization happens when high energy radiation interacts with cells and results in the production of a large number of secondary low-energy electrons, (LEEs). LEEs may resonantly attach to molecules and result in the formation of anions and neutral fragments through dissociative electron attachment, DEA. Similarly, interaction of radiosensitizers, including thiazole-based molecules, with LEEs results in the formation of transient negative ions which further decompose into anions and neutral fragments through DEA [1].

In the present study, we investigated DEA to thiazole, (nominal mass 85 u) and 2-bromo-5-nitrothiazole, (208 u) using a crossed electron-molecular beam system coupled with a quadrupole mass spectrometer. The molecules are shown in Fig. 1 and Fig. 2. The molecules were introduced in the interaction region of a hemispherical electron monochromator, (HEM) and crossed with an electron beam. Ions were formed upon the monochromatized electrons interacted with the molecules. Ions were then extracted and analysed using the quadrupole mass spectrometer. Subsequently, the mass-separated anions were detected using a channeltron-type secondary electron multiplier. Ion yields were measured as a function of incident electron energy. We report that thiazole and 2-bromo-5-nitrothiazole are efficiently decomposed by LEEs in the range of 0–14 eV electron energies. The most abundant formed anions are $C_2H_2^-$ and S^- in thiazole and $C_3HN_2O_2S^-$ in 2-bromo-5-nitrothiazole. This behaviour is very beneficial to our understanding of the physiochemical properties of thiazole and 2-bromo-5-nitrothiazole, which is very important for the synthesis of related drugs and the potential application of radiosensitizers.

Figure 1. Chemical structure of thiazole

Figure 2. Chemical structure of 2-bromo-5-nitrothiazole

The 2-bromo-5-nitrothiazole molecules were also studied using a time-sliced velocity map imaging, (VMI) spectrometer [2]. The experimental setup included an ultra-high vacuum chamber, an electron gun, a Faraday cup to measure the electron beam current, a needle to produce an effusive molecular beam and a time-of-flight based VMI spectrometer. Kinetic energy and angular distributions of both Br^- and NO_2^- produced from DEA to 2-bromo-5-nitrothiazoles at different resonance energies were studied. The main resonance peaks for Br^- and NO_2^- were found at 4.2 eV and 4 eV respectively. The experimental study has also been supported by thermochemical threshold calculations.

References:

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Fig. 1

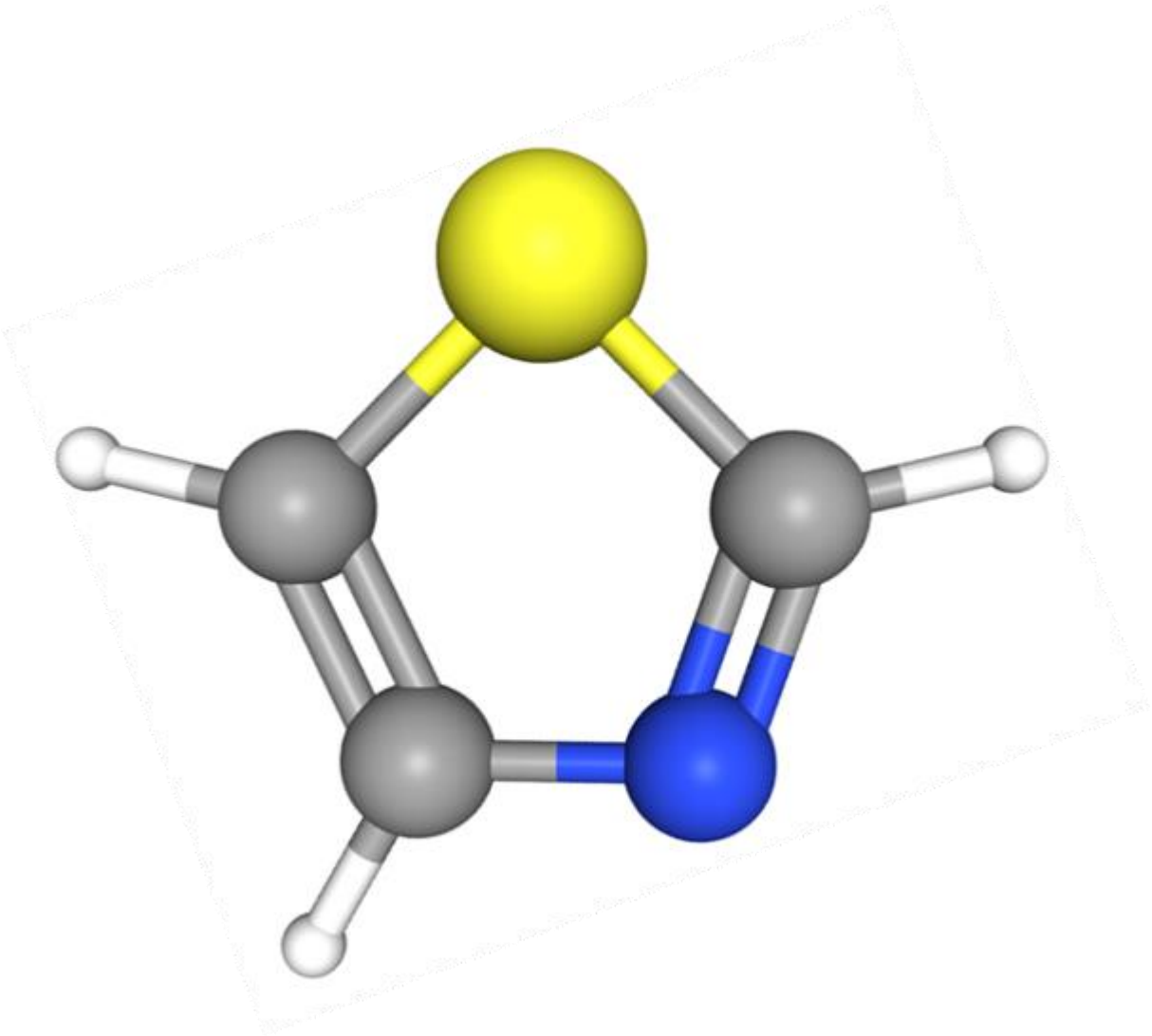
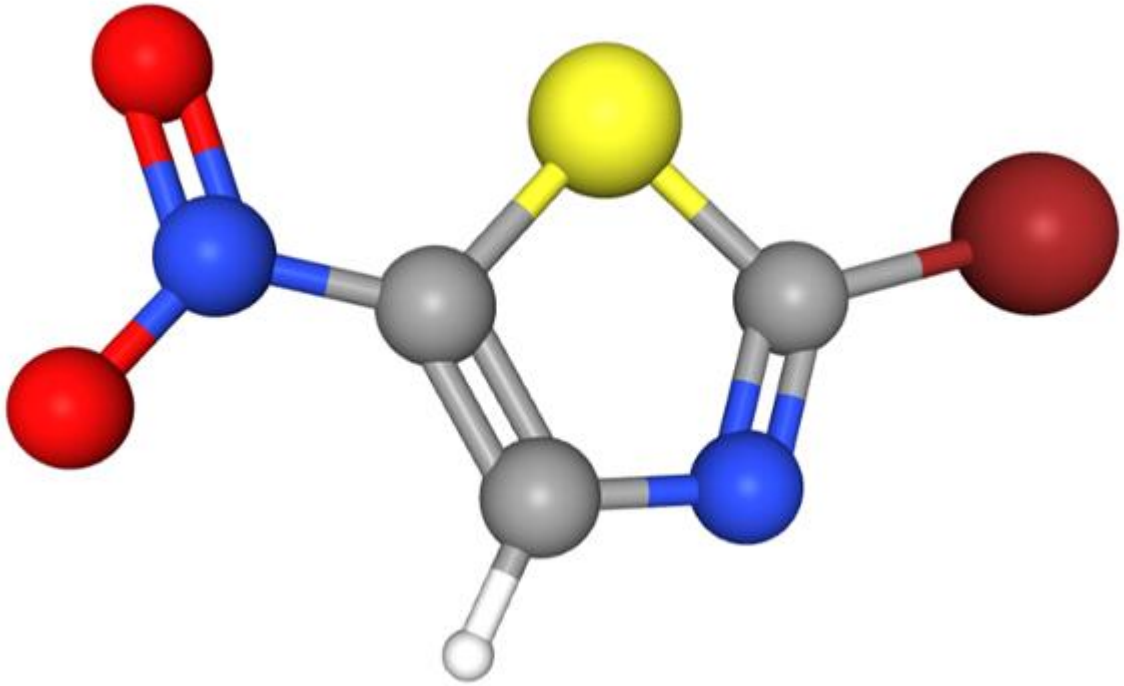


Fig. 2



P 67

Detection of ions generated in an intense pulsed EUV-light beam using different interfaces to a high-resolution TOF-MS

N. Pengemann[1], S. Benter[1], M. Hammelrath[1], J. Rieger[1], F. Schuler[1], A. Elshani[2], I. Gisch[2], L. Nagel[2], H. Kersten[1], S. Brose[2], C. Holly[2], P. Gust[1], T. Benter[1]

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[2]*RWTH Aachen University, Aachen, Germany*

To gain a better understanding of the gas-phase chemistry induced by extreme ultraviolet (EUV) radiation, a commercially available time-of-flight mass spectrometer (TOF-MS), the LTOF (Tofwerk AG, Switzerland), is employed to detect ions generated in the EUV beam path. The examined gas phase usually consists of hydrogen, where a low-density plasma is induced by the EUV radiation.

The employed mass spectrometer contains a time-of-flight mass analyzer with a flight distance of 2700 mm, an ion transfer quadrupole with a rod length of 100 mm, and a custom ion source. This source operates as a transfer stage for ions originating from the plasma region to the flight tube. In addition, it provides an electron ionization (EI) stage for the detection of neutrals. The instrument is operated at the EUV high-intensity exposure (EUV-HIEX) irradiation facility (RWTH Aachen), which provides focused radiation at a wavelength of 13.5 nm. The MS is coupled via different interfaces to the exposure system, at variable distances and viewing angles to the focus of the EUV beam. The exposure stage is operated at pressures up to 5 Pa with different gas compositions, while the TOF analyzer is maintained at pressures below 2E-7 mbar.

Previous studies in this project have revealed unexpected results regarding the recorded ion intensity at different operating pressures. In general, the analysis of EUV-induced plasma ions with a high-resolution TOF-MS is challenging due to the minuscule number of native ions reaching the analyzer region and the large pressure gradient between the EUV chamber and the TOF analyzer. As an orifice is essential to maintain the MS operating pressure, considerable ion losses are inevitable. For a detailed analysis of the ion loss processes, two chamber designs were developed: i) a minimalistic design with the smallest possible volume to couple the MS on-axis with the light beam and ii) a more complex design, which allows to couple the MS at different positions and place a probe in the EUV beam focus. The investigated distances range from 170 mm up to 700 mm from the EUV-focus to the TOF-MS entrance. With each variation of the experimental setup, mass spectra at different exposure chamber pressures and gas compositions are analyzed.

The results from this campaign will be presented.

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Exploring the influence of the number of Ions on peptide fragmentation spectra

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Introduction

Single-cell and low-input proteomics leverage powerful new tandem mass spectrometry (MS/MS) tools for exploring cell biology at a granular level. While improvements in instrumentation and sample preparation methodologies have made single-cell proteomics attainable by many labs, consistently detecting peptides in single-cell experiments remains a challenging endeavor. One way to improve our ability to detect peptides in low-input experiments is to use reference spectra acquired from bulk samples with more input material. Recent reports have suggested that the quality of MS/MS spectra may change as input material decreases, and in this work, we sought to determine how many ions are required for an Orbitrap™ mass analyzer (OT) to produce internally consistent spectra using the same instrument.

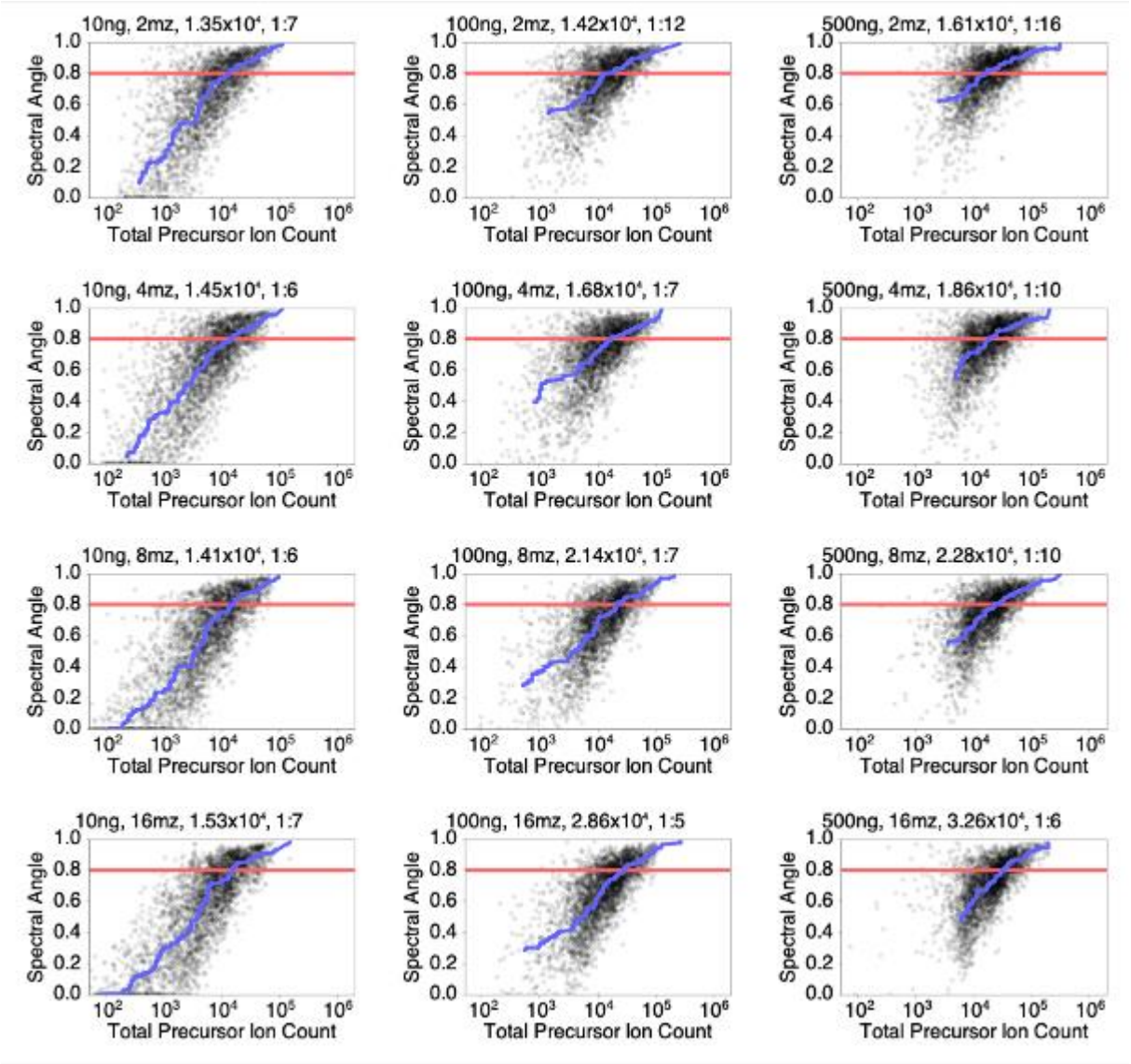
Methods

We configured a Thermo OT to collect data-independent acquisition (DIA) MS/MS of HeLa digest in a "packeting" pattern where for every DIA window, a precursor MS was followed by three MS/MS at Automatic Gain Control (AGC) targets of 50%, 150%, and 250%. By collecting spectra with different AGC targets in quick succession, we are confident that these measurements contain the same rate of ions in the ion beam and the same distribution of peptides (including background). Each packet has a preceding MS to calculate the number of ions in the ion routing multipole (IRM). We performed experiments using different precursor isolation sizes (2, 4, 8, and 16 Th) and at different injection inputs (10, 100, and 500 ng).

Preliminary Data

Rather than modulate sample concentrations to change the number of ions in the IRM, we adjusted AGC to mimic single-cell level measurements by limiting the number of ions. This gave us a unique platform to test the effects of limited ions on the quality of MS/MS, where higher AGC spectra act as controls. Integrating the precursor isolation window from proceeding precursor spectra, we calculated the number of ions within each MS/MS measurement, given the ion injection time. Within each packet, we calculated the spectral contrast angle between the optimal MS/MS (250% AGC) and MS/MS with fewer ions limited by lower AGC targets. We observed that the quality of MS/MS spectra measured in the OT was tied to the ion count of the highest fragment, suggesting that the relationship is governed by ion statistics, rather than total ion count. We found the OT required approximately 1500 ions as the predominant fragment to produce >80% spectral angle (Figures 1 and 2). This corresponded to approximately 14000 total precursor ions, equivalent to only 1.4% of the normalized AGC target in most low-input DIA experiments. Consequently, we believe that previously observed systematic differences in Orbitrap MS/MS between bulk and single-cell measurements are dominated by inconsistent experimental parameters, particularly data acquisition, and collision energy settings.

Fig. 1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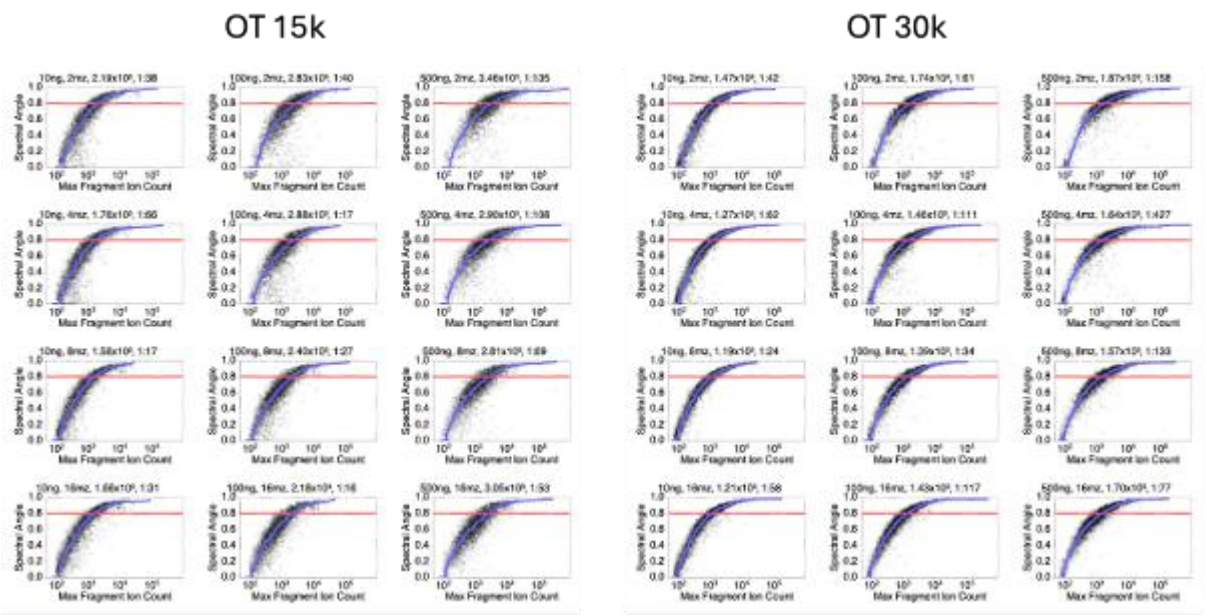
100ng, 8mz, 2.14x10⁴, 1:7
500ng, 8mz, 2.28x10⁴, 1:10
10ng, 16mz, 1.53x10⁴, 1:7
100ng, 16mz, 2.86x10⁴, 1:5
500ng, 16mz, 3.26x10⁴, 1:6
Spectral Angle
Total Precursor Ion Count

Fig. 2



P 69

Investigation of surface-induced dissociation: Molecular dynamics simulations of wall collisions of large droplets formed by ESI

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Electrospray ionization (ESI) produces large, charged droplets which contain the analyte. In common literature, the lifetime of those droplets is often underestimated and the exact dynamics of the droplets on their transfer through the vacuum system of an instrument to the mass analyzer region is unclear. Experimental observations suggest that the droplets can penetrate deeply into mass spectrometric instruments and produce large fragments, which are visible in the mass spectrum [1].The formation of the fragments could possibly be attributed to collision-induced fragmentation of the larger droplets aspirated into the vacuum system of the instrument. However, in supplementary studies of ion trajectory and molecular dynamics (MD) simulations of droplet behavior in a SCIEX Triple Quad 6500, it was found that the larger droplets likely do not obtain enough collision energy through CID processes to fragment significantly. As such, surface-induced dissociation (SID) was proposed as another possible mechanism for the generation of the fragment spectra. In SID the available collision energy in a single collision event is significantly higher compared to CID, which generally leads to different fragmentation patterns of the two methods.

A system to study wall collisions of charged droplets has been set up in the molecular dynamics simulation framework LAMMPS. For this, a stable droplet containing several charges, acetonitrile and water is placed in front of a pure Aluminum wall. The droplet is shot at the wall with different velocities, to reproduce electric accelerations commonly found in mass analyzer instruments like the SCIEX Triple Quad.

First simulations show that the Aluminum wall is able to absorb a lot of the collision energy in its vibrational modes and the droplet mostly sticks to the wall. In consequence, no large fragments were observed. Variations of this simulation have been carried out with an angled wall to simulate grazing collisions of the droplet with e.g. quadrupole rods. Those simulations also show that the droplet mostly sticks to the wall and does not burst into larger fragments. However, with high collision energy, small droplet fragments are ejected into the vacuum from the collision site. Since the behavior of the MD system is sensitive to the applied force field, additional studies have to be carried out where force field parameters are varied to check for plausibility and convergence between different simulations.

We present an overview of the simulation results and a detailed analysis of the sensitivity of the simulation on simulation parameters.

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P 70

Development and characterization of pulsed ion sources coupled to oaTOFs

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[1]*University of Wuppertal, Wuppertal, Germany*

Some ion sources are operating discontinuously, i.e. pulsed, due to their mode of operation. The application of pulsed ion sources in combination with orthogonally accelerating time-of-flight mass spectrometers (oaTOF-MS) may render the performance of such systems when the ion source is not synchronized with the MS. For example, spectra will be recorded at high repetition rates (kHz range) by the MS even though no ions have been ejected by the ion source. This leads to a reduction in sensitivity and an increase in background noise. However, if i) high mass resolution, and ii) sampling of individual full mass spectra in the kHz regime are required, oaTOFs are the mass analyzers of choice. Synchronization of the ion source with the oa-stage also enables time-resolved characterization of the ion source and the ion-molecular gas-phase processes that take place. Thus, in a pulsed plasma ion source, the ionization region, plasma dynamics, and possible ionization mechanisms can be deduced.

The characterization of this synchronization method for further application in pulsed plasma dynamics studies was performed with resonance enhanced multiphoton ionization (REMPI) of nitrogen monoxide (NO). Ions are generated within less than 5 ns wide pulses (repetition rate: 10 Hz) as follows: NO (400 ppm in N_2) at a pressure around 0.05 Pa is fed into a vacuum chamber via a gas chromatography (GC) capillary. A Nd:YAG pumped optical parametric oscillator (OPO) laser system (NT342 Series, EKSPLA, Vilnius, Lithuania) resonantly ionizes NO at 226.2 nm at the capillary outlet. For detection of the ions, the chamber is coupled to a high resolution oaTOF-MS (LTOF, Tofwerk AG, Thun, Switzerland). The LTOF is synchronized with the laser system using the trigger out signal of the Pockels cell driving unit of the Nd:YAG pump laser. The time interval between the laser pulse and the start of the LTOF data acquisition is adjusted by a delay generator (81150A, Keysight Technologies, Santa Rosa, USA).

NO is ionized via (1+1) REMPI in the tight focus of the laser beam. The LTOF acquires a mass spectrum after a set delay. By varying the delay, the flight time and width of the expanding NO^+ plume is determined. Conclusions can be drawn about the velocity distribution of the detected ions and their kinetic energies. When the background pressure in the chamber is increased, fewer ions are detected, and the average velocity of the ions shifts to longer times. By placing an electrode behind the area of ionization, the effect of applying ion accelerating (pushing) and decelerating (drawing) potentials on the ion arrival time can be studied. Also, the electrons produced during ionization can be measured as a pulsed current through the electrode and thus the number of generated ions can be estimated. The experimental results are supported by simulations using SIMION and IDSimF. It is expected that these experiments will be a major step forward in the understanding of pulsed plasma sources.

P 71

Unveiling biomolecular interactions: A quantitative Laser-Induced Liquid Bead Ion Desorption (qLILBID) approach

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Understanding the dynamics of biomolecular interactions is pivotal for elucidating cellular processes. We introduce a novel method, Quantitative Laser-Induced Liquid Bead Ion Desorption (qLILBID), which employs mass spectrometry to examine these interactions. By excitation of water molecules with an infrared laser, we enable the soft release of ions from aequeous sample droplets. A paralell effect of the laser energy transfer can be (partial) dissociation of non-covalently bound complexes. In contrast to LILBID mass spectrometry, qLILBID incorporates a controlled energy transfer onto the sample and subsequent correction for the subsequent dissociation, allowing to determine the equilibrium state of bound and unbound molecules in solution. Initially applied to dissociation constants (KDs) of dsDNA, recent experiments demonstrated adaptability across various substance classes and system, covering affinities from low nanomolar to low micromolar levels. We were able to show, that KDs obtained from qLILBID were in good accordance with those from standard methods such as Isothermal Titration Calorimetry (ITC), Electrophoretic Mobility Shift Assay (EMSA) or melting curves. With its low sample consumption of less than a nanomol and no need for prior altering or separation of the measured complexes, qLILBID has proven to be a consistent and widely applicable method for the determination of binding affinities. Especially with the current development towards affinity measurements for homodimers, qLILBID has the potential to access affinity measurements, that ITC and other titration or separation based approaches struggle with.

This Presentation offers insights into the practical implementation of qLILBID, facilitating a deeper understanding of biomolecular interactions with a top-down approach.

P 72

Steps towards the implementation of Dopant-Assisted MALDI-2 mass spectrometry imaging

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Atmospheric pressure ionization methods such as APCI and APPI build on the increase of ion yields by use of reactive dopants (e.g. acetone), following their single- or resonance-enhanced two-photon ionization (SPI/REMPI). Recently, we demonstrated the usefulness of RF-driven Kr lamps for dopant-assisted (DA) ionization of (semi-)volatile organic compounds in coffee roasts and human breath [1]. In a second study, we showed that acetone dopant can also assist the postionization of phospho- and glycolipids in high-resolving MALDI imaging [2]. Ion formation was in both cases achieved in a dual-ion funnel source, operated at a few mbar of buffer gas. Building on the strong absorption of acetone vapor at 266 nm, here we asked if also laser-based MALDI-2-MSI may benefit from a controlled dopant injection.

To enable a precise control of gas pressures and dopant flow rates, a vapor injection system was developed, sketched in Fig. 1. Acetone is supplied using a syringe pump connected to a system of three PEEK capillaries (IDs: 30, 75, 100 µm). To ensure an effective and complete evaporation, the capillary is connected to a ceramic frit, mounted inside a temperature-controlled metal casing. Dopant vapor is picked up by a stream of nitrogen gas. The pressure in the ion source is monitored with a precision barometric pressure sensor (BMP388, Bosch) and gas supply regulated with a PID-controlled electromagnetic valve. A timsTOF fleX MALDI-2 QTOF mass spectrometer (Bruker Daltonics) was used for the DA-MALDI-2 experiments. In addition, DA-MALDI-2 experiments with a simpler inlet system were conducted using an orbitrap mass analyzer (Thermo Fisher Scientific [1,2]). Polar lipid standards and thin sections of coronal mouse brain were used as test samples and were sublimation-coated with 2,5-dihydroxyacetophenone matrix.

With our setup, acetone flow rates from about 4 to 100 µl/min at a total pressure of about 0.6 to 4.0 mbar in the vacuum vessels could reproducibly be adjusted. To identify optimal DA-MALDI-2 conditions and to obtain a first glimpse into the underlying processes, we varied acetone flow-rates, the pressure inside the ion source and the pulse energy of the Nd:YAG postionization laser (266 nm) of the timsTOF fleX mass analyzer. With high analytical relevance, our first studies already showed a sizable additional boost in signal intensity for several analyte classes; this was achieved with both employed MALDI-2 mass spectrometers. For example, the signals of PC(34:1)+H]+ and [PE(38:6)+H]+ were increased by a factor of about 3 compared to dopant-less MALDI-2.

The described method could be a straightforward means to boost the MALDI-2 ion yields in ionization reactions.

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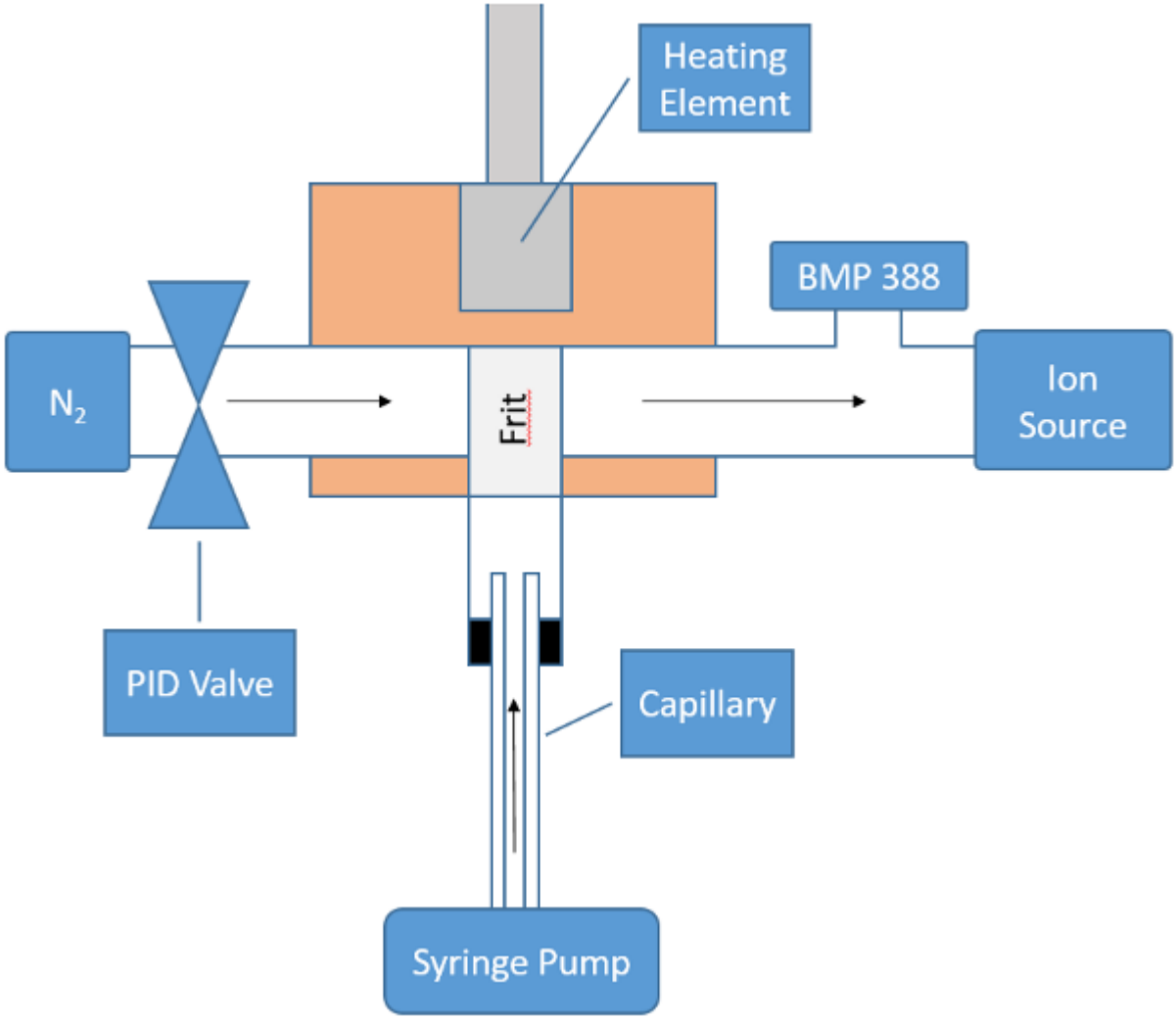
Figure 1: Schematics of the dopant injection system.

Fig. 1


Heating Element
BMP 388
N_2
Frit
Ion Source
PID Valve
Capillary
Syringe Pump

P 73

Ion pairs of cationic Nickel/Gallium complexes and weakly coordinating anions: Comparing LIFDI & ESI MS

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Whereas comparably "hard" ionisation techniques like electron impact (EI) or chemical ionisation (CI) are suitable for organic or biochemical compounds like peptides,1 these methods usually lead to pronounced fragmentation in the analysis of highly sensitive organometallic compounds. Therefore, particularly "soft" methods like liquid injection field desorption ionisation (LIFDI) or electrospray ionisation (ESI) are commonly used in this regard. Both LIFDI and ESI have been successfully employed for the analysis of highly air- and moisture-sensitive species like transition metal complexes2, 3 or clusters.4

Using a setup that combines a LIFDI cell and a Thermo Fisher Exactive Plus Orbitrap mass spectrometer with an inert atmosphere glovebox, sample preparation and MS measurements can be performed under strictly air- and moisture free conditions.5 At the same time, the Orbitrap mass spectrometer features an ESI source, which enters the device from the opposite side. Thus, this setup allows for acquiring high resolution ESI as well as LIFDI MS data of highly sensitive compounds with the same spectrometer (see Figure 1).

Figure 1: Schematic rePresentation of the MS setup comprising an ESI and a LIFDI source combined with a glovebox (modified figure from Ref. 5).

Employing the setup described above, the analysis of the dicationic, highly air- and moisture-sensitive nickel/gallium complex [Ni(GaC5Me5)4Ga(MeCN)2C5Me5]2+ featuring two weakly coordinating tetraarylborate (BArF) anions reveals differences between LIFDI and ESI as ionisation methods (see Figure 2). Both measurements were performed in dry dichloromethane as a solvent with concentrations of 1 mg/mL (LIFDI) and 1 µg/mL (ESI). In order to avoid any contact with air and moisture, samples were prepared inside a glovebox and directly injected from inside the glovebox for LIFDI MS measurements. LIFDI results in comparably strong fragmentation with the fragment ion Ni(GaC5Me5)(MeCN)2(C5Me5)+ as the peak with highest intensity. The molecular ion [M]+(BArF)+ could be detected at low abundance. Similarly, the fragment ion corresponding to the loss of one Cp* ligand (C5Me5) was detected. In contrast, the ESI MS spectrum shows the molecular ion with slightly higher intensity at the cost of an even higher degree of fragmentation.

Figure 2: Molecular structure of [Ni(GaC5Me5)4Ga(MeCN)2C5Me5]2+ with two weakly coordinating BArF anions, including LIFDI and ESI MS spectra.

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Fig. 1

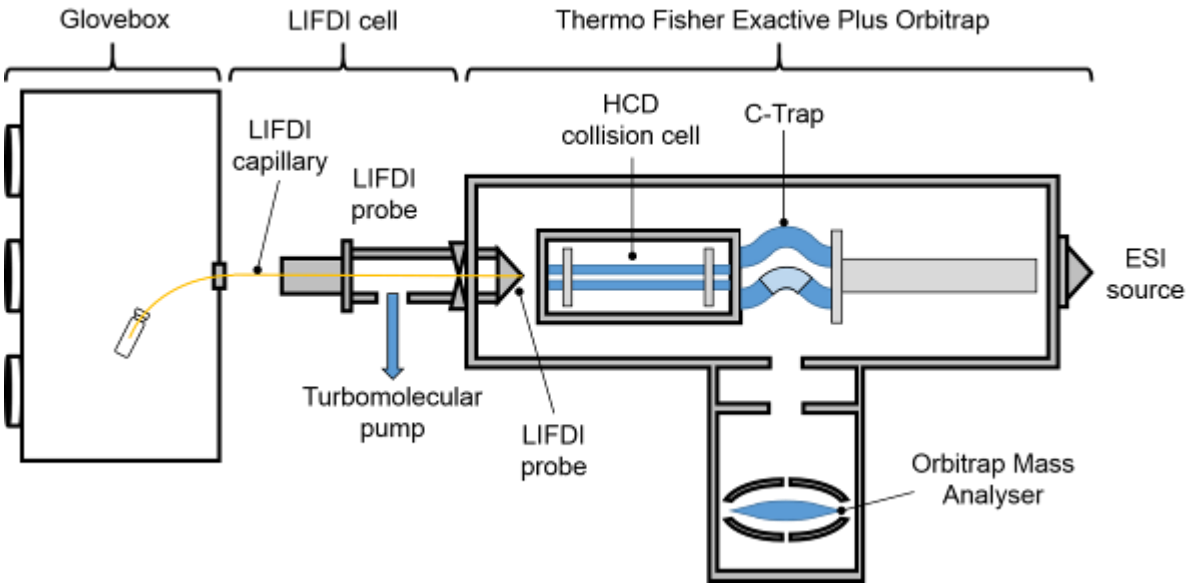
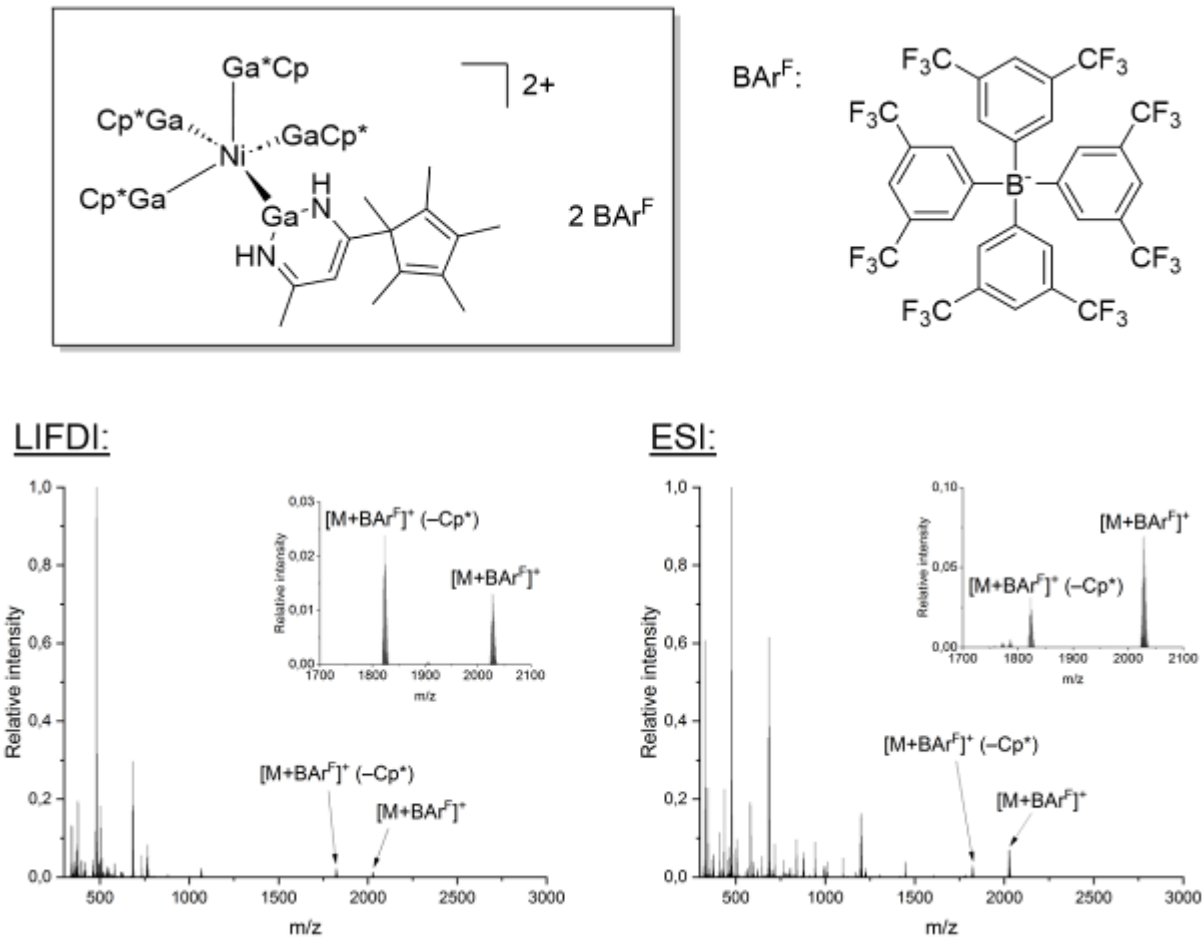

Glovebox
LIFDI cell
Thermo Fisher Exactive Plus Orbitrap
LIFDI capillary
LIFDI probe
HCD collision cell
C-Trap
ESI source
Turbomolecular pump
LIFDI probe
Orbitrap Mass Analyser

Fig. 2


Ga*Cp
2+
Cp*Ga
GaCp*
Ni
Cp*Ga
H
N
Ga
HN
2 BArF
BArF:
F3C
CF3
B-
LIFDI:
ESI:
Relative intensity
m/z
[M+BArF]+ (–Cp*)
[M+BArF]+

P 74

Investigation of the chlorine oxides redox chemistry in ESI

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ESI can be described as a multiphase electrolysis. Under the impression of that picture there is high interest in the investigation of the behavior of chlorine oxides (Chlorine dioxide, Chlorite and Chlorate). This species are known for their complex redox chemistry with complex pathways. But usually the investigation is only conducted in liquid phase or gas phase. Methods like APCI and ESI are a case where both of these environments have to be investigated. That raises many questions especially while the identification of oxidation products of disinfectants. Usually this gets neglected due to a chromatographic separation before the ionization. In the case of the investigation of reactions with low time constants that can not be done. While understanding the impact of different chlorine oxide species in the ionization process that would lead to many possibilities in the investigation of these reactions.

The reactive species and reactions are handled in common Agilent LC-vials. The samples, only soluted, in water were mixed in the mixer built in the Sciex ion source. The chlorine dioxide is purified by a stripping process and chlorite and chlorite are typical technical chemicals due to the risk of explosions. That leads to the problem that sodium chlorite has 20 w% of sodium chloride added. As oxidized species, indole derivatives like tryptophan are used.

The basic setup is hereby a mix of chlorine dioxide and tryptophan with varying ratios.

The main goal of the experiments was first of all the investigation of the ion source temperature on the TIC and the spectra. While looking at the spectra, the relative intensity between the unoxidized species and oxidized species are of highest interest.

This was also investigated with addition of chlorite, chlorate and formic acid in different ratios.

As preliminary result first of all the temperature influence on the reference tryptophan is investigated. As expected the amount of fragmentation products rises. For the oxidized species the same can be said. Furthermore it can be observed that the addition of chlorine dioxide leads to a quite stable signal without the addition of formic acid. At the same time that leads to vastly different spectra.

In conclusion the addition of chlorine oxides leads to a higher ion yield and a more stable signal. Nonetheless for analytical conditions the addition of formic acid is recommended. Furthermore it can be said that a nonpolar portion in the solution that is sprayed is in agreement with the existing literature. Nonetheless the electrochemistry of chlorine oxides as "modifier" is far more complex than that of formic acid.

P 75

Energy-resolved collision experiment of host-guest complexes between angle-strained alkyne-containing cycloparaphenylenes and fullerenes

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Cycloparaphenylenes (CPPs) are strained ring molecules, comprising only sp^2-hybridized carbon atoms. As a result of their both concave and convex extended π arrays, CPPs have been widely employed as ideal supramolecular hosts for fullerenes [1] and CPPs [2].

In this study, a variety of functionalized triazole-containing CPPs with an elliptic lasso-like shape are exploited as hosts for C_{60} and C_{70}. Host-guest complexes of these Lasso-CPPs and closely related [12]CPP with C_{60}/C_{70} are investigated by electrospray ionization mass spectrometry. The mass spectra show that [1:1] complexes of Lasso-CPPs with C_{60}/C_{70} are formed as radical cations and protonated species, while [2:1] complexes mainly exist as protonated molecules. Energy-resolved collision (MS^2) experiments reveal that Lasso-CPP⊃fullerene [1:1] complexes are more stable as radical cations than as protonated species. This is due to the fact that in the radical cation, the positive charge on Lasso-CPPs can be delocalized, thus enhancing the complex stability. Changes in the electron donating/-accepting nature of peripheral substituents on Lasso-CPPs, on the other hand, have little influence on the complex stability. Additionally, MS^2 experiments indicate that [2:1] and [1:1] complexes of Lasso-CPPs with C_{70} are more stable than the corresponding C_{60} analogue, as reported for CPP-based complexes [2]. However, complexes of Lasso-CPPs with C_{60}/C_{70} are found to be more stable than [12]CPP complexes.

Our results suggest that strain-promoted Lasso-CPPs with a series of unique properties are desirable host molecules for fullerenes. Mass spectrometry is a powerful tool for the study of these non-covalent host-guest complexes.

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Fig. 1

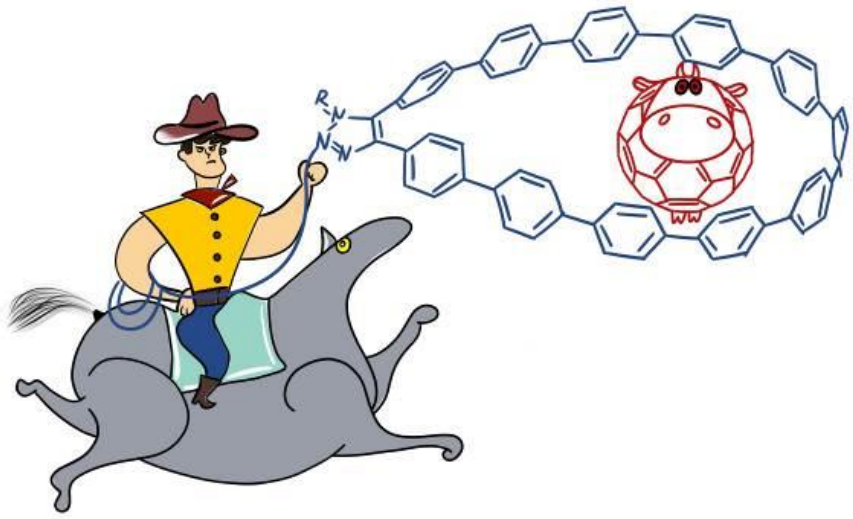
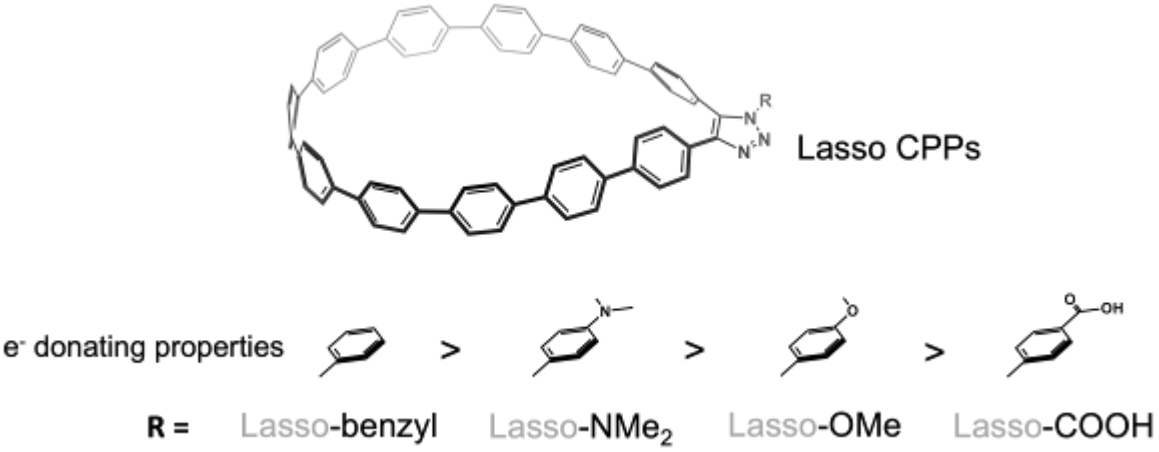


Fig. 2


Lasso CPPs
e⁻ donating properties
>
>
>
R =
Lasso-benzyl
Lasso-NMe2
Lasso-OMe
Lasso-COOH

P 76

Chemistry of charge-imbalanced layers generated by Ion Soft-Landing

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Ion Soft-Landing (ISL) is used to deposit mass-selected molecular ions and their fragments onto surfaces.1 The deposition of multilayers of one type of pre-charged, electronically stable ions (which retain their charge in the soft-landing process) raises fundamental questions about the charge-balancing processes in the formed layers. We demonstrate the formation of counterions (ammonium in the case of anions and small carboxylates in the case of cations) in these charge-imbalanced layers. We show that the exact chemical composition of the counterions is dependent on the deposition parameters (e.g. ion current) which influence the coaccumulation of contaminations present in the background of the vacuum chamber.

Additionally, we show that highly reactive fragment ions formed via CID can bind at the surface to a previously deposited ion of same polarity, resulting in highly charged clusters, which were analyzed by analytical mass spectrometry.2

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P 77

Capabilities of Surface-Assisted Flowing Atmospheric-Pressure Afterglow Mass Spectrometry (SA-FAPA-MS) for Direct Analysis of Polycyclic Aromatic Hydrocarbons (PAHs)

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Ambient desorption/ionization mass spectrometry (ADI-MS) is attractive for direct mass spectral analysis of liquid and solid samples because minimal sample preparation is required and measurements are fast.[1] In this work, a plasma-based ADI source, namely a pin-to-capillary flowing atmospheric-pressure afterglow (FAPA) source[2], is coupled to a high-resolution MS and used to directly probe polycyclic aromatic hydrocarbons (PAHs) from thin-layer chromatography (TLC) plates. TLC plates serve only as sample substrates without a preceding planar separation step. Both cyano (CN)-HPTLC and reversed-phase (RP2)-TLC plates were compared for best performance because they were identified earlier as best suited for TLC-FAPA-MS analysis.[3]

First, FAPA-MS was optimized for best PAH analysis directly from TLC plates. Thirty different PAHs were screened in positive and negative ion mode. Sample preparation was minimal and included only the triplicate spotting of 1 µL of sample onto the TLC plate. CN-HPTLC and RP2-TLC plates proved most suitable and a linear range of up to four orders of magnitude could be achieved. Limits of detection (LOD) in neat solutions of, for example, anthracene, chrysene, and fluorene were determined to be in the µg/L range. Because real environmental samples may contain hundreds of different PAHs in addition to matrix compounds, PAH containing real samples were used to test the applicability of FAPA-MS to screen complex samples. In addition, a standard reference material (diesel particulate extract, NIST SRM 1975) was used for method validation. While further optimization is required, TLC-FAPA-MS is already considered an interesting method for rapid qualitative screening of PAHs in real samples.

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P 78

A novel approach in addressing the challenges of monitoring multi-classes of POPs in a single run by GC-Ion Mobility-HRMS

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Polychlorinated dioxins and furans are bio-accumulative molecules largely formed during combustion and industrial manufacturing processes. Their analysis is complex due to low level regulatory exposure limits and difficult sample matrices. Both classes are considered as persistent organic pollutant (POP) and could be widely found in environmental samples. Severe principal consequences derived from dioxins and furans exposure, even at low concentrations, include cancer, reproduction and growth issues, immune system diseases and endocrine effects. An additional emerging POP family are polychlorinated biphenyls. It consists of 209 congeners, 12 of them known as "Dioxin-like PCBs". These compounds are industrially used as heat transfer fluids, dielectric compounds, and coolant fluids for electrical components. Even though their manufacturing was drastically decreased in the 1960"s due to the proved toxicity, their chemical stability and lipophilicity cause some congeners to still be found today in environmental samples, like soils and sediments. Most reported health effects are dermal and ocular lesions, lowered immune responses, mutagenic effects, breast cancer and poor cognitive development in and motor control problems in children.

All these compound classes are mainly analyzed and quantified in natural abundant matrices by high-resolution sector field mass spectrometry. Proposed here is a novel workflow involving ion mobility as an orthogonal criterion for identification and quantification, coupled to a high-resolution QTOF mass spectrometer. The benefit is a high flexibility for analyzing various classes of compounds all in a single GC run with high sensitivity.

1 uL samples were separated by GC (35 min run time, Restek 60 m 0.25 mm id 0.25 um column, HxCDD and HxCDF separation). A GC-APCI source was coupled to the timsTOF Pro 2 (Bruker) which enables fast and sensitive analysis of dioxins, furans and PCBs in a single GC/HRMS run. Criteria for validation and quantification of compounds were high mass accuracy, retention time, isotope pattern matching, MS/MS qualifiers and collision cross sections (CCS) from ion mobility filtering. Kendrick mass defect plots were applied for the extraction of the specific area of compounds containing Cl or Br from the complete GC/MS chromatogram.

All PCBs have been detected at an LOD of ≤10-20 ppt. The dioxins were detected down to levels of 25-125 ppt, depending on the individual compound. Examples of the analysis of real-life samples like rapeseed oil, milk fat, sludge extract and ash are presented.

P 79

Detecting PFAS beyond the current regulative request: A complete overview of the contamination in water and soil

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PFAS are by now very present in the public news as true threat to mankind and nature. They are "forever chemicals" due to their persistent, bio-accumulative, toxic (PBT) properties and ubiquitous presence in the environment and organisms. The US EPA estimates almost 15.000 species in this group as manufactured compounds, precursors and degradation products. This makes their systematic and comprehensive environmental monitoring an extremely challenging task. Due to the group size, there is a lack of reference standards or spectral libraries, and there are plenty of isomers. Adding trapped ion mobility spectrometry (TIMS) to UHPLC-HRMS allows for comprehensive monitoring of organic micropollutants in environmental matrices such as water and soil without using reference libraries.

Water and soil samples were spiked with sets of PFAS compounds or taken from common household and environmental resources. Data independent acquisition was performed on a high-resolution mass spectrometer equipped with ion mobility. Kendrick mass defect (KMD) analysis filtered potential PFAS from the matrix background, based on the fluorine content (repeating CF2 units). Spectra were compared with the Norman network and NIST suspect lists of 5000 entries for non-targeted analysis. These contain information about the elemental composition and the InChI structures for PFAS which were compared with the four criteria of exact mass, isotope patterns, MS/MS fragmentation and CCS values of the experimental data for an automated and untargeted identification of all PFAS present in the sample. Prediction tools like MetFrag and CCS-Predict were used for creating theoretical values from the InChI structures for the comparison.

The ion mobility feature of the system was utilized for several purposes. First, it can separate coeluting isobars and isomers. Second, the TIMS filter results in higher sensitivity and lower detection limits of the targeted PFAS as well as significantly higher quality of full-scan MS and bbCID MS/MS spectra. Finally, collisional cross sections (CCS) as additional identification criteria enhance the identification confidence with is based on the exact mass, diagnostic fragmentation ions and the isotope pattern fit. The wide-scope suspect screening of real-life samples against the suspect lists proved to be a comprehensive approach for a fast and efficient identification and quantification of PFAS against the total set of 5000 compounds in complex environmental matrices.

P 80

Getting a better perspective of oil spills: Photo-oxidative degradation of oil components

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Oil spills are common and have negative environmental impacts, high economic costs and are hard to clean up. During oil spills different chemical and microbial reactions can take place, one of them resulting in the formation of stable residues such as tar balls, which are widespread and recalcitrant contaminants. Efficient remediation and assessment of the consequences of oil spills requires knowledge of the weathering processes of oil. Several complex processes determine the fate of spilt oil. One of the most important weathering processes is photooxidation, which leads to chemical transformation of petroleum compounds. It has been previously reported that certain oils form a solid phase upon exposure to light. This process has not yet been studied using modern analytical techniques.

The photooxidation of a light crude oil fraction and alkylbenzene as a model compound was investigated in detail in a custom-built photoreactor. Samples were dissolved in water and exposed to visible and UV light at wavelength of 340-720 nm. The samples were analyzed with a 7 T LTQ FT-ICR MS at a resolution of R = 400 k at *m/z* 400 and an LTQ-Orbitrap Elite with a mass resolution of R = 480 k (Thermo Fisher Scientific, Bremen, Germany) using APPI ionization (Krypton VUV lamp, 10.0 eV and 10.6 eV, Syagen, Tustin, CA, USA). Spectral stitching with 30 Da mass windows and 5 Da overlap was used for the oil samples. MSn experiments were performed to investigate the structure of the resulting compounds. Collision Induced Dissociation (CID) experiments were performed using helium as the collision gas.

In the original sample, the majority of detected compounds belong to the hydrocarbons class and some minor amounts of compounds with up to 4 oxygen atoms are detected. The irradiation causes a significant increase in both the oxygenation of the compounds (up to 11 oxygen atoms) and their size. Most of the newly formed polar compounds don"t remain in the oil but partition into the water phase. The formation of higher molecular weight compounds is evident from DBE and carbon number analysis of the samples. The irradiated products tend to have a large condensed aromatic nucleus to which short alkyl chains are attached. Oxygenation occurs on both the alkyl chains and the nucleus. The result of irradiation of the model compound is the formation of oligomers that are highly oxygenated. The oxygen reacts at a number of different points in the molecule. In some of the reactions, a single condensed aromatic nucleus may be formed from simpler aromatic precursors. In addition, even more oxygenated precipitate was formed in the irradiated samples. This illustrates the complexity of photochemical reactions. The results of the photooxidation of crude oil on molecular level will be shown.

P 81

Identification of chemicals with Transthyretin binding properties using effect-directed analysis (EDA) in human serum samples

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Effect-directed analysis (EDA) represents an innovative strategy employed for the identification and assessment of potential risks associated with complex chemical mixtures1. This method combines advanced analytical techniques with *in vitro* bioassay testing to identify components accountable for the detected toxic effects. The typical EDA process involves testing the biological activity of the original extract, followed by fractionation, repeated bioassay testing, and the mass spectrometric analysis of pre-selected active fractions2. In this study, a high-throughput EDA approach was employed to evaluate whether chemical mixtures found in human serum samples are capable of competing with thyroid hormone (TH) for binding the TH distributor protein transthyretin (TTR).

Two serum extracts of different origins (Europe and Australia) were processed for assessment of their capacity to bind to TTR in the presence of the TH T43. Both extracts showed high TTR-binding effects and were suitable to undergo high throughput EDA1. Briefly, extracts and their matching procedural blank were fractionated by high performance liquid chromatography (HPLC) coupled to a FractioMate™ fraction collector (tec4science, Amsterdam, Netherlands). A total of 80 fractions were produced per each extract (13.5 s of chromatographic run per fraction). The activity of the fractions was assessed using the same TTR-binding assay. Non-targeted chemical profiling of the extracts was performed using the same chromatographic conditions coupled to a compact II QTOF (Bruker, Bremen, Germany). Deconvolution of MS(MS)-data and peak identification was done using MetaboScape 4.0 (Bruker, Bremen, Germany). The extracted features were annotated by the software and aligned to different suspect lists in a hierarchical mode. The suspect lists used were EU MassBank; MassBank of North America, and CECScreen4. The quality and confidence level of identification for the identified suspect chemicals present in the active fractions was assigned using a semi-automated in house TAQ-code2.

Several active fractions were detected in all samples considering a 20% effect threshold. Several analogies could be seen between serum extracts. One active fraction was specific for the extract of European origin only. The alignment of the bioassay results with the corresponding LC/MS data for the feature identification will be presented in detail. This study shows that some activity drivers might differ between sample origins.

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P 82

pH-dependent UV-degradation of biocides including qualification and characterization of their transformation products using stable isotope labeled standards

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Biocides are added to ready-to-use building materials to protect them from microbial infestation during storage and after application, e.g. as part of an external wall façade. These included biocides are exposed to the environment and thus continuously degraded and transformed photolytically and hydrolytically by sunlight and precipitation events.

There is no closed mass balance for most biocides after environmental exposure and biocidal transformation products (TPs) have a potential ecotoxicological effect. Therefor the study was designed to detect and describe unknown or poorly characterized TPs first in laboratory and later in a free-weathering trial. For this purpose, Terbutryn and Octylisothiazolinon were dissolved in neutral and basic solutions and irradiated with simulated sunlight for maximum 144 h. For an uncomplicated and reliable detection of potential TPs, the biocides were present as native and isotope-labelled standards so that potential TPs could be identified by a defined mass shift using HR-MS/MS technology. In addition, potential TPs were considered based on their kinetics, by matching MS^2 fragment spectra (stepped collision energies of 10 eV, 20 eV and 40 eV) and their m/z-presence in real samples (exposed render façades). For a potential structure identification, MS^2 fragment spectra were compared with experimental and in-silico databases. Measuring of all samples including the generation of fragment spectra happened by using a HPLC-MS/MS, equipped with an ESI source, a quadrupole and a time-of-flight analyzer.

It was demonstrated that both target biocides are substantially degraded by UV radiation and that most of the detected degradation products are formed photolytically instead of hydrolytically. Although the pH value has no significant influence on the degradation behavior of the precursor components, some degradation products exhibit pH-dependent formation properties. The structural identification of new potential degradation products with the assistance of open accessible databases remains a challenge. Accordingly, experimental validation is still being sought in the project. Nevertheless, new potential TPs were identified for both biocides (approx. 5 per biocide). These TPs show a clear kinetic profile and matching MS^2 fragments. Some of them could also be detected in exposed real samples and are therefore of great importance for toxicological evaluation.

P 83

Utilizing Ion mobility to enhance targeted and non-targeted analysis of PFAS from environmental samples collected at a ski resort

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Perfluoroalkyl substances (PFAS) are a group of commonly used compounds, known particularly for their hydrophobic, non-stick properties. Their unique chemistry led to their use in ski waxes. While competition rules have recently banned the use of PFAS-containing ski wax, the persistence of PFAS means they could still be detected for years. Given the hazards and concern about PFAS contamination, we investigated if PFAS could be detected at a ski area that supports a high-level race program. While previous studies have looked at targeted analysis for known PFAS, this study used high-resolution mass spectrometry (HRMS) and ion mobility to look for new and unexpected PFAS. Samples were collected from a variety of locations within a ski area in New Hampshire to investigate the trends of PFAS in this type of environment. Data collection was done with ion mobility enabled data-independent acquisition on a SELECT SERIES™ Cyclic™ IMS (Waters Corporation). The added dimension of ion mobility can be used for spectral clean-up, which aids in the elucidation of unknown compounds as well as confirmation of known target compounds. Detected peaks were first compared to an internal HRMS PFAS library for identification. In the extracts, several legacy PFAS including perfluoroalkyl sulfonates, perfluoroalkyl carboxylic acids, and fluorotelomer sulfonates were detected. Unknown peaks were selected for further scrutiny based on their detected drift time in the ion mobility dimension. Previous studies have shown that the CCS values of per- and poly-fluorinated compounds are lower than compounds of similar *m/z*. An ion mobility filter was created based on this knowledge and applied to a list of detected peaks to select for possible PFAS in a list of unknowns. Using this filter, a series (C9-C24) of polyfluorinated carboxylic acid compounds with one hydrogen substitution in the carbon chain were tentatively identified. The distribution for this series of compounds was not consistent, with longer chains found in samples from the base of the ski slopes and the shorter chains in the snowmaking retention pond. While authentic standards were not available for many of the tentative identifications, two standards were purchased and compared with experimental data to confirm proposed structures.

P 84

The search for markers for microbiological contamination in surface water

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Surface water plays an important role as a drinking water source in Germany. In recent years, non-target screening (NTS) through liquid chromatography coupled to tandem high-resolution mass spectrometry (LC-HRMS) has emerged as a promising approach for surface water quality assessment. In contrast, the microbiological water quality is still mostly monitored using culture-based approaches, which only have limited selectivity and require several days to give a result.

NTS of surface water also provides information on the metabolome of the microorganisms in the water. Our objective is to use this information to identify marker substances that indicate microbiological contamination in surface water. These markers can encompass metabolites and other substances that correlate with contamination due to their shared origin.

To extract the relevant parts of the NTS data we divided the LC-HRMS spectra into small areas. For each of these areas, we calculated the correlation between the intensity in the area and the amount of different microbiological parameters found in the same samples. Figure 1 shows the result of such a correlation. Afterwards, we applied additional filters to narrow down the candidate peaks.

In a second approach we used features extracted by the enviMass software from NTS data.[1] We correlated these as well with the results of different microbiological measurements of the same samples. Figure 2 shows the result of the correlation with the *E. coli* colony count.

We observed a moderate correlation between valsartan and the concentration of E. coli in river water. Valsartan, an antihypertensive drug, is prevalent in municipal wastewater. Within a waste water treatment plant it is quickly degraded to valsartanic acid.[2] Consequently, its presence can serve as an indicator of wastewater discharge into the river, potentially contributing to the elevated levels of *E. coli*, a fecal bacterium.

In the next step we want to utilize the features extracted by both methods to train a prediction model for microbial contamination solely from NTS LC-HRMS data. This model shall then function as an early warning system for microbial contamination before results obtained through cultivation methods become available.

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Figure 1: Correlation between LC-MS intensity and *E. coli* colony forming units in the sample.
Figure 2: Correlation between features extracted with Envimass from LC-MS NTS data and the colony count of *E. coli* in the same samples.

Fig. 1

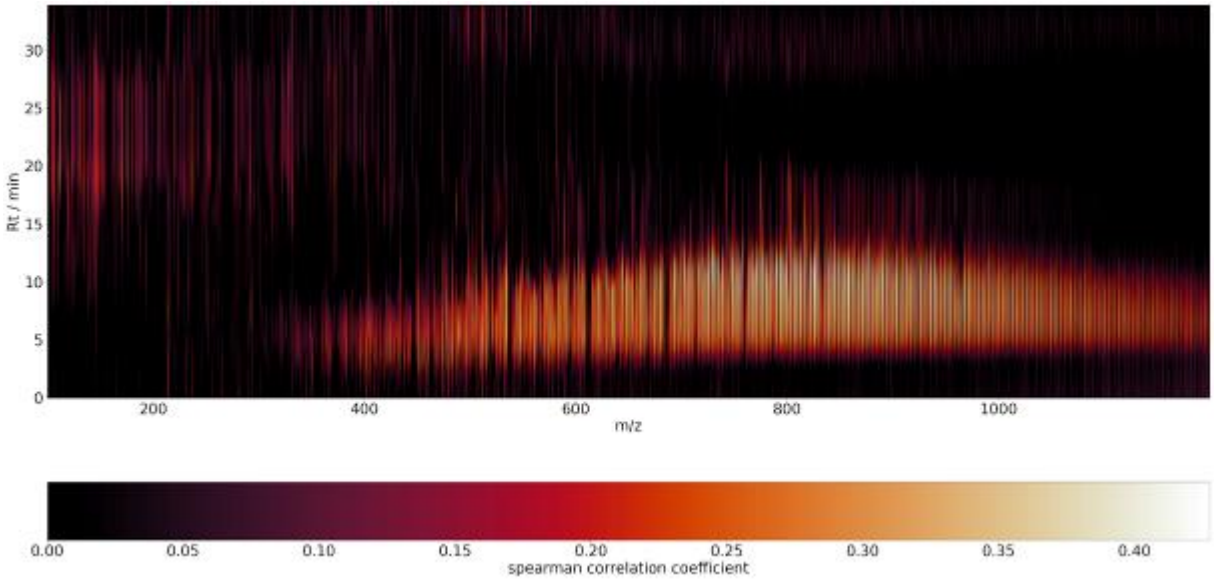
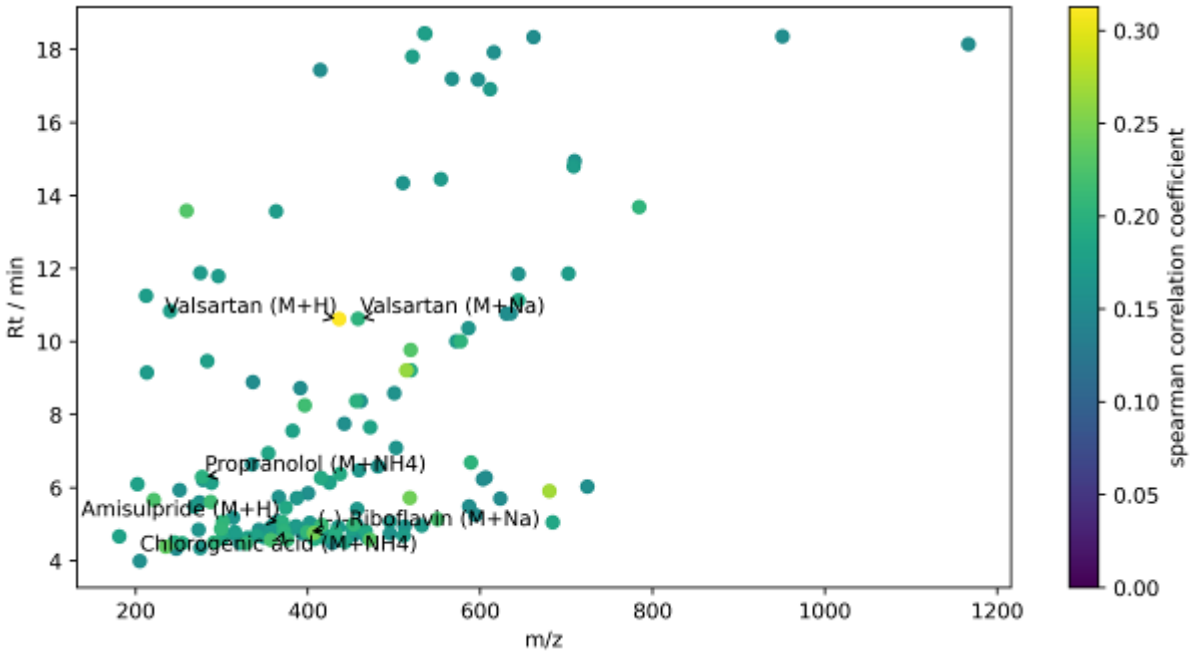

Rt / min
m/z
spearman correlation coefficient

Fig. 2


Rt / min
m/z
Valsartan (M+H)
Valsartan (M+Na)
Propranolol (M+NH4)
Amisulpride (M+H)
(-)-Riboflavin (M+Na)
Chlorogenic acid (M+NH4)
spearman correlation coefficient

P 85

Feasibility study on the adsorption of antibiotics onto fresh and aged microplastics

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Microplastics (MPs) include any type of plastic fragments, fibers, or beads with diameters ranging from 100 nm to 5 mm. The omnipresence of MPs as an environmental pollutant has evolved into a global concern. While primary MPs are used for commercial purposes, e.g., as exfoliants in personal care and cosmetic products, secondary MPs originate from weathering and degradation processes of macroplastic and mesoplastic debris in the environment.[1]

Due to their high surface area-to-volume ratio and hydrophobic nature, MPs can potentially serve as a vector substance for the accumulation and transport of various organic contaminants such as polyaromatic hydrocarbons (PAHs), perfluorinated alkyl substances (PFAS), pharmaceuticals and personal care products (PPCPs), as well as trace metals including silver, cadmium, chromium, and copper.[1] In the environment, MPs aging is a common phenomenon influenced by factors such as temperature, sunlight, oxygen, and chemical substances. The aging process can lead to considerable changes in the physicochemical properties, which, in turn, can affect the adsorption behavior.[2]

This study aims to explore the effect of aging and size on the microplastics potential to act as vectors for environmental contaminants. First, selected microplastics (polystyrene (PS), polypropylene (PP), and polyethylene (PE)) were prepared in-house and aged under controlled conditions for 12, 24, 36, and 48 hours. Second, the MPs were exposed in plastic-free containers to model solutions of antibiotics (namely, amoxicillin and phenoxymethylpenicillin) as potential co-contaminants in wastewater. The adsorption behavior of these antibiotics on in-house manufactured secondary microplastics of various sizes and aging stages at different time intervals were examined using high-performance liquid chromatography (HPLC-UV) and surface-assisted flowing atmospheric-pressure afterglow mass spectrometry (SA-FAPA-MS).

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P 86

Impact of Heat Shock and Salinity pretreatments on Diazotrophic Cyanobacterium *Anabaena* PCC7120

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The study was done to fill in the knowledge gap regarding how pretreatment affects cyanobacteria. The outcome clarifies the synergistic impact of pretreatment toxicity on the cyanobacterium Anabaena PCC7120. In terms of growth pattern, morphology, pigments, lipid peroxidation, antioxidant activity, and molecular modification, cells that had been pretreated with heat and salt had significant and repeatable modifications. When compared to heat shock pretreatment, salinity pretreatment revealed a more than 5-fold decrease in phycocyanin content but a 6-fold increase in carotenoid. Additionally, salt-pretreated samples showed a 3.6- and 1.8-fold increase in FeSOD and MnSOD transcript levels. Morphological and fluorescence studies showed accumulation of reactive oxygen species and alteration in membrane integrity in filaments exposed to stress. Electron microscopic analysis revealed breakage of filaments, wrinkling and elongation of cells along with the secretion of exopolysaccharide and prominent increase in the inter-thylakoid space could also be observed indicating thylakoid membrane damage and reorganisation. Furthermore, 136, 153, and 174 proteins were found using LC-MS/MS analysis in control, heat pre-treatment and salt pre-treatment, respectively. Salinity pretreated cell expressed a conserved hypothetical protein which is uncharacterized and the expression of this putative protein may be to protect the photosynthetic complex against stressors that lead to cellular problems. One could conclude that pretreatment amplifies the negative effect. It also demonstrated that salinity amplifies the negative effects of heat shock however; detrimental effects of salt can be reduced in filamentous cyanobacteria using heat pretreatment, laying the groundwork for increased cyanobacterial tolerance to salt stress which can be applied to various biotechnological approaches.

P 86 a

Pretilachlor-induced physiological, biochemical and morphological changes in Indian paddy field agroecosystem inhabited Anabaena doliolum

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Pretilachlor is a systemic, pre-emergence herbicide applied in the paddy fields to kill narrow and broadleaf weeds. The present study evaluates the toxicity of pretilachlor on the non-target diazotrophic free-living cyanobacterium Anabaena doliolum, commonly found in the paddy fields of eastern Uttar Pradesh (India) and used as a biofertilizer. A.doliolum was subjected to several doses (0, 2, 5, 7, 10, 20 and 40 µg/ml) of pretilachlor and its effects were examined in terms of alterations in cellular morphology, ultrastructure, physiology, and biochemical attributes. The treatment of pretilachlor decreased the growth, total pigment content and photosynthetic efficiency of the test organism in a dose-dependent manner. The decline in growth was observed on 20th day at 2, 5, 7, 10, 20 and 40 µg/ml of pretilachlor concentration by 4, 9, 26, 47, 71 and 92%, respectively. Furthermore, Chlorophyll a and phycocyanin levels were noticeably declined. As a result, the photosynthetic performance also registered a similar decline as measured by chlorophyll fluorescence. However, carotenoid content increased by 13%, 41% and 53% at 5, 10 and 20 µg/ml on 5th day reflecting its protective property. A marked increase in fluorescence intensity and malondialdehyde content by 2.65 and 2.45 folds at 10 and 20 µg/ ml on 7th day was registered. The enzymatic antioxidants (SOD and CAT) and a concurrent increase in glutathione reductase activity were registered (1.75 and 2.11-fold at 20 and 40 µg/ml on 5th day), indicating pretilachlor mediated ROS generation. Moreover, ultrastructural studies done by SEM and TEM revealed plasma membrane and thylakoid membrane damage and fragmentation. These findings have contributed to the broader comprehension of the stress responses triggered by pretilachlor in cyanobacteria. Moreover, they can aid in the evaluation of the detrimental impact of pretilachlor on A. doliolum, given their crucial function as a nitrogen contributor in paddy fields.

P 87

Analysis of the metabolic profile, bioavailability, and elimination behavior of the selective androgen receptor modulators LGD-4033, RAD-140, and S-23 after transdermal application for doping control purposes

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Given their muscle growth-inducing effects, selective androgen receptor modulators (SARMs) are on the Prohibited List by the World Anti-Doping Agency (WADA), disallowing their use at all times[1, 2]. It has been established in the past that orally applied dietary products can be contaminated with SARMs and consequently may lead to an adverse analytical finding (AAF) [3]. Apart from an oral route of administration, there are products (e.g., creams and liquids) containing SARMs, which are designed for a transdermal application. This makes another route of administration as well as contamination scenario realistic, in which an athlete has either intentional or unintentional transdermal exposure to a SARM.

A substance applied to the skin has to overcome different diffusive barriers (e.g., stratum corneum), is affected by enzymatic activity in the skin and, once is diffused into the blood vessels, also avoids the first pass metabolism[4, 5]. Thus, the metabolic profile, bioavailability, and elimination of a drug may differ from that of an oral application [5]. To investigate the aforementioned characteristics, an exploratory study was initiated, where 10 µg of each SARM (LGD-4033, RAD-140, and S-23) were applied onto the forearm of one male volunteer. Urine samples, collected prior to and after SARM application, were processed with and without enzymatic hydrolysis and solid-phase extraction, and analyzed via liquid chromatography-high resolution (tandem) mass spectrometry (HRMS/MS). Samples of a previously conducted micro-dose excretion study using an oral administration were used for comparison.

As all three SARMs were detected via LC-HRMS/MS, it can be adopted that they permeated through the skin and diffused into the blood vessels before renal elimination. Correspondingly, associated metabolites were identified, showing the substances were affected by enzymatic activity. A similar profile of phase I metabolites was observed for LGD-4033 when compared to results of an oral administration study. Initial results indicate though that there may be a difference in the extent of phase II metabolism. A yet unreported metabolite featuring the same mass as RAD-140 but exhibiting a different fragmentation pattern was detected in both dermal and oral post-administration urine samples. In addition, another RAD-140 metabolite previously not described was detected in urine after the oral application of the substance, and this metabolite was not identified in urine after dermal drug exposure. For S-23, we observed another phase II hydroxy-metabolite (M5a), which has not been described so far. As there is little literature on the general metabolism of S-23 *in-vivo*, the phase II metabolism of LGD-4033, and only one subject applied a comparable low dose, these results should be considered with caution and as preliminary. Future studies will be conducted with more subjects and at a higher dose.

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P 88

High-Throughput protein quality attribute monitoring during biotherapeutics development by MALDI-MS

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Biotherapeutic proteins including monoclonal antibodies (mAb) and their derivates are an important class of drugs to treat various diseases (e.g. cancer).

During development several screening steps are conducted like cell line and process (down- or upstream) optimization, producing several hundred of samples which should be analyzed in a short turn-around time to enable data driven decision making and speed up the whole process.

Meanwhile, also high-speed liquid chromatography MS-based methods are very powerful and fast at both the intact and subunit level to monitor quality attributes. Nevertheless, it might be advantageous want to develop matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) on a high throughput level, as it can be even faster.

Desired protein attributes could include e.g. glycosylation and modification information as well as correct assembly of bispecifics. In this regard, the two main challenges to be solved are automated high-throughput sample preparation and resolution as it is limited for larger proteins using MALDI-TOF.

Sample preparation should be fully automated using a robotic liquid-handling system as manual handling takes an enormous amount of time and might cause reduced reproducibility. Automatic preparation of 96 samples includes steps like purification, enzyme digestion, reduction, mixing of sample and matrix-solution and target spotting. Additionally sample acquisition, analysis and data handling have been optimized in respect of automatization and turn-around time.

Up to now only glycosylation information at the Fc/2 have been evaluated due to the resolution capabilities of axial MALDI-TOF system. It might be possible to switch to a quadrupole time-of-flight (Q-TOF) MS with the limitation of the quadrupole and the associated transmission efficiency. To circumvent this obstacle, higher charge states can be generated, and MS parameters can be optimized. This might enable the detection of proteins/subunits at a high resolution by modern MALDI QTOFs allowing to monitor modifications with only minor mass shift which have been out of scope up to now.

By combining high resolution MALDI-MS and automated sample preparation it is possible to monitor therapeutic protein attributes at high throughput/fast turnaround time not achievable before and by this enable data driven decision making.

P 89

Characterization of a LC-HRMS method for differentiation between doping and contamination scenarios concerning the anabolic agent S-23

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Anabolic Agents have been among the most frequently detected prohibited drugs in professional sports. One class of substances known as Selective Androgen Receptor Modulators (SARMs) has been recently showing a growing prevalence. Although the novel substance class has been undergoing clinical investigations for several years, no approved drug has been commercialized, and yet these substances are available mostly via Internet-based suppliers. Consequently, there is a potential for a growing misuse in sports. Thus, the 2021 Anti-Doping Testing Figures Report released by the World Anti-Doping Agency (WADA) revealed the rise of the novel SARM S-23. It is therefore of great relevance for doping controls to expand knowledge and understanding of the novel SARM. Furthermore, SARM S-23 contaminations have been observed in dietary supplements. Therefore, in addition to the primary doping scenario, a contamination scenario is conceivable. This distinction is significant, as an adverse analytical finding (AAF) can have severe consequences for athletes.

To obtain a comprehensive understanding of potential metabolites of SARM S-23, an *in-vitro*-study using human liver microsomes was conducted. Thereafter, a human *in-vivo*-administration micro-dose-study was conducted with a 50 µg dosage of S-23 involving a cohort of 5 adult males. Thus, an understanding of the excretion profile after micro-dosing and the potential detection window was gained. Metabolites that were not previously described for S-23 were found and associated with characteristic product ions, obtained by collision-induced dissociation. These additional metabolites could improve the result interpretation of S-23 findings in sport. Further administration studies are planned to examine the impact of reduced dosages. Additionally, the distribution of Phase 1 and Phase 2 metabolites was analyzed. The data obtained in this study could support the interpretation of S-23-related AAFs in doping controls in the future.

The urine samples were prepared using liquid-liquid extraction and enzymatic hydrolysis and analyzed using liquid chromatography with tandem mass spectrometry. Assay validation was performed regarding lower limit of detection, selectivity, recovery, linearity, carryover and robustness.

P 90

Single-cell investigation of the influence of aflatoxins on the lipid composition of liver cells by means of MALDI-2 mass spectrometry imaging

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Aflatoxins are mycotoxins found in molds, which are produced by the mold species *Aspergillus flavus* and *Aspergillus parasiticus*. These toxins are among the most potent toxins found in nature and have hepatotoxic properties. Since aflatoxins can be ingested by humans via foods such as corn, cereals, peanuts and spices, it is of major importance to investigate the effects of these toxins on the human organism. Immortalized human liver tumor cells are used for this purpose. Here we aim to analyze the influence of aflatoxins on the lipid composition of HepG2 cells by fluorescence microscopy and MALDI-2-MSI using single cell analysis.

In order to achieve meaningful and comparable results, the sample preparation and methods were first optimized. Isolated growing cells facilitate single cell analysis at a pixel size of 5 µm and above. A cell density of 1x104 cells has proven to be optimal for this purpose. Because HepG2 cells tend to form agglomerations, drawing up the cell solution in a pipette with the highest possible pressure acting was established as the best possible method for cell separation. For pre-MALDI staining, the dyes *CellMaskTM Green Actin Tracking Stain*, *FITC coupled Wheat Germ Agglutinin* and *Alexa FluorTM 488 Phalloidin* for cell membrane as well as *DAPI Nucleic Acid Stain* and *Hoechst 33342* for cell nuclei were tested, with all dyes proving to be suitable. For single cell analysis, fluorescence images of isolated cells were segmented with a convolutional neutral network by using *Cellpose*. Concentration dependent cytotoxicity of aflatoxin was assessed using established assays using on crystal violet as a dye. Based on these results, single cell MALDI-2-MSI measurements were performed at different toxin concentrations. Results of these measurements enable the analysis of effects of aflatoxin B1 (AFB1) on the lipid distribution of HepG2 cells. Acute toxication with AFB1 lead to a reduction of some lipid signals starting at 5 µM concentrations. Nevertheless, no obvious trend with increasing AFB1 concentration was observed. In addition, not all expected lipids could be detected within the cells, which is why a method optimization of the workflow is still necessary. Spatial analysis of the MSI results revealed, that aflatoxin affects cells within a cluster differently as compared to isolated cells.

The future goals of this work is to optimize the sample processing and to carry out a detailed assignment of the detected signals using MS/MS measurements. In addition, a single cell analysis will be performed by overlaying the fluorescence image with the image of the MALDI-2-MSI measurement in order to analyze the behavior of the cells as well as possible morphological and molecular changes within a cluster. Furthermore, recovered cells after a sub-lethal dose will be examined.

P 91

Rapid chromatography-free screening of stimulant drugs in human urine by DART-TQ

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Immunoassays (IA) are commonly used forensic toxicology test methods for drugs of abuse in Urine Drug Screening (UDS), generating rapid results with ease of automation. However, IA may generate false positives by cross-reactions with other ubiquitous co-analytes. Therefore, a positive IA result must be confirmed by another analytical approach, typically a chromatography-based method like LC/MS and GC/MS, which are used due to their high sensitivity, specificity, and accuracy. LC-based approaches are well established and commonly achieve sub-ng/mL detection limits, but they often rely on costly carrier gases and solvents and are limited in throughput. We developed a chromatography-free method using DART (Direct Analysis in Real Time) coupled to a triple quadrupole. An assay of four typical illicit phenylethylamine drugs (amphetamine, methamphetamine, 3,4-MDA, and 3,4-MDMA) was used as a test batch since their detection suffers from interferences in IA urine screens. The optimized DART-MS workflow achieves a throughput rate of 96 samples in 40 minutes, similar to IA but not using expensive kits and consumables. It meets the low limits of detection and RSD in urine matrices and avoids interference from matrix or co-analytes.

500 µL of certified drug-free urine and 300 µL DI water were added to each well in a 96 deep-well ToxBox® customized Stimulants Validation plate (PinPoint Testing, Little Rock, USA). The ToxBox custom drug panel contains reagent solutions A-C, a preloaded 96 well plate with selected analytes for an 8-point triplicate calibration curve, triplicate QC samples, along with sample and calibration blanks. 1 µL aliquot from each well was transferred onto a Bruker DART QuickStrip HTS-96 screen. After spots were fully dried, the prepared QuickStrip screen was loaded onto the automated XY transmission stage of a JumpShot DART source mounted to an EVOQ triple quadrupole (both Bruker) and analyzed in pulsed gas mode via MS/MS. The analysis speed is 20s/sample.

DART-MS analysis of the panel of compounds resulted in a linear correlation of $R2 > 0.99$. The Limit of Quantitation (LOQ) was 125 ng/mL for each of the four analytes, indicating that this simple chromatography-free workflow is sufficient in detecting these compounds at levels at or below the common cutoffs in urine. The performance of this workflow is as good as or better than commonly used UDS immunoassays, without the high rate of false positives, and demonstrates the utility of DART-MS in rapid quantitative drug screening for urine as a viable alternative to current UDS assays. The chromatography-free workflow is faster, more accurate, quantitative and eliminates the need for expensive and time-consuming chromatography based confirmatory tests.

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Introducing a game changer for fast and reliable forensic and clinical analysis

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Forensic analytics face increasing demands, driven by new psychoactive substances (NPS), faster response times and higher efficiency for an increased number of caseloads. This demands faster preparation and analysis time while maintaining the needed high sensitivity and selectivity. DART (Direct-Analysis-in-Real-Time) matches these requirements. Adding trapped ion mobility spectrometry (TIMS) to DART provides another dimension of separation, allowing to differentiate drugs including isomers without LC, and enabling CCS (collision cross sections) as an orthogonal criterion for the reliable identification. Publicly available libraries (NIST, MMHW etc.) can be used to generate reports within 15 seconds after sample introduction, making it highly efficient. As a result, this feature improves the workflow, reduces cycle times, and enhances screening accuracy.

A DART JumpShot source coupled to a timsTOF Pro 2 (both Bruker Daltonics) was used to run a typical drug mixture including isomers and the LC/MS Forensic Toxicology Test Mixture (Agilent) with 21 different drugs. 3 µL sample were applied onto the DART QuickStrip cards. Alternatively, a small quantity of drug powder could be placed on a glass stick and ionized directly. No additional sample preparation or chromatographic separation is needed. The DART source was operated in pulsed gas flow mode using helium at a temperature of 350°C. The timsTOF mass spectrometer acquired full scan MS spectra as well as MS/MS spectra in PASEF mode for positive ions. Processing was performed with the DataAnalysis 6.1 software (Bruker).

With DART-HRMS, accurate masses, isotopic patterns and MS/MS fragments are captured supporting the identification of the tested samples. The acquired spectra were run through an automated library search. It was possible to separate and identify the drugs of the LC/MS Forensic Toxicology Test Mixture with a single shot DART analysis. TIMS enabled the separation of isomeric pairs like morphine / norcodeine, hydromorphone / norhydrocodone and 6-acetylmorphine / naloxone with an ion mobility resolution of 100. With nearly no sample preparation and fast analysis times in the range of 15-30 sec per sample, DART-TIMS-PASEF is a valuable tool for the high throughput analysis and fast identification of isomeric drugs and their mixtures.

P 93

Integrative single-plaque MS imaging analysis reveals signature Aβ and lipid profiles in the Alzheimer's brain

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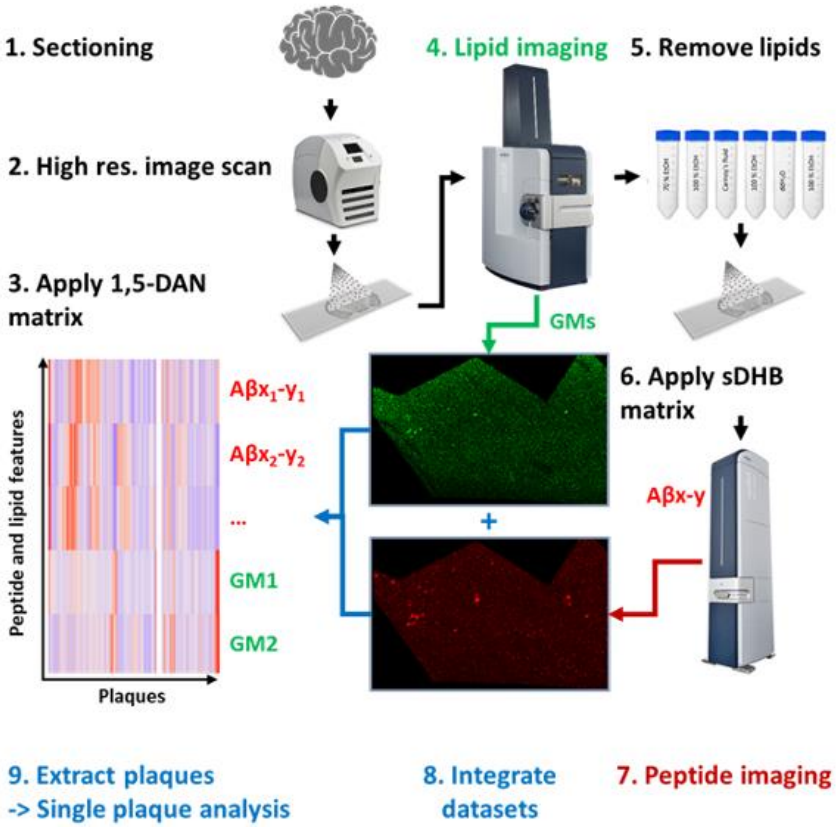
Utilizing multimodal mass spectrometry imaging (MSI) combined with machine learning techniques, this study investigates the molecular heterogeneity of amyloid-β (Aβ) plaques and associated lipid profiles in post-mortem brain samples from Alzheimer"s disease (AD) and amyloid-positive cognitively unaffected (AP-CU) individuals. Our analytical approach permitted single-plaque level MSI investigation, revealing distinct populations of amyloid plaques characterized by differential Aβ and lipid compositions. Notably, the integration of MSI data with machine learning based feature extraction enabled the identification of Aβ38 and ganglioside GM1 as molecular markers differentiating AD from AP-CU amyloid pathology. These findings suggest that heterogeneity in Aβ metabolism and lipid homeostasis, specifically gangliosides, play differential roles in the pathogenesis of AD. The application of MSI and machine learning based feature extraction in this context exemplifies a progressive analytic strategy to unravel complex biochemical phenomena, offering potential pathways for the refinement of diagnostic tools and deepening the understanding of neurodegenerative diseases from an analytical chemistry perspective.

Fig. 1



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Development of an efficient purification method for clinical samples to quantify antibiotics in sepsis therapy using LC-MS/MS

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Introduction: Sepsis is an acute, life-threatening organ dysfunction that triggers a massive inflammatory reaction throughout the body. Effective antibiotic therapy is crucial for treating this disease, but individual dosing of antibiotics for each patient and thus a sufficient antibiotic concentration in the blood is not always guaranteed. LC-MS/MS is the preferred method for therapeutic drug monitoring to solve this problem. It allows the simultaneous quantification of the beta-lactam antibiotics meropenem, piperacillin and the beta-lactamase inhibitor tazobactam used in critically ill patients. However, the complex matrix of the clinical samples presents a challenge for sample preparation due to its many different components. Therefore, methods for the effective minimization of column contamination and aging caused by incomplete separation of these matrix components are presented.

Methods: To optimize the processing of the clinical sample material, drug-free human serum was spiked with the three analytes and three different approaches were tested: The first approach involved protein precipitation by adding various organic solvents at different incubation times. The second option was to combine protein precipitation with a cartridge to remove phospholipids (Phree Phospholipid Removal, Phenomenex) and the third was to test paramagnetic nanoparticles with different surfaces. The recovery of the analytes during sample preparation was determined by adding deuterated substances (meropenem-d6 and piperacillin-d5) as internal standards. Data were analyzed using Freestyle (Thermo Scientific) and Skyline.

Results: For protein precipitation, all solvents showed effective results with acetonitrile performing best. An incubation time of 12 hours compared to 20 minutes showed no difference in the amount of protein removed. The combination of phospholipid extraction cartridges with protein precipitation slightly improved the impurity eluting at 100 % organic content, but resulted in a deterioration of the background noise and a significant impurity in the area where the analytes elute. Additionally, the quantification was also reduced. The first results of the nanoparticle clean-up showed a sufficient recovery of all three analytes and a chromatogram with minimal interfering signals.

Conclusion: The analysis of blood samples is often impeded by the accumulation of sample matrix on the column. To address this issue, protein precipitation, phospholipid removal and nanoparticle application were evaluated. Although precipitation reduced the problem, it still introduced a time-consuming rinsing step, resulting in a threefold increase in analysis time per sample. The combination of protein precipitation and a phospholipid removal cartridge can be a rapid option for efficient sample purification but needs further method development due to the additional contamination. Initial tests with nanoparticles showed promising results for improved analyte isolation.

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Investigation of elimination profiles of microdosed dehydrochloromethyltestosterone (DHCMT) and methylclostebol by GC-MS/MS

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Anabolic-androgenic steroids (AAS) belong to the most abused substance class in professional sport, primarily because of their muscle-building effect. Dehydrochloromethyltestosterone (DHCMT), known under the brand name Oral Turinabol, gained particular attention through the East German doping program and still leads to numerous adverse analytical findings in doping control programs. After long-term metabolites for DHCMT have been identified in administration studies, among others by Sobolevsky and Rodchenkov, and combined with modern and sensitive analytical techniques, minute amounts of the substance can be detected in the urine of athletes, allowing a detection window of up to several weeks. However, the distinction between intentional doping and unknowing exposure, for example via contaminated dietary supplements, can be challenging. In addition, certain metabolites of DHCMT are also observed for other structurally related anabolic steroids such as methyclostebol. If one of these metabolites is detected, an assignment to the primarily ingested substance is not feasible. Therefore, data on urinary elimination profiles are required to support the interpretation of positive findings in doping samples in the future. For this purpose, various administration studies with microdoses were conducted with 4 male participants each, simulating minimal contaminations with the oral intake of 10 µg and greater contaminations with the intake of 200 µg. In order to investigate accumulation effects after multiple doses, further studies were conducted with both concentrations with applications on 5 consecutive days. All studies were carried out with both substances, DHCMT and methylclostebol. Following administration, urine samples were collected, hydrolyzed and derivatized, and eventually analyzed using gas chromatography and tandem mass spectrometry. To achieve the utmost sensitivity, a triple quadrupole was used with several optimized multiple reaction monitoring (MRM) transitions for each analyte. Based on these data, elimination profiles were generated for both substances after the administration of different concentrations. In addition to the intact substances, these also included various known metabolites of the substances. This provided insights regarding the differences in elimination profiles based on the concentration ingested and whether DHCMT and methylclostebol show different elimination profiles, for example in terms of the ratios of produced metabolites.

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Simultaneous acquisition of molecular and elemental profiles of human body fluids by HPSEC-DAD/ICP-MS/ESI-MS for the identification of novel biomarkers for neurodegenerative diseases

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Introduction:

Neurodegenerative diseases (NDs) such as Alzheimer"s or Parkinson"s result in loss of personal independence and lead to increased morbidity and mortality. Early diagnosis and knowledge of disease progression in NDs is critical since intervention in the early stages can extend independence via symptomatic care. Available biomarkers of NDs are often protein-based but they can lack specificity and can only be used for initial diagnosis.[1] It is therefore essential to intensify and diversify the search for novel biomarkers for diagnosis, monitoring of disease progression and treatment response of NDs. Given the interplay between proteins and metals, with more than a third of proteins requiring a metal co-factor[2], some interest has turned to metal signatures in body fluids.[3] While the total metal content does not yield a statistical significant correlation to any of the studied NDs the hyphenation of high-performance liquid chromatography (HPLC) or capillary electrophoresis to inductively-coupled plasma mass spectrometry (ICP-MS) shows more promise.[3] Nevertheless, even greater diagnostic potential could be gained from simultaneous acquisition of molecular properties.

Methods:

We present a newly-developed setup that allows simultaneous acquisition of time-resolved molecular and elemental mass spectra. We employ high-performance size-exclusion chromatography (HPSEC), in parallel, coupled to a high-resolution ICP-MS (Element XR, Thermo Fisher Scientific) and a high-resolution electrospray ionization (ESI) MS (Q Exactive, Thermo Fisher Scientific). The ICP feed is introduced via a desolvating nebulizer (Aridus II). The ICP-MS is operated in medium resolution (R=4000) with platinum cones and oxygen as an additional gas. The ESI-MS is equipped with a heated ESI (HESI) source and is operated in positive ion mode at a resolution of R=140000.

Results:

Four different SEC columns were studied with respect to their separation capabilities and background signal in both MS. Subsequently, a standard mixture of known proteins was used to optimize column performance and minimize contamination across both MS. At higher volatile salt content, a post-column dilution with MQ water was introduced for the ESI-MS feed using a second HPLC pump to maintain a stable spray current in the HESI source. The optimized setup was applied to serum and cerebrospinal fluid samples with elemental chromatograms deconvoluted by non-linear least squares fitting.

Conclusions:

The optimized setup and computational approach allow for a multidimensional analysis of body fluids yielding many parameters than can be correlated. This offers a high chance to discover new biomarkers for NDs.

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P 97

Development and validation of a UPLC-MS/MS method for evaluating the intracellular concentration of the PARP inhibitor olaparib

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Olaparib is an inhibitor of poly (ADP-ribose) polymerase protein (PARP), an enzyme involved in sensing and repairing DNA damage. PARP inhibitors, including olaparib, are used in therapy for breast and ovarian cancer patients with BRCA1/2 mutation. Development of resistance to chemotherapy is a common treatment-limiting factor for patients treated with olaparib. Understanding of molecular basis underlying cancer cells resistance to chemotherapeutics remains a major challenge. Several possible mechanisms of how the resistance to a drug is aquired by cancer cells are possible, including enhanced drug efflux or elevated drug metabolism. Both mechanisms result in decreased intracellular olaparib concentrations. For this reason, we established an UPLC-MS/MS analytical method to investigate the intracellular concentration of olaparib using triple quadrupole MS/MS with positive electrospray ionisation in the selected reaction monitoring mode. The olaparib mass transition of m/z 435.2 to m/z 367.2 was used for quantification and stable isotopically labelled 2H4-olaparib standard was used for signal normalization. The calibrated olaparib concentration range was 1 (LLOQ) to 300 ng/mL (ULOQ). The method was subsequently validated according to EMA and FDA guidelines with regard to cellular determinations. Accuracy and precision were within the range of +/-20% for LLOQ and +/-15% for quality control levels at 3, 100, and 275 ng/mL. The evaluation of the olaparib uptake (intracellular levels) into ovarian cancer cell lines OC12 with and without developed olaparib resistance allows further understanding of intricacies behind the drug resistance mechanism.

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Fast LC-MS based identification of common respiratory viruses in human saliva

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Moder viral diagnostic methods are highly specific to individual viruses and, thus offer no simple way to simultaneously test for various respiratory viruses leading to time-consuming test procedures or a diagnosis purely based on symptoms and experience of the treating doctor. In the case of respiratory infections this circumstance is further aggravated by the fact that many viral respiratory infections cause very similar symptoms, especially in the early phases of infections. Shortcomings like these could be addressed using state of the art mass spectrometry (MS) analysis tools.

We aim to develop an easy-to-use MS-based tool to improve modern diagnostics and make early differentiation between dangerous pathogens and "the common cold" easy, fast and cost effective.

By optimizing reaction settings and establishing detection limits, we generated a relibale protocol for the detection of SARS-CoV-2, methods and databases were then gradually extended to detect common respiratory viruses such as influenza viruses, rhinoviruses and human respiratory syncytial viruses. In the next steps of the project LC-MS parameters will be optimized to further push the limits of detection and ensuring reliability over a wider range of pathogens.

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Identification of disulfide-adducts of small molecule thiols in human plasma resulting from exposure to malodorants

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Mercaptans, e.g. *n*-butanethiol (S^{n}Bu), are characterised by their intense and unpleasant smell, which can be perceived easily at low exposure levels and urge exposed humans to evade their stench [1]. Therefore, mercaptans may find application as malodorants in civilian, law-enforcement and military use and as incapacitants or means of crowd control [2,3]. Prolonged exposure may cause minor health effects such as headache and nausea or – in extreme cases – major health effects such as unconsciousness or coma [4].

Thiol-containing endogenous small molecules in plasma like cysteine, homocysteine, N-acetylcysteine or cysteinylglycine are expected to react with the thiol group of mercaptans to form a disulfide-adduct. These adducts may be targeted by bioanalytical methods for medical and toxicological purposes. Herein, we present a new micro-liquid chromatography triple-quadrupole tandem-mass spectrometry (µLC-MS/MS) method to document exposure to malodorants by analysis of plasma.

Human plasma was incubated *in vitro with* S^{n}Bu to simulate exposure of a person. Plasma was processed by protein precipitation and hybrid solid-phase extraction (HybridSPE) to remove proteins and phospholipids from the sample [5]. Afterwards, the disulfide-adducts were identified by µLC-MS/MS. S^{n}Bu was proven to form disulfide-adducts with endogenous small molecule thiols. In initial stability and reliability experiments, these adducts appear as robust bioanalytical markers for exposure to malodorants.

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P 100

Targeted absolute quantification of a panel of neuroinflammation protein markers in human cerebrospinal fluid

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Neuroinflammation has a prominent role in the pathogenesis and progression of neurodegenerative diseases such as Alzheimer''s Disease (AD). Robust quantitative tracking of molar concentrations of neuroinflammatory proteins in cerebrospinal fluid (CSF) can improve clinical diagnosis and treatment efficacy. We developed a sensitive and accurate multiprotein targeted mass spectrometry assay for the absolute quantification of 20 key neuroinflammation markers in human CSF. The panel was developed on the basis of the FastCAT method [1]. We selected 66 quantotypic peptides to quantify 20 recognized neuroinflammation proteins. Quantotypic peptides were distributed and concatenated into three individual chimeric protein recombinant standards and used as the stable-isotope labeled internal calibrants. Chimeric protein was spiked into the neat CSF, which was digested with trypsin and analyzed by LC-MS/MS in parallel reaction monitoring (PRM) mode.

The panel allowed us to quantify neurologically relevant apolipoproteins: ApoA1, ApoD, ApoJ and ApoE, including all of its three isoforms, a common genetic risk factor for AD. Other markers included complement proteins (C1q, C3, C4), osteopontin and alpha-1-antichymotrypsin, together with microglial and astrocyte activation markers (YKL-40, TREM2 and GFAP) as well as the two TAM receptors (Axl and Tyro3) and the MIF immunoregulatory cytokine. Altogether, the quantified marker proteins spanned 10,000-fold molar concentrations ranging from 20 pM to 200 nM and were detected in a single LC-MS/MS run with excellent repeatability (median CV ~6%) and inter-peptide agreement (typical r >0.98). We propose that this panel of molecular markers will contribute to personalized diagnostics by monitoring inflammatory signatures of CSF in the broad spectrum of neuroinflammation diseases.

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P 101

Normalization strategies of human urine in untargeted metabolomics using HILIC LC-MS/MS

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Urine is well suited matrices to screen or quantify metabolites in health and disease related research. However, urinary metabolomic profiles suffer from huge variability e.g. dietary habits. This variation needs to be controlled because it could affect the data quality of metabolomics studies. Therefore, different urine normalization strategies are existing, including pre- or post-acquisition methods to account for variability. We have collected twenty urine samples from ten healthy and ten proteinuria patients to test both normalization approaches on metabolomics data. We have analyzed urinary metabolites by hydrophilic interaction liquid chromatography coupled to mass spectrometry (HILIC LC-MS/MS) in positive and negative ionization mode. Our goal was to compare four pre-acquisition methods including dilution according to creatinine, specific gravity, UV-absorption values to a simple uniform dilution. Three post-acquisition methods were applied to the metabolite data, derived from uniform dilution including MS Total Useful Signals (MSTUS), normalization to creatinine after analysis or normalization to creatinine feature. We evaluated non- and normalized metabolomics data for variability, number of detected features, and discrimination ability. We applied supervised multivariate statistical analysis to find metabolome differences between healthy and diseased subjects and the impact of normalization techniques on discriminatory power. Preacquisition normalization (dilution factor of 5-6) resulted in a reduced number of detected features, compared to uniform-dilution (dilution factor of 4). Specific gravity normalization decreased the number of features by 29%, followed by creatinine normalization (-17%). UV-absorption did not impact the number of detected features in urine samples. We could observe a huge variability of urinary features in the twenty subjects, especially in data pre normalized to specific gravity, which is result in decreased number of features and imputed zero values. A simple uniform dilution of urine resulted in significant model between healthy and proteinuria subjects built by PLS-DA and 186 features had a VIP over 1.5, meaning their valuable contribution to the separation of both groups. All preacquisition normalization techniques resulted in a loss of the discrimination power and none of the built PLS-DA models showed significant p-values or VIP values over 1.5. In summary, a pre-acquisition normalization of urine prior metabolomics analysis is recommended to decrease variability but is accompanied by laborious work, and the supply of multiple urine aliquots for analyzing normalization values. In our work, we could not observe an essential advantage of pre-acquisition normalization methods on urine in discriminating healthy or proteinuria subjects based on metabolomic profiles.

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Development of a rapid and specific MALDI-TOF mass spectrometric assay for SARS-CoV-2 detection

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We developed a method for detecting severe acute respiratory syndrome coronavirus type 2 (SARS-CoV-2) using matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF-MS). We have already established the detection of SARS-CoV-2 nucleoprotein by MALDI-TOF-MS in a previous study [1]. As the instruments are available in many clinics, MALDI-TOF-MS can be used for rapid, sensitive and cost-efficient diagnostics as an alternative or complementary to quantitative reverse transcriptase polymerase chain reaction (RT-qPCR) assays. To obtain specific peptides that are identified in MALDI-TOF mass spectra, proteolysis with trypsin is performed prior to MS-analysis. This work aims at extending our previous method by performing an on-target tryptic digestion to further reduce sample preparation time. By using only small volumes (1-2 µl) on the MALDI-target, the time of proteolysis can be reduced to 5 minutes. The on-target digestion is performed using an incubation chamber designed for MALDI-targets. The humidity inside the chamber as well as the temperature of the sample plate holder and the cover plate can be varied. Parameters like protein concentration, protease to protein ratio, time and humidity during proteolysis were optimized to increase signal intensities of peptides derived from the SARS-CoV-2 nucleoprotein. Different sampling methods are currently being tested to develop a robust method for the detection of SARS-CoV-2 nucleoprotein in nasopharyngeal swabs, gargle solutions and saliva.

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Influence of maternal BMI and breastfeeding on infant blood amino acid and acylcarnitine profiles: Implications for later childhood obesity risk – A longitudinal cohort study

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The global rise of obesity has reached pandemic proportions, imposing a significant burden on healthcare systems due to its association with various health risks. Addressing this challenge requires urgent measures for early risk prevention. To achieve this goal, a comprehensive understanding of metabolic changes leading to obesity development in early life is crucial. Early risk factors, such as a high maternal preconception body mass index (BMI) and limited breastfeeding, have been identified as influential in affecting long-term health outcomes for offspring, including the development of metabolic disorders and overweight. However, the underlying metabolic mechanisms are not yet fully described, notably due to analytical challenges arising from low metabolite levels. This study aims to unravel these metabolic alterations, providing insights into the pathways linking early-life factors to obesity, thus paving the way for effective preventive strategies.

To thoroughly understand metabolic processes associated with obesity and nutrition, we developed and validated a sensitive multi-analyte hydrophilic liquid interaction chromatography (HILIC)-MS/MS method for infant serum analysis. Employing micro volumes of serum (25 µL), we implemented a derivatization-free sample preparation technique suitable for efficient high-throughput analysis. A total of 62 serum metabolites, including 40 amino acids and their derivatives, as well as 22 acylcarnitines, were accurately quantified within a 20-minute run using isotopically labeled standards and multiple reaction monitoring.

Utilizing the developed profiling method, we examined the energy metabolic signature, with a specific emphasis on amino acids and acylcarnitines, in 1246 serum samples obtained from three-month-old infants participating in the prospective mother-child cohort study, Programming of Enhanced Adiposity Risk in CHildhood-Early Screening (PEACHES). Our approach revealed distinct amino acid and acylcarnitine patterns in infants influenced by maternal preconception BMI and breastfeeding. Specifically, valine, 2-aminobutyric acid, and 2-aminoadipic acid emerged as predictors for a higher-than-normal BMI development at preschool age (3-5 years). Furthermore, our results underscore valine levels' causal role in infants mediating the influence of breastfeeding status on BMI development. Integrating metabolic, clinical, and demographic data obtained early in infancy enabled individual risk prediction of BMI development during pre-school age. In summary, these findings emphasize the involvement of amino acid and acylcarnitine metabolism in early BMI development and provide a basis for identifying infants at high risk for later overweight and metabolic disorders at a very early stage of life.

The authors declare no competing financial interest.

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Advancing clinical peptidome profiling in plasma with a robust high-throughput workflow

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Introduction: Endogenous peptides perform various functions and are widely used for medical diagnosis or therapy. However, their untargeted identification in plasma is still limited due to considerable challenges in sample preparation, data acquisition and data analysis. The main challenge in sample preparation is the dominance of highly abundant proteins, complicating the detection of low-abundant peptide biomarkers. Additionally, the low percentage of identified mass spectra implies the presence of non-tryptic or modified peptides overlooked by standard data analysis approaches. We aim to develop a robust and high-throughput workflow to facilitate the identification of endogenous peptides in plasma and apply it to a large clinical cohort of ischemic stroke patients, where an early diagnosis is crucial for tailored treatment.

Methods: Combining methanol-based precipitation for abundant protein removal (e.g. albumin, globulins), ultrafiltration (10kDa MWCO) for peptide separation and SCX/ C18-purification, we achieved a robust and high-throughput workflow. Resulting peptides were analyzed by Orbitrap LC-MS/MS and searched using MSFragger. Prosit-rescoring[1,2] improved the overall peptide identification. For clinical applicability, we applied the method on plasma samples from a cohort of 24 ischemic stroke patients (collected at Day 1,3 and 7 post-stroke) and 24 healthy controls, acquiring both proteome and peptidome data.

Results: We established an optimized workflow that successfully identified 6,000+ endogenous plasma peptides with high reproducibility. It revealed an overrePresentation of low-abundant circulating small proteins and peptides, such as protease inhibitors, peptide hormones, and neuropeptides, compared to standard proteomic measurement. Notably, in the ischemic stroke cohort, an overall and strong abundance increase of various peptides was observed. Among them, peptides derived from proteins associated with the coagulation cascade, such as Fibrinogen (A, B & G), Coagulation Factor IX, and Serum Amyloid A1. Mapping the peptidome to the amino acid sequence of the underlying proteins revealed a preference for individual peptide termini, suggesting stroke-specific cleavage events induced during the acute phase. Importantly, the overall full proteome remained largely unaffected, with only minor abundance changes, predominantly driven by Serum Amyloid A1 and C-Reactive Protein. This highlights the impact of peptidomic data acquisition on unravelling specific molecular changes in the context of ischemic stroke.

Conclusions: We have developed an optimized workflow for clinical peptidome profiling in plasma, applicable to large cohorts and other biofluids. Peptidomics profiling in ischemic stroke patients identified numerous enriched endogenous peptides, underscoring peptidomics' potential as a valuable biomarker resource.

References: [1]PMID: 31133760, [2]PMID: 35074002

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Evaluation of Extracellular Vesicle Isolation Methods from human biofluids for HD Biomarker Identification

Chr. Papadopoulos, M.J. Aaltonen, O. Shatnyeva, E. Kunold, T. Wild, D. Staudt, C. Kluger, C. Schaab, D. Hartung, K. Schneider, N.Olszok, F. Aqel, P. Beuzer, H. Wilkinson, J. Rosinski, J. Chen, T. F. Vogt, R. Lee

Evotec SE

Introduction: Endogenous peptides perform various functions and are widely used for medical diagnosis or therapy. However, their untargeted identification in plasma is still limited due to considerable challenges in sample preparation, data acquisition and data analysis. The main challenge in sample preparation is the dominance of highly abundant proteins, complicating the detection of low-abundant peptide biomarkers. Additionally, the low percentage of identified mass spectra implies the presence of non-tryptic or modified peptides overlooked by standard data analysis approaches. We aim to develop a robust and high-throughput workflow to facilitate the identification of endogenous peptides in plasma and apply it to a large clinical cohort of ischemic stroke patients, where an early diagnosis is crucial for tailored treatment.

Methods: Combining methanol-based precipitation for abundant protein removal (e.g. albumin, globulins), ultrafiltration (10kDa MWCO) for peptide separation and SCX/ C18-purification, we achieved a robust and high-throughput workflow. Resulting peptides were analyzed by Orbitrap LC-MS/MS and searched using MSFragger. Prosit-rescoring[1,2] improved the overall peptide identification. For clinical applicability, we applied the method on plasma samples from a cohort of 24 ischemic stroke patients (collected at Day 1,3 and 7 post-stroke) and 24 healthy controls, acquiring both proteome and peptidome data.

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References: [1]PMID: 31133760, [2]PMID: 35074002

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Fast and selective extraction of data from DIA-MS raw files for subsequent analysis by a machine learning system for virus quantification

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Although there exist numerous liquid chromatography-mass spectrometry (LC-MS) studies in which viral proteins and virus-related modulations of biomarkers have been successfully identified and quantified in patient samples, a workflow for LC-MS-based diagnostics of viral infections has not been established yet. A promising approach for comprehensive virus diagnostics using LC-MS is data-independent acquisition (DIA), which aims to measure the entire proteome of a sample in an untargeted manner. The proteins are quantified by calculating so-called label-free quantification values (LFQs) from normalized intensities. In the case of viruses, this not only makes it possible to detect a pathogen based on a large number of different peptides, but also to take into account the changes in the host proteome caused by the infection.

Conventional analysis approaches for DIA-MS data iterate over an entire raw data file in a largely unbiased manner. As a result, the analysis of many individual samples is associated with redundant processing and thus low overall time efficiency. This currently hinders the use of DIA-MS for efficient clinical applications. We try to overcome this limitation by analyzing only small parts of the raw data sets in a supervised machine learning system.

First, we performed DIA-MS measurements of several dilution series of inactivated SARS-CoV-2 particles in saliva using a timsTOF Pro. We analyzed the acquired raw data sets with DIA-NN to obtain protein identification and LFQ reports for each sample and to generate an experimental spectral library. The reports showed that there is a clear linear relationship between the LFQ value of a protein and the virus concentration in plaque-forming units for all detected SARS-CoV-2 proteins. For further analysis, in addition to the detected viral proteins, we selected all abundant salivary proteins that were stable in their LFQ values.

Based on the experimental spectral library, we determined the retention time and m/z selection windows containing intensity information for the selected proteins. We then extracted the corresponding parts of the raw datasets using a modified version of the open-source Python package AlphaTims. Our goal is to use the extracted data after minimal treatment as input values for a machine learning system to predict virus concentrations in saliva samples. We are currently investigating if a multiple regression system is already sufficient for this task or whether more advanced technologies such as neural networks are required.

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Prosit_PTM: One model to predict them all

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Introduction

Over the past few years, the scientific community has increasingly focused on predicting the intensities of peptide fragments. However, most studies in this area have overlooked post-translational modifications (PTMs), often limiting the supported PTM repertoire to a small set. In this study, we introduce a novel Prosit model capable of predicting indexed retention time and fragment ion intensities for both singly and multiply modified peptides. Notably, our research demonstrates the ability to forecast the fragmentation behavior of peptides containing PTMs that were not included in the training dataset.

Data

To train our model, we employed peptides from ProteomeTools, encompassing 60 distinct peptide classes that span unlabeled, labeled, tryptic, non-tryptic peptides, as well as approximately 15 PTMs. We systematically explored various methods of encoding PTMs to ensure generalizability to unseen modifications. Multiple features were considered, including atom counts for moieties gained and lost, as well as the atom closest to the modification site. Additionally, we investigated the replacement of sequence encoding with a SMILES rePresentation of the complete peptide. We devised a novel data augmentation strategy, where we encode artificial amino acid substitutions as if they were PTMs.

Results

Our new Prosit model exhibited remarkable agreement between predicted and experimentally acquired spectra for observed modifications, achieving normalized spectral angles (SAs) of 0.9 for HCD and 0.86 for CID. Additionally, we evaluated the model's performance on 15 modifications not present in the training data, yielding average prediction performance scores of 0.87 for HCD and 0.84 for CID.

For the indexed retention time model, we trained it on over 1.5 million modified peptides and tested it on more than 400,000 modified peptides. The model exhibited high precision in predicting retention time for observed modifications, with a $\Delta t95\%$ of approximately 105 seconds. Furthermore, we tested the model on 15 unseen modifications using over 4,000 peptides, resulting in a modest drop in performance to a $\Delta t95\%$ of around 150 seconds.

We also explored using percoltor to PTM localization. When comparing our model to MaxQuant in localizing challenging cases where modifications could shift by only one amino acid, our model achieved an improved localization accuracy, increasing from 73% to 92%.

By incorporating PTMs, the generated predicted spectral libraries become more comprehensive. Integration of these libraries into a data-independent acquisition (DIA) search engine should lead to more accurate identifications. Furthermore, incorporating these predictions into a rescoring pipeline will enhance identification rates and improve PTM localization accuracy.

Conclusion

Incorporating predictions of MS/MS spectra for rescoring modified peptides, increases the number of identified and improves PTM site localization accuracy.

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Simulations of arrival time distributions of reacting systems in Ion mobility spectrometr

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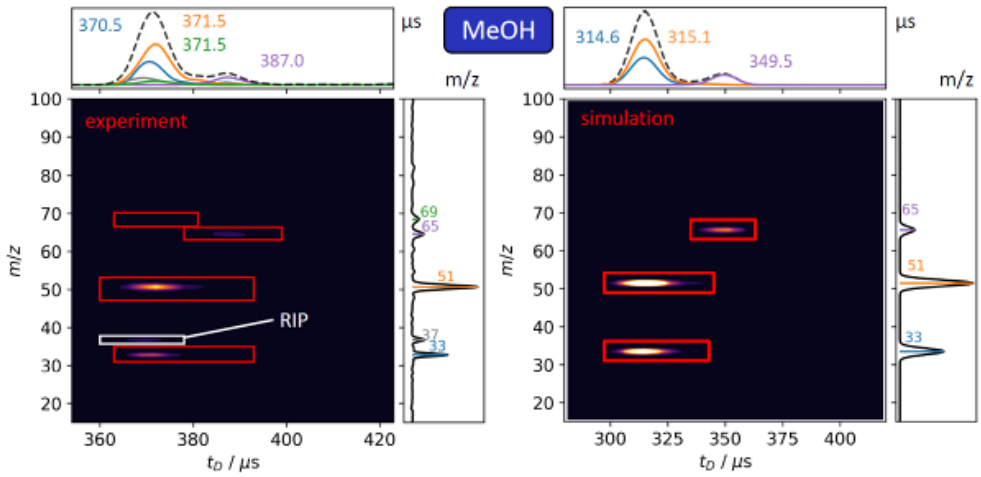
Introduction: Ion mobility spectrometry (IMS) has emerged as a powerful separation technique that is readily coupled to mass spectrometry (MS). Its applications range from trace gas analysis to biochemical samples. The advantages over other separation techniques include its fast measurement times and the possibility to obtain structural information from the observed arrival time distribution (ATD). Specifically, the arrival time can be converted into a collision cross section (CCS), allowing for the identification and characterization of even very similar structural isomers. Moreover, the shape and width of the arrival time distribution can also be used to harness information on the chemical dynamics of the analyte, e.g., its conformational flexibility, its tendency towards clustering or its stability toward fragmentation. However, except a few examples, only qualitative conclusions are usually drawn from the shape of the ATD. This is especially true for the most widely used techniques in the field, namely trapped IMS (TIMS) and travelling wave IMS (TWIMS).

Methods: In order to allow for a more quantitative correlation between ion dynamics and the observed ATD we developed the simulation framework TAURUS for the direct simulation of the ATD from first principles. Being able to simulate the ATD for a given system and instrument allows the direct comparison to experimental observations and to draw important conclusions about the dynamic nature of the system. In TAURUS, we combine density functional theory (ORCA) and CCS (MobCal-MPI 2.0) calculations with home written python code to obtain data for reaction dynamics (reaction rate constants) simulations as well as field-dependent ion mobility and ion diffusion data for ion trajectory calculations. The combination of reaction and collision dynamics in a Monte-Carlo based approach allows for a direct computation of the ATD of a dynamic system at any field strength.

Results: Here we present TAURUS and validate it against experimental data obtained from the High Kinetic Energy IMS (HiKE-IMS), a drift tube IMS that allows for variation of the electric field strength up to 120 Td, coupled to a time-of-flight MS. Specifically, we investigate three different scenarios: 1) non-reactive systems showing the effect of peak broadening through field-dependent ion diffusion, 2) peak broadening through dynamic clustering with background moisture, and 3) non-typical peak shapes caused by ion fragmentation. The simulations show good agreement with experiment and reveal how ion dynamics can affect the ATD.

Conclusions: TAURUS proofs to be a useful tool to study ion dynamics in IMS instruments and how these are affected by higher field strengths. These results have important implications on TIMS and TWIMS instruments, where high field strengths and long transit times allow ion dynamic effects to unfold.

Fig. 1


370.5
371.5
371.5
387.0
µs
MeOH
m/z
experiment
RIP
69
65
51
37
33
m/z
t_D / µs
314.6
315.1
349.5
µs
m/z
simulation
65
51
33
m/z
t_D / µs

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Combining SIMSI-Transfer and Prosit leads to a synergistic increase in identifications

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Introduction

While missing values are rare in single-batch TMT experiments, combining multiple TMT experiments causes a rapid increase in missing values. To address this challenge, SIMSI-Transfer was published in 2022, which employs MS2 spectrum clustering to group spectra based on similarity and transfer peptide identifications across TMT batches. SIMSI-Transfer only transfers identifications across batches and is therefore unable to generate new identifications. Conversely, the fragment spectrum intensity prediction tool Prosit is capable of generating new IDs compared to an ordinary MaxQuant run, making it an ideal complement to SIMSI-Transfer. Combining SIMSI-Transfer with Prosit proved to effectively benefit from the distinct methodologies of the two tools, and we are therefore aiming to find the best workflow combining the two to minimize missing values in multi-batch TMT experiments.

Methods

To evaluate and compare the performance of SIMSI-Transfer and Prosit rescoring, we conducted individual runs of both tools on an identical dataset of three TMT batches. We analyzed where the two tools agree on the same peptide-spectrum matches (PSMs), where they disagree, and where only one of them managed to identify a spectrum. Additionally, we assessed the combined workflow of SIMSI-Transfer and Prosit by applying Prosit to re-score a 100% FDR MaxQuant search and subsequently running SIMSI-Transfer, evaluating changes and increases in the identifications gained.

Results

Applying SIMSI-Transfer and Prosit resulted in similar gains, yielding an overall PSM increase of 13% with SIMSI-Transfer and 15% with Prosit. Notably, the difference in identified spectra indicates an orthogonal identification, with only 20% of the spectra identified by SIMSI-transfer also being identified by Prosit and vice versa. Among the spectra identified by both tools, approximately 75% were identified as the same peptide, while the remaining 25% showed strong indications of chimerism, with each tool identifying one of the two peptides. In cases where the identification varied between the two tools, the top 5 MaxQuant peptides did not include the peptide identified by SIMSI-Transfer 95% of the time, making it impossible for Prosit to generate the same identification. Running SIMSI-Transfer after Prosit enhanced the PSMs gained by using each tool individually; although 6% of the exclusive IDs from SIMSI-Transfer were lost, the combination with Prosit yielded a 20% increase in PSMs acquired by SIMSI-Transfer.

Conclusion

The integration of SIMSI-Transfer and Prosit in a multi-batch TMT experiment demonstrated their synergistic potential to mitigate missing values. While SIMSI-Transfer excelled in transferring identifications across batches, Prosit's ability to generate new identifications complemented it effectively, resulting in a combined workflow that significantly increased peptide-spectrum matches and addressed challenges inherent in multi-batch TMT experiments.

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Assessing the reactivity of N- hydroxysuccinimide ester-based cross-linkers towards hydroxy-containing residues by retention time prediction of 'dead-end' products

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Cross-linking mass spectrometry (XL-MS) is as a robust technique to probe the 3d-structure of proteins and disentangle the dynamics of protein-protein interactions across diverse levels of sample complexity [1]. Currently, the most widely used cross-linkers possess two N-hydroxysuccinimide (NHS) esters as reactive warheads. They covalently bridge primary amines of lysine (K) and N-terminus of proteins (Fig. 1, upper panel). Nevertheless, these cross-linkers have also been demonstrated to react with hydroxy-containing amino acids like serine (S), threonine (T) and tyrosine (Y), albeit at a lower rate [2]. The reactivity ratios between lysine-lysine (K-K) and lysine-hydroxy-containing residues (K-STY) remain unknown for NHS-ester-based cross-linkers [3, 4]. The ambiguity of relevant fragment ion spectra and the challenges associated to the false discovery rate control further complicate the matter.

We developed a new method that relies on the retention time (RT) of abundant "dead-end" cross-links (also known as mono-links or type 0 cross-links) to predict the RT boundaries of elusive STY-links (Fig. 1, lower panel). This enables assessing the depletion of STY-links in both the solution and gas phase, influencing the apparent reactivity of NHS ester-based cross-linkers [5, 6]. Furthermore, we streamlined data processing by leveraging information-lacking "dead-end" peptides for the prediction and validation of cross-links. This strategy greatly simplifies the validation of cross-link spectra, using a workflow that comprises data clustering of peptide retention times to calibrate regression models of cross-link RTs [7, 8].

Fig. 1: Graphical rePresentation of NHS-ester crosslinker reactivity and our new, innovative workflow

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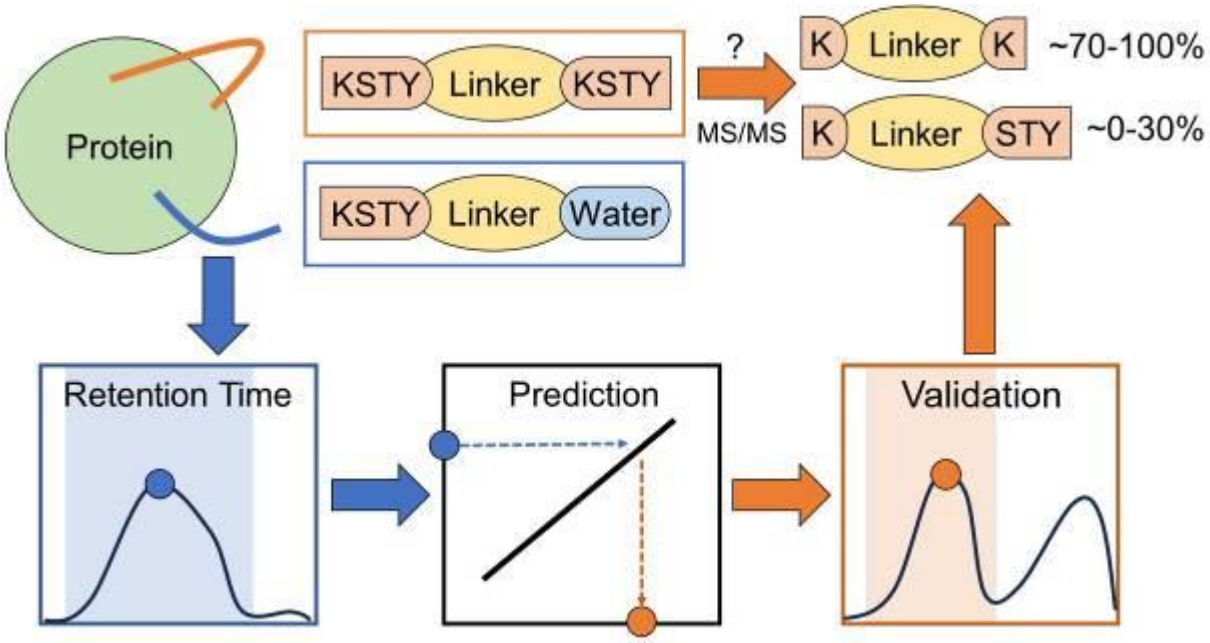
Fig. 1


Protein
KSTY
Linker
KSTY
KSTY
Linker
Water
?
MS/MS
K
Linker
K
~70-100%
K
Linker
STY
~0-30%
Retention Time
Prediction
Validation

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Koina: Bringing machine learning to the community

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Introduction

Recent developments in machine learning (ML) and deep learning (DL) have proven immense potential for applications in proteomics, such as optimized spectral library generation or improving peptide identification. Although new ML/DL models for various applications and peptide properties are frequently published, only few are readily available and thus adopted by the community. Two models that are widely used are Prosit and MS2PIP. We attribute this success not only to the excellent prediction accuracy, but also to the existence of user-friendly web servers. To make full use of state-of-the-art models, ease of use and reusability in existing packages is required. To facilitate this, we developed the model hosting platform Koina, a generic, open-source, and online-accessible prediction service that enables ML/DL model usage in any pipeline by serving ML/DL models.

Methods

Koina is based on Nvidia Triton, a state-of-the-art open-source model hosting platform that can be used to retrieve predictions from ML models trained with a variety of frameworks. Source code for Koina is available at github.com/wilhelm-lab/koina. A Docker image is provided to streamline deployment of new servers.

Results

We developed Koina with a focus on incorporating the FAIR4RS principles, to improve findability, accessibility, interoperability, and reusability of ML models. To improve model interoperability, we defined a common interface based on the ProForma PSI. Requests can be sent via either HTTP for ease of use or gRPC when performance is critical, ensuring optimal accessibility for various use cases. A rich documentation is available summarizing model capabilities and limitations, improving model findability, as well as ensuring that models can be reused effectively within the limitations of their development. Multiple public servers already hosted Koina, whereas koina.proteomicsdb.org serves as the central access point and distributes load across all connected public instances, resulting in an estimated <100 ms latency across Europe.

We believe that the open-source and federated design will lead to widespread community adoption, by developers and users alike, potentially resulting in Koina becoming a common standard for publishing ML models in proteomics and thus facilitating model adoption and supporting model performance comparison. To illustrate its flexibility, we already provide access to popular ML models, such as AlphaPept (PyTorch), DeepLC (TensorFlow), MS2PIP (XGBoost), and Prosit (TensorFlow). We invite all developers of ML models to improve the impact of their work by adding their model to Koina.

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Mass spectrometric non-target screening – Interdisciplinary solutions and data handling

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Mass spectrometric non-target screening (NTS), non-target analysis and untargeted screening are synonyms for the fact that mass spectrometric driven ion extraction, fragmentation and fragment detection is leading to new insights into very complex samples (sometimes without former molecular knowledge of the analyst, performing the analysis).

In our days, the analytical performances are challenging in using various chromatographic separation techniques, (ion mobility) as well as (tandem)mass spectrometric detection. On the other hand, these systems are mostly quality-assured and robust useable. The subsequent data evaluation and data interpretation steps are ongoing research topics to realize flexible but reproducible data handling. Workflow steps like the so-called peak picking, componentization, alignment and others [1] as well as their combination are under development but on a good way.

However, new challenges in NTS come up, if one wants to answer specific questions in different disciplines which is mostly very application specific and needs adjusted but robust holistic solutions. However, there are solutions available that can be used in interdisciplinary context.

This lecture will present and include the similarities and differences of NTS concepts and workflows in different disciplines. Such NTS data handling pipelines are presented for real life solutions in environmental analysis (with the identification of emerging compounds like PFAS in water samples [2]), in plant metabolomics samples (with the molecular reflection of metabolomics pathways [3]), in food analysis (with focus on authenticity check), in process analysis (with salty waters) and in clinical analysis (with a view on disease biomarkers).

The state-of the art reflection gives direct consequences for the future of NTS in research and in practice; both will be discussed.

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P 123

Novel, real-time acquisition logic prevents fragmentation of uninformative precursors in metabolomics studies

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Question: Confident metabolite annotation requires high-quality fragmentation spectra. Analyzing complex, biological samples using short gradients, co-elution of sodium and other adducts of lower intensity can lead to competing fragmentation events. Resulting MS/MS spectra are often not as suitable for library-based annotation as those originating from protonated precursors. Thus, it is desirable to select exclusively protonated precursors to generate informative fragmentation spectra for metabolite annotation.

Methods: A Bruker timsTOF Pro 2 system was equipped with a novel, real-time capable precursor selection logic in the APIo interface of timsControl software. The real-time de-adducting (RTDA) algorithm, implemented in Python code and embedded in a dedicated timsControl acquisition method, prevents precursors representing unwanted ions, specifically sodium or potassium adducts or singly charged dimers, from being fragmented.

The method was applied to the analysis of an ethanolic tobacco leaf extract. Nine technical replicates of these samples were measured with a default PASEF method for small molecules and the new RTDA method, respectively. Data processing was performed using the T-ReX® 4D workflow including 4D feature finding, de-adduction, de-isotoping and retention time alignment functionality provided by the Bruker MetaboScape® software.

Results: The RTDA method significantly reduced the number of sodium and potassium adducts being fragmented and improved the ratio of protonated precursors while maintaining a consistent number of detected features. Odds ratios of obtaining an informative MS/MS spectrum were confirmed to be strongly in favor of protonated precursors based on the comparison of the conventional vs. the RTDA method.

Conclusions: The Real-time de-adduction method effectively prevents fragmentation of uninformative precursors while maintaining a high number of detected features in a metabolomics analysis of a complex plant extract. The RTDA plugin facilitates higher rates of informative MS/MS spectra and supports deeper analysis of compounds found in the biological sample.

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CHIMERYS: Spectrum-centric analysis of data-dependent (DDA) and data-independent acquisition (DIA) data

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Background

DIA spectra are inherently chimeric as they contain fragment ions from multiple peptide precursors, some of which will be shared. Peptide-centric approaches typically remove these shared fragment ions based on correlations with precursor ions over retention time. We have previously developed a technique to deconvolute chimeric DDA spectra that does not rely on such correlations and can distribute shared fragment ion intensities between precursors instead of discarding them. This approach extends to any chimeric MS2 spectrum, ensuring consistent DDA and DIA data analysis.

Methods

CHIMERYS™ is a spectrum-centric, library-free algorithm for analyzing chimeric MS2 spectra. It deconvolutes by distributing shared fragment ion intensity, quantifying peptide contributions over retention time, and controlling false discovery rates using Mokapot. Post-processing utilizes Thermo Scientific™ Proteome Discoverer™ 3.1 software.

Results

We compared our algorithm to DIA-NN and Spectronaut® on public DIA data. CHIMERYS™ matches state-of-the-art software in identifying peptide precursors. Entrapment experiments demonstrate well-calibrated error rates, and shared peptides show high quantitative similarity across software solutions. While our algorithm misses low-abundance peptides, it exhibits comparable quantitative precision in recovering known ratios from mixed-species samples. Achieving low coefficients of variation even with independent searches, CHIMERYS™ enables head-to-head DDA and DIA measurements of the same sample with the same algorithm.

Conclusion

A novel, spectrum-centric algorithm for the analysis of DIA data and head-to-head comparison of DDA and DIA data.

P 125

CHIMERYS server: deploying the power of the cloud in your basement

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Background

CHIMERYS is a cloud-based search engine on Amazon Web Services (AWS) with a client interface for Proteome Discoverer software (PD). While AWS offers scalability and accessibility, transferring large data or user preference for in-house data storage may limit cloud service practicality. To address this, we adapted CHIMERYS as an on-premises solution for single servers and HPC environments, combining the best of both worlds.

Methods

The current service utilizes containerized, distributed applications orchestrated by Kubernetes (K8s) and communicates through high-performance protocols. To simplify deployment on single servers, we replaced cloud-specific services with compatible interfaces and transitioned to K3s, a lightweight Kubernetes distribution. We also developed a CLI for direct job submission to CHIMERYS for streamlined integration into custom workflows.

Results

The goal was to port CHIMERYS to a local server featuring off-the-shelf hardware, capable of processing of 16 raw files in parallel. To simplify server deployment, we utilized pre-built virtual components and automated hardware setup with PXE-boot-based auto deployment. The server ran a minimal Debian with a GUI for KVM hypervisor or Ubuntu on bare metal. The GPU was directly passed through to an Ubuntu virtual machine in a virtualized setup. CHIMERYS' microservice architecture was modified to fit this deployment, trimming unnecessary cloud functionalities, and using compatible interfaces like Argo workflows for processing queues. RAM limitations were addressed by reducing Mokapot's[2] RAM footprint and streaming data from fast NVMe drives. We developed a CLI and browser-based GUI for easy integration of the local CHIMERYS service into customizable workflows. Benchmarking showed the server can analyze 16 HeLa 1h Q-Exactive HFX raw files in 30 minutes, making large-scale experiments feasible on locally deployed infrastructure.

Conclusions

Porting the cloud-native CHIMERYS software to a local server allows performant processing of data as a full-featured computation package, avoiding bandwidth bottlenecks from large file uploads while maximizing data privacy.

Figure Legends

Figure 1. Left: Example hardware configuration for server using off-the-shelf components. Right: Automated deployment routines based on Hypervisor allowing hands-free system setup.

Figure 2. Resource consumption of Percolator[1] v3.0.5, Mokapot[2] v0.74 and Mokapot including performance fixes on a large input file containing 8.4 M PSMs.

Figure 3. Benchmarking of local server using a 1 h Q Exactive HFX HeLa raw file. Runs are submitted in 30 second intervals. Left: Timelines of 16 concurrently running CHIMERYS jobs. Right: RAM consumption of running jobs.

References

[1] The et al 2016 DOI: 10.1007/s13361-016-1460-7

[2] Fondrie, Nobel 2021 DOI: 10.1021/acs.jproteome.0c01010

Fig. 1

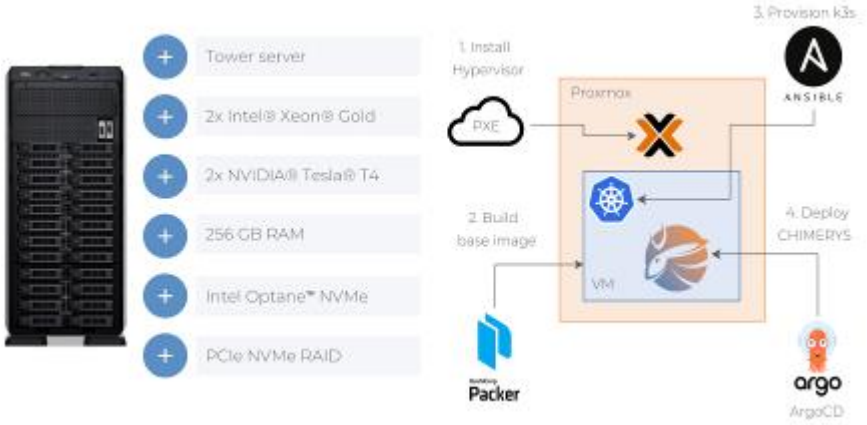


Fig. 2

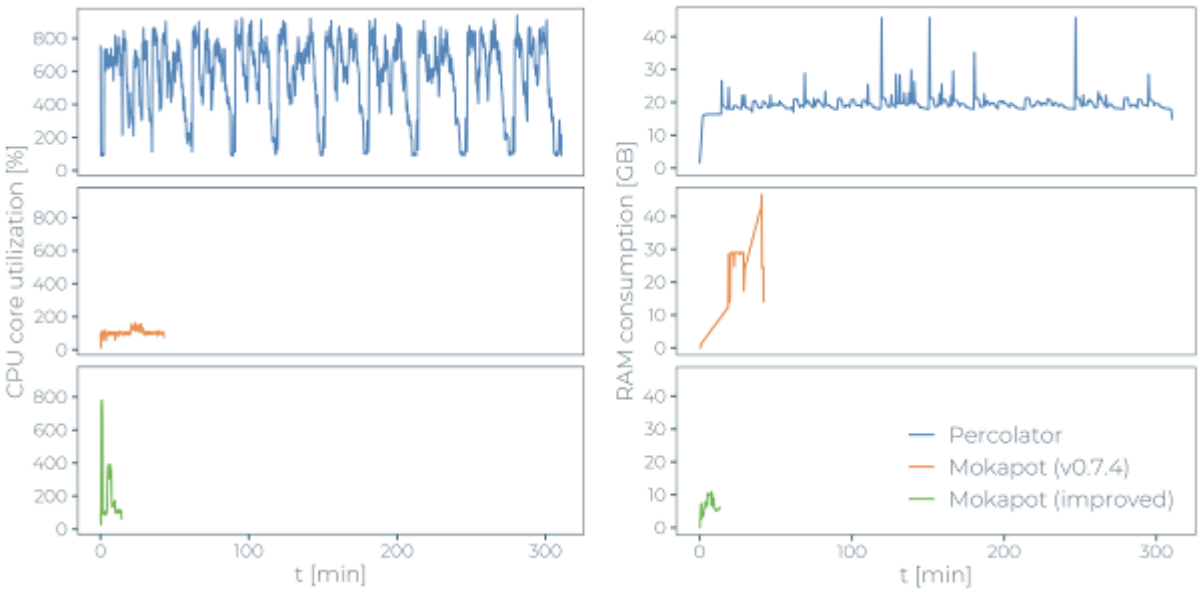
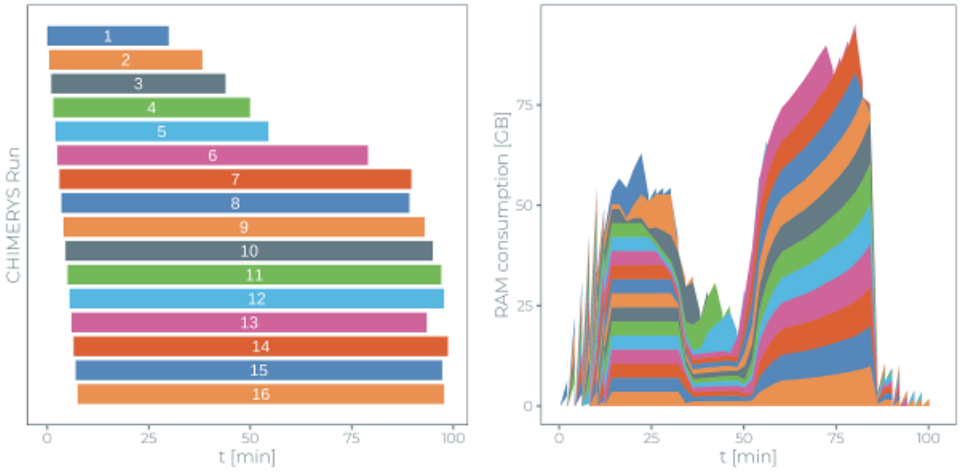


Fig. 3


CHIMERYS Run
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
0
25
50
75
100
t [min]
RAM consumption [GB]
75
50
25
0
0
25
50
75
100
t [min]

P 126

ProteomicsDB: Connecting proteomes across species

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ProteomicsDB, initially established in 2014 as a protein-centric in-memory database for exploring the first draft of the human proteome [1], has evolved into a multi-omics and multi-organism resource for life sciences research encompassing more than 198 projects totaling over 29k LC-MS/MS experiments. Over the years, it has grown substantially, incorporating data from various omics sources and expanding its functionalities, such as integrating diverse omics data, introducing new APIs for systematic data access, enhancing user interfaces for better data visualization, and integrating deep-neural-network Prosit for peptides" fragments prediction, ultimately enabling reevaluation of stored search engine results with state-of-the-art methods to boost data utilization and identification rate in proteomics studies [2]. This continuous development and integration aligns with its commitment to improving data accessibility, interoperability, and usability while broadening its content to encompass various human biology experiments and supporting additional organisms [3]. Most recently, we integrated the proteome of Triticum aestivum (common wheat plant) acquired using a timsTOF Pro machine into ProteomicsDB, expanding the list of file formats and vendors supported by the database.

ProteomicsDB is set to integrate two groundbreaking projects into its framework: a proteomics map of the bacterial kingdom, encompassing data from approximately 400 bacterial strains, and "The Proteomes that Feed the World" project, aiming to provide comprehensive tissue-resolved data for almost 100 most important plants for human nutrition. These projects are characterized by their suitability for cross-species analysis, with the former extending ProteomicsDB's reach to the bacterial kingdom and the latter, shedding light for the first time on many important plant species" proteomes. ProteomicsDB will soon provide statistics and visualizations for cross-species analysis which empowers the community with unprecedented information. These integrations will significantly enhance ProteomicsDB's capabilities, fostering comprehensive insights into diverse species and facilitating cross-species comparisons for in-depth scientific exploration.

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[2] Samaras et al., 2020 (doi.org/10.1093/nar/gkz974)

[3] Lautenbacher et al., 2022 (doi.org/10.1093/nar/gkab1026)

P 127

Unraveling the unknown glycation space using a mass spectral library from *in vitro* model systems

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The non-enzymatic reaction between amino acids and reducing sugars, also known as the Maillard reaction (MR), is the primary source of free glycation products *in vivo* and *in vitro*. The limited number of MS/MS records for glycation products (GPs) in public libraries hinders the annotation and investigation of non-enzymatic glycation. To address this issue, we present a mass spectral library containing experimental MS/MS spectra of diverse GPs from model systems. Based on the conceptional reaction processes and structural characteristics of products, we classified GPs into common glycation products and modified amino acids. A workflow for annotating GPs was established based on the structural and fragmentation patterns of each GP type. The final spectra library contains synthetic model system information, retention time, precursor m/z, MS/MS, and annotations. As a proof-of-concept, we demonstrated the usage of the library for screening glycation products in human plasma and urine. The amino acids with C6H10O5 modification, fructosylation from Amadori rearrangement, were the most found GPs. With the help of the model system, we confirmed the existence of C6H10O5-Valine in human plasma by matching both retention time, MS1, and MS/MS without reference standards. In summary, our GP library can serve as an online resource to quickly screen possible glycation products in an untargeted metabolomic workflow, further with the model system as a practical synthesis method to confirm the identity.

P 129

Deciphering molecular signatures in spinal cord pathology: MALDI mass spectrometry imaging of multiple sclerosis

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Question: Multiple sclerosis (MS) is a complex autoimmune disease that is characterized by demyelination and neuroinflammation in the central nervous system (CNS)[1]. Over time, the cumulative damage to the myelin and axons leads to neurological dysfunction, manifesting as a diverse array of symptoms, including fatigue, impaired mobility, sensory disturbances, and cognitive deficits[1]. Understanding the molecular alterations associated with MS pathology is crucial for unraveling its complex pathogenesis.

Methods: In this study, we employed matrix-assisted laser desorption ionization mass spectrometry imaging (MALDI MSI) to compare the spatial distribution of molecular species in spinal cord tissues obtained from post-mortem MS patients (n=5) and non-MS control (n=5) samples. All MS patient samples have areas of complete white matter demyelination and varying degrees of incomplete demyelination or unaffected white matter. We focused on detecting lipids and small metabolites in negative ion mode, as sulfatide depletion[2] is a well-described feature of MS. Areas of depleted sulfatides, as well as Luxol Fast Blue staining of adjacent tissue sections annotated by physicians familiar with MS pathology, were then used to accurately localize MS lesions.

Results: Our study revealed significant alterations in the distribution of multiple lipid classes and free fatty acids that correlate with the degree of demyelination. Similarly, we observed changes in the inositol phosphate pathway.[3]

Conclusion: Through this method, we achieved precise spatial characterization of spinal cord demyelination in human MS samples, offering valuable insights that contribute to an enhanced understanding of the disease.

Figure 1: A) Luxol fast blue staining of Control (1) and MS (2) spinal cord section and MALDI MSI ion images of adjacent sections depicting the special distribution of C24:1 Sulfatide (3,4) and FA 16:0 (5,6).

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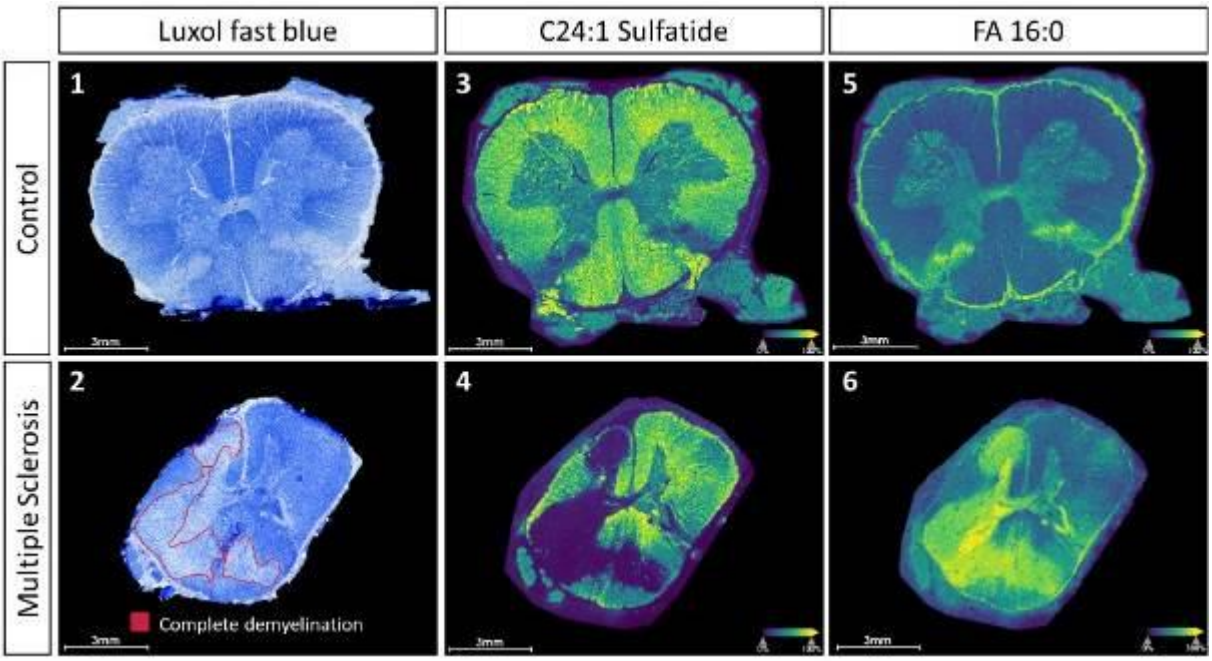
Fig. 1


Luxol fast blue
C24:1 Sulfatide
FA 16:0
Control
Multiple Sclerosis
1
2
3
4
5
6
Complete demyelination
3mm

P 130

Visualizing neutrophils in head and neck cancer tissue using t-MALDI-2-MS imaging combined with fluorescence microscopy

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In cancer, not only the number of systemic neutrophils is expanding, but they are also recruited into the tumour tissue. In fact, a high frequency of tumour-infiltrating neutrophils (termed tumour-associated neutrophils, TAN) is strongly associated with poor outcome in the majority of cancer types, including head neck cancer (HNC). Neutrophils are known to promote carcinogenesis and tumour disease progression in different ways [1]. To visualize neutrophil infiltration into cancerous tissues and their interaction with the tumour microenvironment, classically fluorescence microscopy is used. Although theoretically forming a perfectly comprehensive imaging modality, for example to determine the lipid make-up of antibody-stained cells, due to limitations in spatial resolution (mostly >10 µm) the use of MS imaging (MSI) has so far been mostly limited to distinguish between different tumour regions, only. A new approach to obtain the desired single-cell resolution is transmission-mode (t-) MALDI-2-MSI [2]. However, increasing the spatial resolution to the micrometer pixel size comes with substantial challenges concerning sample preparation protocols and instrumental setups.

Here we optimized these protocols towards the correlative imaging analysis of human HNC tissues. Specifically, we identified individual TANs using immunofluorescence (IF) microscopy with neutrophil-specific antibodies (CD66b) and standard histological staining and recorded the lipidomic profiles in the so identified different tumour regions from adjacent tissue sections.

The t-MALDI-2-MSI experiments were conducted with a Q Exactive plus Orbitrap mass spectrometer (Thermo Fisher Scientific) equipped with a modified dual-ion funnel MALDI/ESI injector (Spectroglyph) at a pixel size of 2 µm [2]. 2,5-DHAP matrix was applied using a sublimation chamber build in-house. IF microscopy with a specific CD66b antibody was performed on consecutive tissue sections.

t-MALDI-2-MSI identified a series of lactosylceramides (LacCer) as neutrophil markers at a resolution enabling the differentiation of individual immune cells. The simultaneously registered lipid profiles moreover enabled to distinguish between different tumour regions. LacCer plays a pivotal role in signalling pathways and as a precursor in the biosynthesis of other glycosphingolipids. With further optimization on multiplexed methods, our approach could in the future help to better understand the molecular changes that occur in different TAN subsets that have been linked to pro-tumour functions.

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P 131

Improvements in a multi-reflecting Tof mass spectrometer to enhance mass spectrometry imaging specificity

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Mass spectrometry imaging (MSI) provides a method to visualise the spatial distribution of molecules across a surface, with MALDI and DESI being the most common techniques used.

The complexity of MSI data can make accurate identification of analytes a challenge due to overlapping species. Therefore, it is desirable to acquire data at the highest mass spectral resolving power possible to reduce interference. However, MSI experiments that use high spatial resolution often require long acquisition times and can be further limited by the scan speed of the mass spectrometer. Here we employ a method of extending the flight path to enable multiple passes of an MRT analyser that increases the resolving power by >50% enabling >300,000 FWHM at acquisition rates >10Hz.

Experimental and Results

A healthy wild-type mouse kidney and brain were axially cryo-sectioned onto standard glass slides at a thickness of 16 µm.

The sections were analysed by DESI MS on a multi-reflecting Q-ToF mass spectrometer in duplicate employing either a single pass of the analyser or by a novel modification of the analyser to allow a method of extending the flight path to multiple passes of the analyser. Acquisitions were performed in full scan MS for positive and negative ionisation modes.

Data were processed using imaging software to generate segmentation and analyte identification for the different regions of the kidney and brain.

Initial experiments for both tissue types were carried out by DESI imaging in positive ion mode. Mainly glycerophospholipids and triglycerides were detected with a mix of potential cation types such as H+, Na+ and K+, increasing the complexity in lipid identification. Further interrogation of the single pass multi-reflecting Tof brain section data revealed a broad peak at m/z 865.6, the image for the spatial location of this ion shows a higher abundance in the hippocampal region of the brain and a similar abundance in the rest of the brain, no identification of the analyte could be performed. When a consecutive section was analysed with the mass spectrometer modified to allow a longer flight path through a second pass of the multi-reflecting Tof analyser, resulting in a resolving power >300,000 FWHM, two ions at m/z 865.6 were partially resolved. These ions were putatively identified as a potassium adduct of an 13C isotope of HexCer(42:2;O3) and a sodium adduct of SM(42:3;O4), the image of the former ion shows strong localisation to the hippocampus, whereas the latter has a similar abundance across the entire brain. A composite image of the two ions showed identical localisation as obtained for the unresolved ion observed in the data acquired with a single-pass of the analyser.

The use of high resolving power (>300,000 FWHM) to improve DESI MS imaging specificity has been presented. Ions that were previously unresolved can now be separated resulting in increased imaging specificity and excellent mass accuracy (ppb level).

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Ultra-High lateral resolution AP-SMALDI mass spectrometry imaging for organoid analysis

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Introduction

Organoids, 3D models of organs cultivated in laboratories from stem cells, have emerged as pivotal tools in scientific research. They offer unique research tools for, e.g., disease modeling, drug discovery, and personalized medicine. The evolution of mass spectrometry imaging (MSI) technology has significantly enhanced analytical capabilities, especially in studying biological samples like tissues and single cells. In this study, we present a methodology and results that provide the metabolite spatial distribution in targeted organoids using an atmospheric-pressure scanning microprobe MALDI (AP-SMALDI) MSI setup with an oversampling-free lateral resolution of 1.5 µm at a mass resolution of more than 100,000.

Methods

Human cerebral and mouse intestinal organoids were generated and comprehensively characterized. The intestinal organoids were treated with the proinflammatory cytokine tumor necrosis factor-α (TNF-α), with a concentration of 100 ng/ml and incubation time of over 3 days, compared to untreated ones. Several sample preparation steps were optimized for the cryosectioning of organoids, maintaining the samples" integrity morphologically. The sections were thawed in a desiccator and subsequently covered with 2,5-dihydroxybenzoic acid (DHB, positive-ion mode) or 9-aminoacridine (9AA, negative-ion mode) matrix. MSI measurements were performed using an ultra-high lateral resolution prototype AP-SMALDI ion source (TransMIT GmbH, Giessen, Germany), coupled with a Thermo Scientific Q Exactive mass spectrometer (Thermo Fisher (Bremen) GmbH, Germany). Maintaining the morphological integrity of samples in their native form after sectioning, we determined that fixing the organoids in 4% paraformaldehyde (PFA) and embedding them in 15% gelatin before flash freezing and cryo-sectioning is optimal. Next, samples were frozen either by suspension in liquid-nitrogen-cooled isopropanol or by brief exposure to -20°C in a cryotome.

Results

Employing a mass accuracy better than ±2 ppm, we identified and observed over a hundred lipid species in both positive- and negative-ion modes. Lipid class distribution exhibited variations across organoid sections. Notably, in TNF-α-treated intestinal organoids, ceramide (Cer) species were abundant in the lumen in positive-ion mode while being undetectable in untreated samples, indicating that TNF-α triggers a metabolic pathway leading to elevated endogenous ceramide levels, potentially activating the intrinsic apoptosis pathway. In brain organoids, phosphatidyl-ethanolamine (PE) and phosphatidyl-choline (PC) species were evenly distributed, while several phosphatidyl-serine (PS) species were localized only around the organoid's outer edges. These high-resolution lipid profiles of distinct organoids underscore the potential of our AP-SMALDI methodology for advancing research on the localization of cell-type-specific metabolites and their functions within organoid environments

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In-depth characterization of single cells by high-resolution MALDI-MSI

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Recent developments in MALDI-MSI instrumentation allows for lateral resolutions on a sub-cellular scale. Using this technology, we thrive to demonstrate its applicability in the characterization and differentiation of single cells. For this purpose, we are using a commercially available setup, further strengthen the idea of future routine analysis in research and clinical settings. However, this stresses the importance of pre-analysis steps, such as sample treatment and matrix application. In earlier studies, our group was able to differentiate cell lines and stimulation conditions for microglia cells.[1] To expand the methodological concept, we will present first results of two ongoing research projects: a) Differentiation of cell cycle states, b) cancer-induced changes of the lipidome.

For all projects, cell cultures were fixed in paraformaldehyde (PFA) and stored in phosphate-buffered saline (PBS) before analyses. To ensure a homogenous matrix coverage with sufficiently small crystals, while preserving cell morphology, 2,6-Dihydroxyacetophenon (DHAP) applied by sublimation in vacuum was chosen as matrix for AP-MALDI experiments. Measurements were performed on a Q Exactive HF (Thermo Fisher Scientific, Bremen) equipped with an AP-SMALDI5AF ion source (TransMIT GmbH, Gießen). Mass spectrometric images and data extraction for further analysis was done using Mirion (TransMIT) and LipostarMSI (Molecular Horizon, Bettona). Annotation of lipid species was performed using metaspace.

Modification of cells with fluorescence ubiquitination cell cycle indicators (FUCCI) reporters allow the determination of cell cycle states by unique fluorescence readouts. In our studies we analysed FUCCI4 modified U2-OS cells derived from human osteosarcoma to establish a microscopy and AP-MALDI MSI-based workflow for visualization of lipids and assignment to corresponding cell cycle states. Therefore, cell cultures were first imaged by high-resolution fluorescence microscopy using a confocal laser scanning microscope (Leica TCSSP8, Wetzlar) and optical microscopy (Keyence VHX-7000N, Osaka). After optimization of sample preparation steps, we were able to collect AP-MALDI-MSI data at lateral resolutions ≤ 5 µm allowing for the identification of single cells with preserved morphology. Co-localization of U2-OS(FUCCI) cells in defined cell states and corresponding MS imaging results are used as a prototype system to correlate lipidomic cell profile with phenotypic cell states.

In order to demonstrate the capabilities of the methodology, human melanoma cells (WM3734) with and without upregulation of H3K4 demethylase KDM5B/JARID1B are used to investigate the lipidomic heterogeneity of these cells in cell culture and correlate these lipidomic changes to KDM5B expression level changes.

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P 134

MALDI imaging of tryptic peptides: Improvements in spatial resolution and reproducibility in mammalian tissue and first applications in plant science

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MALDI imaging can visualize proteins directly under *in situ* conditions. However, the mass range is typically limited to <50 kDa and identification of proteins is challenging. Here, on-tissue tryptic digestion can extend the range of proteins which can be analyzed by MALDI imaging. Conditions for sample preparation need to be balanced carefully in order avoid migration of tryptic peptides and yet provide efficient digestion[1]. In addition, most of the available protocols have only been optimized for mammalian tissue. This work is focused on optimizations to achieve high spatial resolution MS images of tryptic peptides in mouse brain. Moreover, this workflow has been adapted and optimized to visualize tryptic peptides in plant tissue[2].

12 µm mouse brain sections were used. Lipids and salts were removed from tissue by several washing steps. Digestion was performed by spraying trypsin sequentially on tissue (10 cycles) using a custom-built sprayer. Between each spray, tissue sections were placed inside a custom-built digestion chamber at 40 °C and incubated for 2 h after the last spray. DHB matrix was applied using a pneumatic sprayer. In case of plant material, samples were soaked in ethylene glycol, embedded in gelatin & sectioned at 25 µm. After washing, sections were first sprayed with water followed by trypsin using an HTX-M5 sprayer (HTX Technologies, Chapel Hill, USA). MALDI imaging was performed using an AP−SMALDI 5 AF (TransMIT GmbH, Giessen, Germany) ion source attached to a Q Exactive HF (Thermo Scientific, Bremen, Germany).

For mammalian tissue, trypsin application was optimized to achieve high spatial resolution data of tryptic peptides. Several parameters were investigated including the cycle number, flow rate, volume & the nebulization of the trypsin solution. Our results revealed that the spatial distribution of tryptic peptides and the digestion efficiency are strongly influenced by the trypsin spray. Our optimized workflow allows reproducible imaging of tryptic peptides in mouse brain at 10 µm pixel size. In addition, high mass resolution and -accuracy allows protein identification in combination with LC-MS/MS data. Through increased digestion efficiency, we could confirm several peptide sequences by on-tissue MALDI-MS/MS. In a parallel effort we adapted our workflow to plant samples. The sectioning and trypsin application were adapted and resulted in MALDI images of tryptic peptides at 65 µm pixel size. These results constitute the first MALDI imaging of tryptic peptides with accurate mass for plant tissue. The application of this workflow to the toxic protein abrin-a in crab"s eye vine seed will be discussed.

In conclusion, our results show reproducible 10 µm imaging in mouse brain and the first MALDI images of tryptic peptides with accurate mass in plant tissue.

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P 135

Wound healing in *Bacillus subtilis* biofilms monitored by MALDI-2 mass spectrometry imaging

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Bacterial biofilms are multicellular communities containing various differentiated cell types and featuring a noticeable division of labor and resilience to external stresses. Comparisons between biofilms and potentially more intricate eukaryotic tissues are becoming more frequent. A prominent example to this end is the "wound healing" process in colonies of the Gram-positive soil bacterium *Bacillus subtilis* after physical damage. However, the regrowth of *B. subtilis* biofilms is almost unexplored. Here we used a Bruker timsTOF fleX MALDI-2 mass spectrometer to amend these data on a molecular level.

Following the protocol developed by Brockmann et al. [1] biofilms of wild type *B. subtilis* strain NCIB3610 were grown on mixed cellulose ester membranes, here placed on minimal medium (MSgg) agar at 30 °C for 24 h to promote biofilm formation. After this first incubation step, about half of the colonies were damaged and macroscopically removed from the membrane using a spatula, so that there was no residue left in this area visible to the eye. The injured biofilms were subsequently incubated for 24, 48 or 72 hours to allow for the healing of the wound. Following a brief fixation and deactivation step with 15% formaldehyde for 30 min at RT, samples were coated with 2,5-dihydroxyacetophenone MALDI matrix using a SonoTek ultrasonic sprayer and a mixture of acetonitrile, methanol, trifluoroacetic acid and water as spraying solution. Positive ion mode MSI data were acquired at a pixel size of 50 µm.

In the *m/z* range ≥1000 next to various isoforms of surfactin and plipastatin lipopeptides, the signaling epipeptide EPE [2] was detected by MALDI-(2-)MSI. In the *m/z* range below 1000, next to structural phospholipids, a substantial number of 224 ion species (by mass presumably lipids) and low mass analytes are detected. A wide variety of molecular distributions occurred, with some analytes being expressed more strongly in the area of the wound while others were found more enhanced in the area of within the intact biofilm. At the time of this submission, a detailed annotation and identification was still pending Despite of this shortcoming, our MALDI-2 imaging data notably reveal a reproducible spatial variation of various compounds in the regrowing area. In line with the visual film morphology, the MSI data reflect that the healing process is essentially complete after 72 h. Or in other words, molecular profiles within the healed and non-damaged areas of the biofilm resume to exhibit the same radial symmetry across the films.

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P 136

MS, tMS2, ddMS2 approaches for signal identification with an AP-SMALDI Orbitrap MS instrument

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Introduction
Coupling Atmospheric-pressure scanning microprobe MALDI (AP-SMALDI) MSI with Orbitrap-based mass analysis provides high resolution in mass and space. Although this technology allows for detailed insights into biochemical and physiological processes in tissues and cells, it lacks tools for direct and automated structural identification. Here, we present a workflow to acquire full-MS data in parallel with MS2 data and image data processing for visualising the spatial distribution of precursors and diagnostic fragments.

Method
Sample slicing, matrix spraying and the principle of data acquisition for MS Imaging are described elsewhere[1]. Experiments were performed using an AP-SMALDI5 AF ion source (TransMIT GmbH, Giessen, Germany) coupled with a Thermo Scientific Orbitrap Exploris 480 mass spectrometer (Thermo Fisher Scientific (Bremen) GmbH, Germany). The Method Editor of Thermo Scientific Orbitrap Exploris Series Instrument Control Software was used to acquire MS1 data (R = 240,000 FWHM at *m/z* 200) and targeted or data dependent MS2 data (R = 30,000 FWHM at *m/z* 200) in consecutive scan events with a sampling area of 20×20 µm2. Ion images of MS1 and MS2 data were generated using MIRION and METASPACE.

Preliminary Data
Depending on the experimental design, MS1 and MS2 data can be acquired from the same or from neighboring pixels (in x direction). Resulting from material ablation from a given pixel by the MS1 precursor analysis, MS2 acquisition from the fresh, neighboring pixel typically was found to be more appropriate in terms of analytical sensitivity but comes with a reduction in lateral resolution. Depending on the analytical question to address, MS2 data were acquired from either the same or the neighboring pixel.In a **targeted** approach, we investigated tissue sections of drug-treated *Fasciola hepatica* parasites in positive-ion mode.[2] Imatinib was detected by MS1 and confirmed by MS2 fragmentation data. The MS1 drug signal was highly intense in vitellarium and intestine of the parasite; its distribution was superimposable with resulting MS2 fragment ion data.In a **data dependent** approach, MS2 was employed to image and identify small metabolites in coronal mouse brain tissue sections. METASPACE was used to annotate signals based on accurate mass. For signal confirmation, individual MS2 spectra were further evaluated, to identify specific fragment ions. Utilizing the high sensitivity of our AP-SMALDI MSI setup, we identified metabolites related to various biological pathways (e.g. glycolysis, TCA cycle) in a spatially resolved manner across different brain regions.

Novel aspect
AP-SMALDI5 AF + Orbitrap MS: MS and MS2 imaging within one experiment and subsequent data processing, enabling MS/MS2 overlays.

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P 137

From Spectra to Molecules in Spatial Metabolomics: Data processing and metabolite annotation of AP-SMALDI-Orbitrap data using METASPACE

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Coupling atmospheric-pressure scanning microprobe matrix-assisted laser desorption/ionization mass spectrometry imaging (AP-SMALDI MSI), using an AP-SMALDI5 AF ion source (TransMIT GmbH), with Orbitrap technology (Orbitrap Exploris 480, Thermo Fisher Scientific), enables spatial metabolomics with an unmatched combination of high lateral resolution and high mass resolution while providing high mass accuracy and high sensitivity. A key challenge in MSI in general is metabolite identification, which we achieved using an automated spatial metabolomics pipeline via FDR-controlled metabolite-annotation and data processing in METASPACE. 1

Here, we analyzed mouse kidney tissue, which is known to have high metabolic activity and a complex morphology, making it an ideal target for spatial metabolomics with high resolution in mass and space. Spatially resolved metabolic networks were confidently identified and images of respective molecules were generated automatically. Annotation confidence was ensured using an automated screening for multiple co-localized adducts of the same analyte, metabolite-signal match (MSM) scoring or target-decoy false-discovery-rate (FDR) estimation, yielding an unprecedented reliability for MSI data annotation.

METASPACE provided up to 98 (48, 25) individual metabolite annotations employing an FDR of 20% (10%, 5%) for the mass range of *m/z* 110-440, utilizing the CoreMetabolome database. Metabolites were grouped based on the similarity of their spatial distribution via a co-localization filter in METASPACE. It was found that for example glucose, citric acid, glutamate, aspartate, or glycerol-phosphate were presented at increased levels in the medulla of the kidney, whereas malate, pyroglutamate, phosphoglycerate or maleic acid were intensely detected in the cortex of the kidney. This indicates a spatial organization of biochemical pathways, even within commonly known metabolic cycles.

Additionally, and for the first time, it was possible to demonstrate the usability of METASPACE metabolite identification in stable-isotope-labelled spatial metabolomics. To this end, mice were infused with [U-13C] glucose to visualize tissue-dependent metabolic activity in kidney tissue.2

To evaluate the performance of the FDR control mechanism, we tested various mass-resolution settings and found that high mass resolution and high mass accuracy are essential for an automated and confident annotation of metabolites in MSI data.

In summary, this study showcases the seamless integration of FDR-controlled spatially resolved metabolite annotation by METASPACE using the AP-SMALDI-Orbitrap MS imaging workflow with various experimental settings for confident metabolite annotation. All data was made publicly available to expand the pool of community-based knowledge accumulated via METASPACE towards a comprehensive metabolite atlas.

1 Palmer et al, Nature Methods 2017

2 Wang et al, Nature Methods 2022

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imzML Analyzer – an open source Python application for the rapid MSI data quality evaluation: Application to the ecotoxicological model organism *Dreissena bugensis*

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Aim: In this work, the imzML Analyzer, a tool for the fast assessment of MSI data quality is introduced. To demonstrate its potential use in the data analysis pipeline, a key feature of the tool is applied to a high mass resolution MALDI-MSI measurement of the ecotoxicological model organism *Dreissena bugensis.*

Methods: The imzML Analyzer was written in Python 3.7 and comes as a standalone application with a GUI. For parsing the imzML file, methods of the imzML Parser1 are employed. The tool will be made available as open source software. *D. bugensis* mussels were collected from the River Regnitz near Pettstadt, kept in an aquarium & were fed daily with green algae. For cryosectioning, the shell was removed. The mussel was sectioned at -20°C using a Leica CM3050S (Wetzlar, Germany). The measurement was conducted on a Q Exactive HF (Thermo Fisher, Bremen, Germany) coupled to an AP-SMALDI-AF5 ion source (TransMIT, Gießen, Germany).

Results & Discussion: In mass spectrometry the reliable identification of molecules is only possible if both a high mass resolution & a high mass accuracy are provided. A straightforward approach to check the mass measurement accuracy (MMA) is to use reference masses of known sum formula. In case of MALDI-MSI measurements matrix clusters can be used.2 This work proposes a combination of MMA plots & RMSE values of reference matrix clusters such as DHB to quickly evaluate the MMA of the mass analyzer in proximity to the analytes. In the next step, the MMA of the analyte is evaluated to assess the analyte annotation. In addition, the importance of checking the MMA of annotated analytes is exemplified. To choose the appropriate bin size for ion image generation, tools such as MMA plots & MMA images were combined. In conclusion it is shown, that an inappropriate bin size can lead to misinterpretations regarding the spatial distribution and identification of analytes.

To perform the proposed checks in a rapid & easy manner, the imzML Analyzer was developed. To perform the evaluation in an automated fashion, an imzML Analyzer key feature constitutes the fast generation of Quality Reports from a list of preselected peaks. The report displays important quality parameters such as MMA plots, ion images & RMSE values to quickly assess the mass accuracy of each selected peak. This provides a rapid overview over the data quality. These considerations & imzML Analyzer features are discussed on the example of a MALDI-MSI measurement of lipids at 35 µm pixel size showing histological *D. bugensis* structures such as the colon, stomach, gills & the digestive gland.

Conclusion: Accurate statistical analysis of MSI data strongly depends on a sufficient mass accuracy & high-quality ion images. We introduced the imzML Analyzer, an open source Python based tool to quickly evaluate the quality of MSI data in the imzML format.

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2) Treu, A. & Römpp, A. 2021 https://doi.org/10.1002/rcm.9110

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MALDI-2 Imaging enhances the detection sensitivity of anthocyanins in wheat seed samples

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Introduction

The characteristic hue of pigmented kernels is related to the presence of anthocyanins and carotenoids. These compounds occur to a minor extend in conventional wheat varieties but are more consistently present in the pigmented forms. The interest in these bioactive compounds is emerging because these phytochemicals have antioxidant activity and thus implications in human health. Several studies indicate that anthocyanins may have a potential effect in reducing the incidence of cardiovascular disease, cancer, hyperlipidemias, and other chronic diseases. In planta, these specialized metabolites are involved in multiple roles, like protection from frost, excessive UV exposure, osmotic disbalance and drought. Anthocyanins are known to occur in the outer layers of the grain kernels. Traditionally, distribution investigations were performed by analyzing the content in pearling fractions. Here, we developed a method using MALDI Imaging to localize anthocyanins in wheat seeds.

Methods

Wheat seeds originated from blue (AF Oxana) and purple (Rosso) varieties grown in sandy loam soil in Italy were embedded in 10% gelatine and frozen. Cross sections of 14 µm thickness were cut on a cryostat and mounted on IntelliSlides (Bruker Daltonics) using a tape method. Samples were covered with 2,5-Dihydroxyacetophenon (DHAP) or 2,5-Dihydroxybenzoic acid (DHB) matrix for positive mode MALDI Imaging using a M3+ sprayer (HTX Technologies). MALDI Imaging data were acquired on a timsTOF fleX instrument (Bruker Daltonics) equipped with a MALDI-2 post-ionization laser. Spectra were recorded with 20 µm pixel size in the mass range 150-1500 Da with trapped ion mobility spectrometry (TIMS). Data were analyzed with the software SCiLS Lab 2023b. CCS-values of the anthocyanins were predicted with MetaboScape 2023b.

Results

In accordance with previous results [1], the six most common anthocyanins were putatively detected in both wheat seed varieties, namely, cyanidin, dephinidin, malvidin, pelargonidin, peonidin and petunidin. MALDI-2 post-ionization enhanced the detection sensitivity from five out of the six anthocyanins 2.5 to 8-fold and allowed visualizing the distribution of the phytochemicals at high spatial resolution. The anthocyanins were localized in the outer layer of the kernel cross sections. Corroborating previous findings [1], higher anthocyanin contents were detected in the red wheat variety Rosso. With the help of the ion mobility dimension of the mass spectrometer, TIMS, isobaric and isomeric compounds were separated. This was crucial for extracting individual ion images enabling to visualize true distributions of the putative anthocyanins. In case of isobaric overlays, the CCS-prediction feature of MetaboScape helped assigning individual ion signals to the anthocyanins of interest confirming the ion annotation.

Reference

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P 140

Enabling integrative spatial multi-omics data analysis via dedicated data structures and interactive, web-based visualizations

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Introduction:

Rapid advances in spatial omics technologies have enabled detailed molecular analyses in tissue context, leading to parallel requirements for the integration of multiple spatial omics readouts for a holistic multi-omics view. Due to its unique ability to target classes of analytes such as drugs and metabolites, mass spectrometry imaging (MSI) can bring insightful integrated information when combined with other spatial omics. However, spatial multi-omics data analysis is a daunting bioinformatics task as the data is typically acquired on serial sections, at different spatial resolutions, in a variety of different data formats and requires expertise across multiple domains to obtain the best results from each individual technology. In this work, we present a suite of bioinformatics tools dedicated to enabling spatial multi-omics data analysis.

Method:

The software enables efficient integration and joint visualization of readouts from different assays, as well as multiple common downstream multimodal analysis pipelines. Our approach towards enabling spatial multi-omics data analysis consists of multiple steps. First, we provide accurate, non-rigid image registration to translate between serial sections' coordinate systems. Second, several data integration approaches are provided to create an integrated data structure across assays of different spatial resolutions. This data structure acts as a foundation for downstream data analysis and overall joint visualization. Finally, we provide examples of downstream analysis, including spatial correlation between analytes across assays, multi-omics tissue segmentation and differential expression analysis.

Results:

The broad applicability of our approach and the associated data visualizations are illustrated via two different spatial multi-omics use cases which combine MSI with complementary assays.

Several Imaging Mass Cytometry (IMC; Hyperion, Standard Biotools) readouts on a single lymph node section were stitched together and compared with a MALDI-IHC (rapifleX, Bruker) measurement from a serial section. These IMC and MALDI-IHC measurements were used to investigate similarities and differences between both assays for readouts of equivalent antibodies.

Molecular and cellular alterations associated with amyloid plaques in a mouse model of Alzheimer"s Disease were examined via a trimodal approach consisting of stable isotope-encoded MS peptide imaging (rapifleX), spatial transcriptomics (GeoMx DSP, NanoString) and immunofluorescence microscopy.

For both use-cases, a web-based, interactive spatial multi-omics data visualization was constructed. The use-cases illustrate that the integrated spatial multi-omics data structures at the heart of our approach support a wide range of downstream data analyses.

Novel Aspect:

We provide a framework to integrate MSI data with a variety of popular spatial omics assays. This work addresses the need for spatial multi-omics bioinformatics solutions.

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Dermal penetration efficacy of nickel determined in *ex vivo* porcine ear skin model

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Nickel allergy, one of the most prevalent forms of contact dermatitis, is characterized by an adverse immune response to nickel ions and complexes. Despite its widespread prevalence, the mechanisms underlying nickel-induced allergic reactions remain incompletely understood. This research project aims to bridge this knowledge gap by investigating the penetration of nickel ions and their complexes into the skin, as well as their impact on the metabolomic composition of the skin and associated immune response.

An *ex vivo* porcine ear skin model was employed for this study. Skin was subjected to 1, 2.5, 5, and 10% nickel sulfate hexahydrate solutions. After a penetration time of 3 h, nickel-treated and control tissues from 3 biological replicates were fresh frozen, and cross-sections were stored for further analysis. The metabolomic alterations induced by nickel treatment were analyzed by atmospheric-pressure scanning microprobe matrix-assisted laser desorption/ionization mass spectrometry imaging using an AP-SMALDI5 AF ion source (TransMIT GmbH, Giessen) coupled to Q Exactive HF (Thermo Fisher Scientific, Bremen). After metabolomic MS imaging analysis, tissue sections were stained with dimethylglyoxime (DMG) to visualize penetration of nickel ions into the skin. The pink complex formed by nickel and DMG was analyzed in tissue using light microscopy. After the nickel-DMG readout, the same tissue section was subjected to H&E staining for morphological characterization of the tissue layers.

Nickel was found to be predominantly located in the stratum corneum after 3 hours penetration time. The semiquantitative performance of the developed DMG staining method is shown for different nickel concentrations and biological replicates. Metabolomic analysis revealed changes not only in the stratum corneum but also in the living epidermis. Specifically, nickel treatment resulted in a concentration-dependent downregulation of arginine and histidine in stratum corneum and ceramides and sphingomyelins in epidermis compared to control skin, suggesting a perturbation in skin barrier function and potential exacerbating effects on nickel-induced skin reactions. In addition, several potential tissue-related nickel adducts/complexes were detected in the skin top layers. The findings from this study will provide valuable insights into the molecular mechanisms underlying nickel-induced allergic reactions, contributing to a better understanding of the skin's response to metallic allergens. This knowledge can potentially lead to the development of improved diagnostic tools and personalized treatment strategies for nickel-allergic individuals.

Acknowledgement

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Conflicts of interest

BS is a consultant of TransMIT GmbH, Giessen

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Coupling AP-SMALDI MS imaging technology with an orbitrap exploris MX mass detector

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INTRODUCTION

Atmospheric-pressure scanning microprobe matrix-assisted laser desorption/ionization mass spectrometry imaging (AP-SMALDI MSI) combined with Thermo Scientific Orbitrap technology provides unmatched performance regarding mass resolution, spatial resolution and sensitivity. Recent studies demonstrated the spatially resolved analysis for numerous classes of biomolecules using Thermo Scientific Orbitrap Exploris 120, 240 and 480 mass spectrometers. Here, we describe a novel instrumental MS imaging setup by coupling the AP-SMALDI5 AF ion source with a Thermo Scientific Orbitrap Exploris MX mass detector. We demonstrate AP-SMALDI-Orbitrap MSI performance regarding spatial resolution, mass resolution, mass accuracy and sensitivity while showcasing technical reproducibility and experimental flexibility, ranging from high-speed to accurate 3D-surface MSI analysis.

METHOD

Tissue samples were sectioned at –20 °C using a cryotome and thaw-mounted on regular glass slides. MALDI matrices were applied using a SMALDIPrep device (TransMIT GmbH, Giessen, Germany). All measurements were performed using an AP-SMALDI5 AF ion source (TransMIT GmbH, Giessen, Germany) coupled to a Thermo Scientific Orbitrap Exploris MX mass detector (Thermo Fisher Scientific (Bremen) GmbH, Germany). The mass spectrometer was operated using Xcalibur software in positive- and negative-ion mode. The mass range, mass resolution and pixel size were set in accordance with the selected MS imaging measurement mode. Internal lock mass correction by EASY-IC enabled mass accuracies below 1 ppm. Ion image generation and metabolite annotation was performed using METASPACE.

PRELIMINARY DATA

The performance of our new AP-SMALDI MSI instrumental setup is demonstrated using different experimental designs. First, we conducted high-speed MSI analysis of Schistosoma-infected hamster liver to generate a spatial lipidomic profile for the complete tissue section within a few hours of measurement time. Based on this overview, we determined tissue areas that exhibited specific lipidomic changes, to guide high-resolution MSI of these regions for the adjacent tissue section. Mapping the metabolome with its spatial information in tissues and cells represents a major challenge for MSI techniques due to low intrinsic ionization efficiencies. Thus, we demonstrate high sensitivity for spatial metabolomics applications by ablating the full pixel combined with pixel-wise autofocusing to ensure optimal and identical ionization conditions for the whole MSI experiment. Applied to mouse kidney, we spatially resolved hundreds of small metabolites. In conclusion, our novel MSI setup based on the Orbitrap Exploris MX mass detector delivers fit-for-purpose, proven AP-SMALDI hybrid-Orbitrap performance and experimental versatility for spatial multi-omics applications in life sciences.

Novel aspect

Extending the AP-SMALDI hybrid-Orbitrap technology successfully to Orbitrap Exploris MX mass detector.

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Combining single-cell MALDI mass spectrometry imaging with fluorescence microscopy to investigate differentiation in THP-1 derived macrophages

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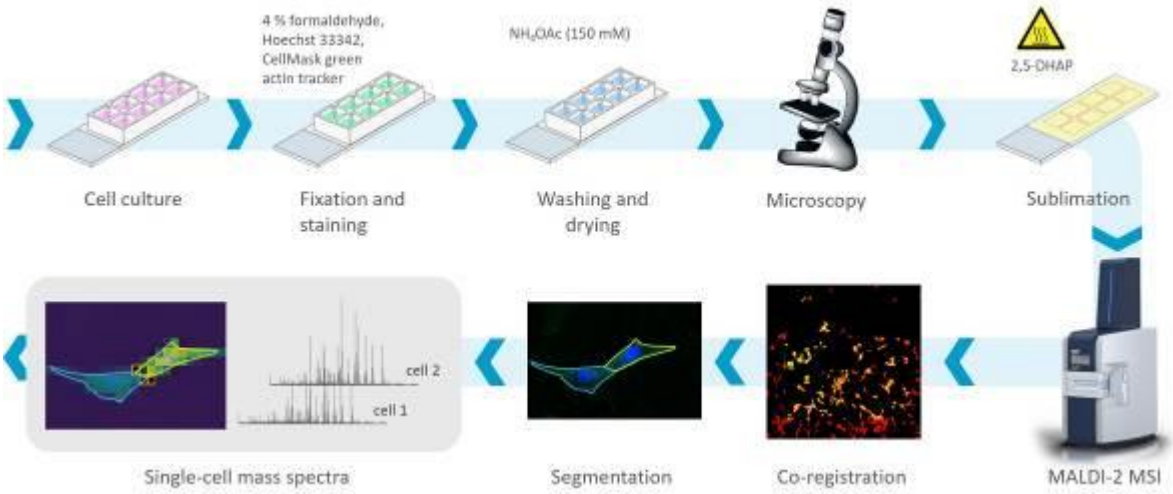
THP-1 is a human monocytic leukemic cell line that is commonly used as a model to mimic human monocyte-derived macrophages (hMDM). By stimulating THP-1 cells with phorbol 12-myristate 13-acetate (PMA) they differentiate into naive macrophages (M0) within 3 days. The aim of our research is to investigate changes in the molecular profile during this differentiation on a single-cell level using MALDI-2 mass spectrometry imaging (MALDI-2 MSI) while combining the molecular data with a morphometric analysis acquired by fluorescence microscopy.

For our experimental setup, the cells were cultured in 8-well chamber slides, differentiated using PMA for 24 h, 48 h and 72 h and fixed with 4% formaldehyde. The membranes and nuclei were stained with CellMask Green Actin Tracking Stain and Hoechst33342 respectively and Lipid droplets were stained using LipidSpot 610. Afterwards the cells were washed with ammonium acetate. Then, fluorescence and bright field images of the samples were captured using a Zeiss LSM980 Airyscan-2 microscope. Subsequently, the slides were sublimated with 2,5-DHAP and measured with a timsTOF fleX MALDI-2 at 5 µm pixel size. Average mass spectra for each individual cell (single-cell spectra) were then generated using a python based in-house built Fluorescence Integrated Single-Cell Analysis Script (FISCAS). In a first step, the script co-registers microscopy and MALDI images. The aligned region is then used to perform a cell segmentation based on the fluorescence microscopy using Cellpose. In the final step, generated cell masks are overlayed with MALDI-MSI results and used to compile single-cell mass spectra of their corresponding cells. Data evaluation is performed using MetaboAnalyst and Python (figure 1).

Using this approach, we were able to investigate changes in the lipid regime of THP-1 cells differentiating into several morphological and molecular subtypes. With a precise segmentation at hand, we examined correlations between the abundance of lipid droplets and several primary and secondary morphological form factors with changes in the molecular profile. With that, we were able to generate a more precise classification of differentiated THP-1 macrophages that may be provide a more precise status of differentiation status as compared to bulk analysis MS methods.

Fig. 1


4 % formaldehyde,
Hoechst 33342,
CellMask green
actin tracker
NH4OAc (150 mM)
2,5-DHAP
Cell culture
Fixation and staining
Washing and drying
Microscopy
Sublimation
MALDI-2 MSI
Co-registration
Segmentation
cell 2
cell 1
Single-cell mass spectra

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Large-scale metabolite imaging gallery of mouse organ tissues to study spatial metabolism

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Introduction

Mass spectrometry imaging (MSI) has enabled the visualization of small molecule metabolites (SMMs) in tissues, but there is a lack of comprehensive SMM imaging databases for different organs. Broader scientific applications therefore may miss the potentials MSI can provide. We present a large-scale SMM imaging gallery for mouse brain, kidney, and liver, covering over 300 SMMs across nine metabolite superclasses and more than 40 metabolic pathways.

Methods

Preliminary experiments were performed on cow liver homogenate for on-tissue effects from vapour pressure drying on metabolite standard intensities before matrix application. For full section imaging, mouse organs were cryosectioned at 12µm onto ITO slides and MALDI matrix was applied with a SunChrom sprayer. NEDC was utilized as the matrix, with a spiked isotopic internal standard (d4-succinic acid) included for data normalisation. MSI was carried out on a Spectroglyph elevated pressure MALDI (EP-MALDI) system coupled to a Thermo Q-Exactive HF for high resolution data. Data was collected at 30µm pixels for the mouse organs, with mass range m/z 50-550 at 120k RP and processed using LipostarMSI. Data were imported in imzML file format and visualised for ± 3ppm mass error. Images were generated using internal standard normalisation. Complimentary histological stains were also performed.

Results

By using EP-MALDI and manual curation of the imaging datasets across the mass range analysed revealed novel insights into organ metabolism, including unexplored spatial distribution patterns. For brain sections (sagittal and coronal) we observed endogenous neurotransmitters across different brain regions including GABA and glutamic acid, with no need for chemical derivatization strategies. Interestingly, glutathione intensities were also noted in the kidneys to have correlation with structural features of nephrons, helping to explore the metabolism and localisation of these molecules. Liver tissue was also analysed, and we highlight the alanine-aspartate-glutamate pathway involved as a vital metabolic organ. We spatially recognise the importance of hepatocytes in the liver and surrounding areas of blood vessels.Using these datasets, we then explored the potential of displaying such images as a resource for broader application fields. Most annotation or visualisation resources for MSI datasets require the user to upload or search the data for various molecules, such as METASPACE. We plan to make the data readily available on METASPACE but have also successfully uploaded image files to the EBI BioImage Archive resource, with annotations, so that researchers can easily access these images. As it is rare for these images or molecular distributions to be displayed in publications, this approach could help broaden the scope of SMM MSI. Developing such a resource could also bring rise to the advancement in machine learning models and AI based methods in further analysing large MSI datasets.

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Visualizing host-parasite interactions with AP-SMALDI MSI revealed altered lipid distribution and abundances in infected organs

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Parasitic infections induce significant metabolic alterations in host cells and tissues: parasites are dependent on their host species for nutrient acquisition while hosts strive to protect themselves against potential damage. Enhancing our comprehension on parasite-host interactions is imperative for identifying not only potential novel targets for parasite treatment but also to better understand pathogenesis. Simultaneous imaging of both, host´s and parasite´s metabolism, provides crucial insights into complex parasite–host interactions occurring *in vivo*. To achieve this, two distinct models were employed: a) bovine skin tissue infected with the apicomplexan *Besnoitia besnoiti*, and b) the liver of infected hamsters by *Schistosoma mansoni* blood flukes.

MSI measurements were performed on a Thermo Scientific Q Exactive HF Orbitrap mass spectrometer (Thermo Fisher Scientific (Bremen) GmbH, Germany), equipped with an AP-SMALDI5 AF ion source (TransMIT GmbH, Giessen, Germany). Pixel size was 10 µm, mass resolution R = 240,000 at *m/z* 200, and cryosections were 20 µm thick. For matrix application (2,5-dihydroxybenzoic acid and 1,5-diaminonaphthalene for positive- and negative ion mode, respectively) an ultrafine pneumatic sprayer (SMALDIPrep, TransMIT) was used. LC-MS/MS measurements of lipid extracts of *S. mansoni*-infected hamsters were performed on a Dionex Ultimate 3000 UHPLC (Thermo Fisher Scientific) equipped with a reversed-phase column, coupled to a Q Exactive HF-X system. LC-MS/MS data were evaluated using LipidMatch Flow. MSI data of bovine skin containing *B. besnoti*-cysts were uploaded to METASPACE platform for annotation and quick visualization. Statistical analyses were done by Perseus or MetaboAnalyst.

In the skin of *B. besnoiti*-infected cattle, parasite-formed cysts were visible by the naked eye. MS images revealed both the enrichment and depletion of distinct lipid species inside these cysts. Furthermore, infection markers specific to *B. besnoiti*-cyst walls, cyst content, and the surrounding unaffected tissue were identified.

In the context of schistosomiasis, up to 50% of *S. mansoni* eggs remain inside the host body. Most of these eggs are deposited in the host liver leading to granuloma formation around the eggs, inflammation, and finally liver fibrosis. Leveraging the high-lateral-resolution capability of the AP-SMALDI5 AF ion source, we successfully visualized *S. mansoni* eggs trapped in hamster livers and observed changes in lipid distributions within granulomas especially hamster hepatocytes. Also, we found evidence for a yet unknown substructure within the formed granulomas, with e.g. ether-phosphatidylethanolamines predominantly localized in the outer regions of the granulomas.

Acknowledgement

Financial support by the LOEWE Center on neglected tropical diseases (DRUID) is gratefully acknowledged.

Conflicts of interest

BS and CGG are consultants and SG is a part-time employee of TransMIT GmbH, Giessen, Germany.

P 146

Sample preparation steps towards high-resolving MALDI-2 mass spectrometry imaging of thin sections across *Bacillus subtilis* biofilms

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Bacillus subtilis is a Gram-positive soil bacterium known for its ability to form robust biofilms on various surfaces. Next to its numerous applications, for instance as probiotic dietary supplement, the "microbe of the year 2023", selected by VAAM*, also serves as an important model organism in microbiology research. To improve our understanding about the complex regulation processes occurring during biofilm formation, MALDI-2 mass spectrometry imaging (MALDI-2-MSI) can be used. In previous studies we demonstrated this potential at the example of whole bacterial colonies [1,2]. Here we aimed to advance our method toward the analysis of thin sections prepared from *B. subtilis* biofilms.

B. subtilis produces endospores that are highly resistant to heat and UV-light. To be able to include genetically modified (GM) strains in our projects, the first task was to develop an MSI-compatible inactivation protocol. Biofilms of the wild type NCIB3610 strain were grown for 72 h at 30 °C on mixed cellulose ester filter membranes placed on minimal medium MSgg, similar to [1]. Following removal from the agar, membranes were placed in sealed Petri dishes and subjected to the gas phases of different inactivation agents at a set of inactivation times and temperatures. Microbial inactivation was determined with standard culturing tests.

Our tests showed that the biofilms were robustly inactivated using 18 % formaldehyde solution at 80 °C for 1 h with no morphological changes of the biofilms visible afterwards. Sterilization was also achieved upon use of 30 % peroxyacetic acid at 40 °C for 1 h. However, this led to biofilms with a "wet" and lightened appearance, indicative of possible washout and oxidation effects.

In a second step, the embedding and sectioning protocols were optimized in order to conserve the biofilm morphology on a low micrometer scale. Our currently preferred method includes the use of carboxymethyl cellulose gel along with two freezing steps and subsequent cryotome-sectioning at ~10 µm thickness.

Thirdly, we conducted first MALDI-2-MSI analyses of *B. subtilis* thin sections using a Bruker timsTOF fleX MALDI-2 mass spectrometer at a pixel size of 5 µm. Based on our preliminary results and a comparison with microscopic images, intricate structures constituting the biofilm are distinctly observable. The rich chemical information obtained is indicative of spatially highly differential expression profiles of certain analytes in three dimensions.

With future method optimizations, such as an optimized MALDI matrix-application, inclusion of GMs and use of t-MALDI-2-MSI, our method could help to provide new insights into the multicellular differentiation of *B. subtilis*, and potentially that of bacterial biofilms in general, on a micrometer scale.

* Vereinigung für Allgemeine und Angewandte Mikrobiologie

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[2] Brockmann EU *et al.*, *J Am Soc Mass Spectrom* 2019, 32, 1053.

P 148

Application of ion mobility – tandem mass spectrometry for improving specificity of targeted analyses of tuberculostearic acid containing lipids in translational tuberculosis research

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The cell wall of *mycobacterium tuberculosis* (*Mtb*) contains the unique fatty acid 10-methylstearic acid (10-Me-18:0), called tuberculostearic acid (TSA) in different lipid species but is absent in eukaryotic cells. Phosphatidylinositol (PI) 35:0 (16:0_19:0) was identified as the major phosphoglycerolipid of several *Mtb*-lineages [1]. In previous work we could show that this lipid can be used as a marker for mycobacterial burden in preclinical model systems and as a metabolic tracer [1,2]. However, it became clear that there is a need for further discrimination between mycobacterial derived PI 16:0_19:0 (TSA) and species comprising isomeric FA 19:0 that were detected in mice lung homogenates, macrophage cell culture and PBMCs of healthy humans [1].

We tested an IMSn approach using the Cyclic IMS (Waters, Manchester, UK) to detect the small differences in collision cross section (CCS, D < 0.7%) of biochemically reasonable isomers 16-Me-18:0, 17-Me-18:0, 2-Me-18:0 and nonadecanoic acid (NDA) from TSA. Several strategies to build multi-pass sequences for cyclic IM (cIM) were evaluated to improve specificity and retain sensitivity for targeted analysis [3]. With the help of FA standards, the cIM was optimized and 2-Me-18:0 was separated at baseline from the other isomers after about 42 cycles (ca. 481 ms) and could be eliminated from the array by the ejection function. Further separation time by about 45 cycles (ca. 503 ms) for the remaining isomers resulted in distinct arrival time distributions, which allows specific detection of TSA. Finally, quadrupole precursor selection for PI 16:0_19:0 (*m/z* 851.5) and all ion fragmentation were performed in front of the cIM array to characterize biological matrices (mouse lung tissue, murine bone marrow derived macrophages, human blood plasma). To demonstrate the specification by IMS2, different bacterial extracts (*M. canetti, M. tuberculosis* H37rv / H37ra, *M. bovis BCG*) were spiked into biomedical relevant matrices and were successfully analyzed. The next step will be the application of this IMS2-based assay in preclinical models and clinical samples.

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2. Brandenburg, J. et al. *WNT6/ACC2-induced storage of triacylglycerols in macrophages is exploited by Mycobacterium tuberculosis.* J Clin Invest 2021
3. Giles, K et al. *A Cyclic Ion Mobility-Mass Spectrometry System*. Anal Chem, 2019

P 149

Utilizing the SICRIT® LC-Module for development and optimization of lipidomic analysis

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[2]Plasmion, GmbH, Augsburg, Germany

The study focuses on advancing lipidomics through the use of a DBDI-based ionization source, specifically the SICRIT® LC-Module, to address challenges in untargeted analysis of metabolomic studies. Traditional methods, such as HPLC-MSMS, face issues like coelution, in-source fragmentation, and adduct formations, making compound identification challenging. The proposed approach involves targeted studies with a range of lipid subclasses to ensure compatibility with different polarities in both positive and negative modes. A library of lipids is then constructed with MS1 and MS2 spectra, followed by untargeted studies to identify known and unknown lipids, utilizing BioTransformer for unmatched lipids.

Successful lipidomic analyses using the SICRIT ionization source have been conducted with MALDI-MS, and the development of the specialized SICRIT® LC-Module extends the applicability to LC-MS for metabolomic studies. The advantages of SICRIT over ESI include minimal adduct formation and fragmentation, simplifying molecular weight calculations. Optimal parameter optimization across replicates allows visibility of targeted lipids even in cases of coelution, ensuring distinct peaks for each compound due to the soft ionization.

The study leverages an existing compound library with MS1 and MS2 spectra, including in-silico components containing biotransformations and unidentified compounds. Future steps involve expanding lipid classes in targeted studies to assess SICRIT® LC-Module capabilities in terms of sensitivity and ionization efficiency. Subsequently, non-targeted studies combining the SICRIT source and the compound library aim to identify a majority of metabolites, with results to be presented at ASMS.

By combining the advantages of the SICRIT ionization source and a comprehensive lipid library, the study seeks to enhance lipid identification, offering biological insights in addition to improving the ease of analysis.

Liu, Qinlei, et al. "High-Throughput Single-Cell Mass Spectrometry Reveals Abnormal Lipid Metabolism in Pancreatic Ductal Adenocarcinoma." Angewandte Chemie, vol. 133, no. 46, 2021, pp. 24739–24747., https://doi.org/10.1002/ange.202107223.

Fig. 1: EIC of Triolein (ESI vs. SICRIT)

Fig. 2: MS1 of Triolein (ESI vs. SICRIT)

Fig. 3: MS2 of Triolein (SICRIT)

Fig. 1

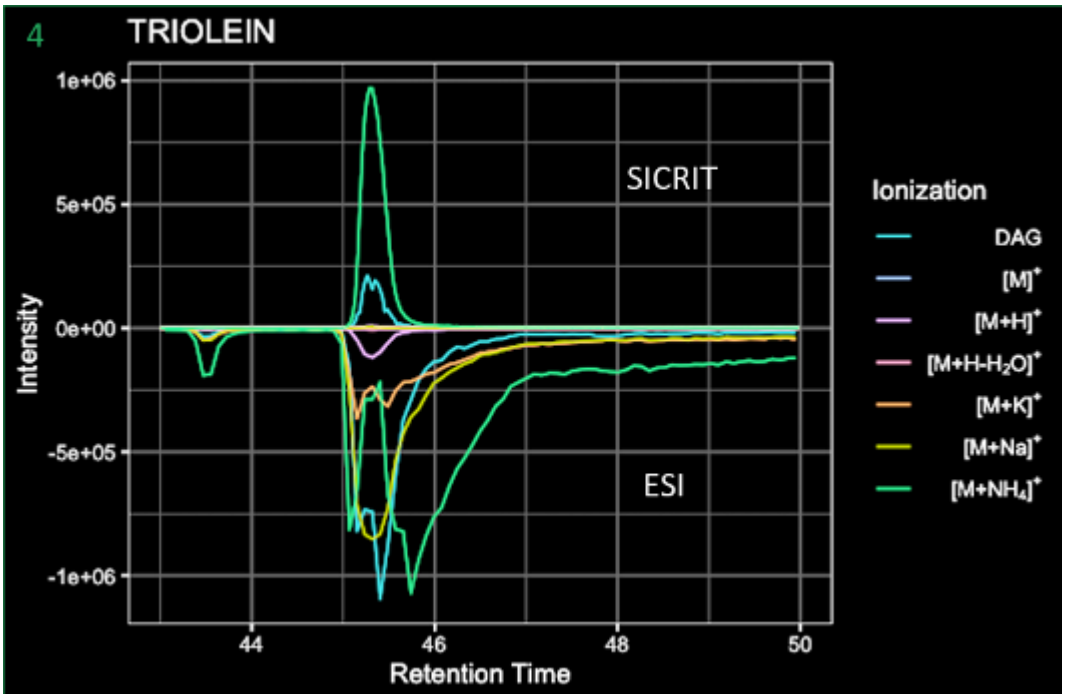

4
TRIOLEIN
1e+06
5e+05
0e+00
-5e+05
-1e+06
Intensity
SICRIT
ESI
44
46
48
50
Retention Time
Ionization
DAG
[M]+
[M+H]+
[M+H-H2O]+
[M+K]+
[M+Na]+
[M+NH4]+

Fig. 2

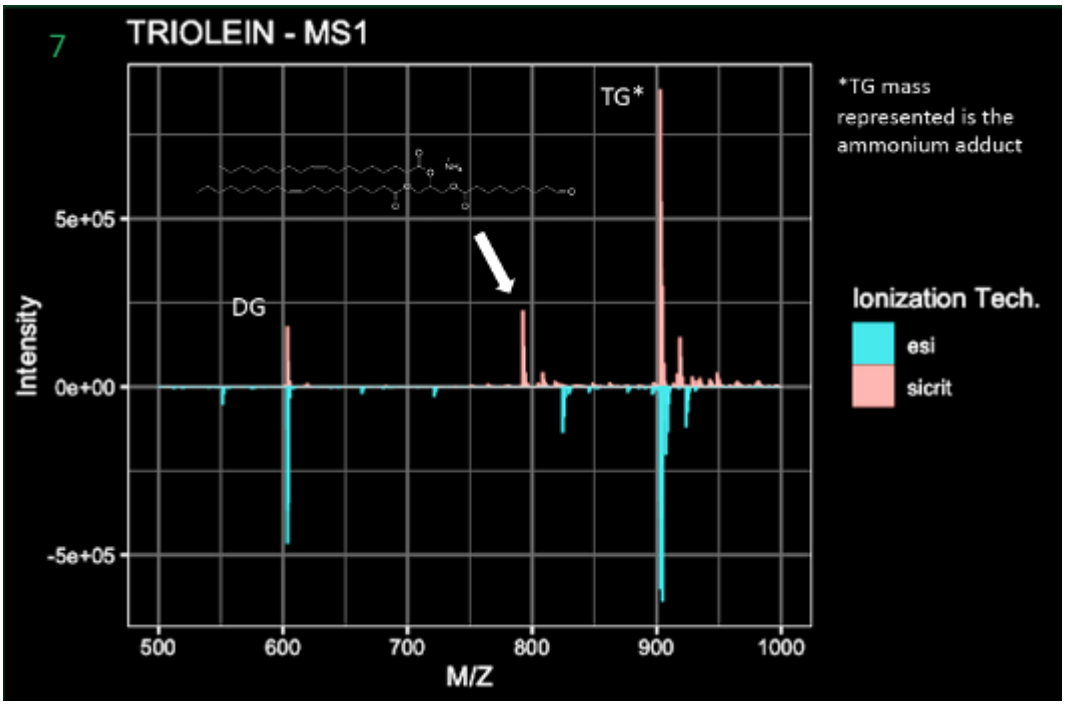
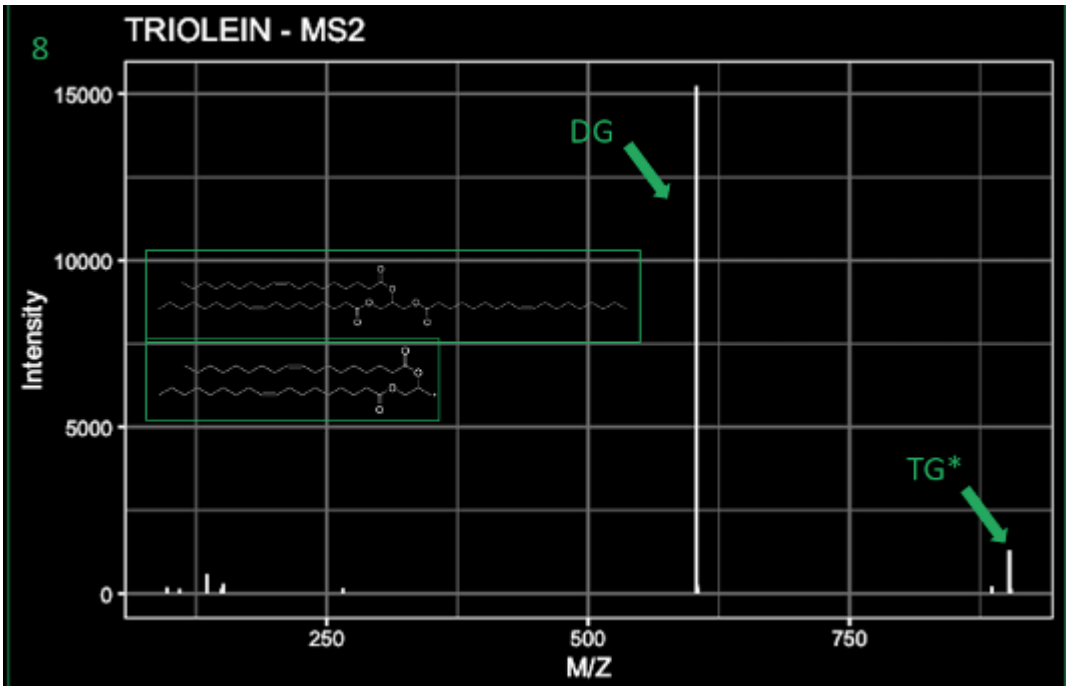

7
TRIOLEIN - MS1
TG*
DG
5e+05
0e+00
-5e+05
Intensity
500
600
700
800
900
1000
M/Z
*TG mass represented is the ammonium adduct
Ionization Tech.
esi
sicrit

Fig. 3


TRIOLEIN - MS2
8
15000
10000
5000
0
Intensity
DG
TG*
250
500
750
M/Z

P 150

Changes in the lipid composition of colorectal carcinoma cells with mutations in the acyl-CoA synthetase long-chain family member 5 investigated by mass spectrometry

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Background: Colorectal carcinoma (CRC) is the third most common cause of cancer-related death, with an increasing incidence among younger adults, associated with smoking, increased dietary fat uptake and obesity. Dysregulated metabolic pathways in CRC have been often reported, resulting in abnormal glycolysis, glutaminolysis, and lipid synthesis. Lipid metabolic reprogramming, especially abnormal fatty acid metabolism, has been reported to drive tumor development and progression, correlating with poor prognosis in CRC patients. Long chain fatty acids, most abundantly present in dietary lipids, must be activated by coupling with Coenzyme A (CoA) by enzymes called long chain acyl-CoA synthetases (ACSLs) to be further involved in anabolic and catabolic processes.

Aims of this study: To determine the clinical implications of ACSL5, the impact of isozymes and respective mutants on the lipid metabolism in different colorectal carcinoma cells (SW480 and HT29) was investigated.

Methods: We established a gain-of-function cell model by stable transfection of SW480 cells exhibiting a low endogenous ACSL5 expression. ACSL5 activity was determined by Western Blot and ACSL activity assay by ^{3}H-palmitic acid supplementation of the cell culture medium. An shRNA approach was used to silence ACSL5 expression in HT29 cells. The ATP production of ACSL5-tranfected cell lines and non-transfected cell lines was monitored using the Seahorse analyzer. Potential changes in the lipid composition of the different cell variants were investigated by coupling high-performance thin-layer chromatography (HPTLC) and electrospray ionization mass spectrometry (ESI MS).

Results: Our data showed an increased ATP production in ACSL5-transfected colorectal carcinoma cells. Furthermore, HPTLC revealed an altered lipid profile of ACSL5-transfected cell lines and mutants compared with non-transfected cells. This applied particularly regarding the total amounts of cholesterol esters, free cholesterol and triacylglycerols, which was in line with an increased gene expression of the fatty acid synthase FASN. A deeper characterization of the separated lipid classes by HPTLC-MS coupling showed significant differences in the degree of fatty acyl unsaturation as well as the chain length in phospholipid species between the different cell types.

Conclusions: Changes in the expression and activity in ACSL5 have a significant impact on the lipid composition of tumor cells. Blocking ACSL5 might suppress tumor cell proliferation, migration, and invasion and have an impact on the fluidity of the cellular membranes.

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Characterization of Glycoglycerolipids in Gram-Positive Bacterial Cell Walls: Insights from *Streptococcus suis*

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The Gram-positive *Streptococcus Suis* causes different swine diseases worldwide. To date, 35 different serotypes are described whereas the serotype 2 is the most frequently isolated. This serotype is classified as a zoonotic agent which can lead to meningitis [1]. The cell wall of *S. suis* comprises a macromolecular peptidoglycan layer which encloses the cell membrane. Embedded to this thick peptidoglycan layer biopolymers like lipoteichoic acids (LTA) are anchored to the cell membrane via glycoglycerolipid-anchors. These glycoglycerolipids (GL) are composed of a glycerol backbone esterified with fatty acids at the *sn*-1 and *sn*-2 position. The carbohydrate moieties, which can differ in quantity and linkage, are connected via a glycosidic bond at the *sn*-3 position. They are located in the outer membrane leaflet and are important for membrane-associated properties like elasticity and curvature [2]. Most interesting, GL function as a stimulus to the C-type lectin-receptor (CLR) *mincle* which senses pathogen-associated molecular patterns as ligands to trigger the production of different cytokines like TNF-α and IL-6 in dependence of NFκB. In *S. suis* the GL α-D-Glucopyranosyl-(1,2)-α-D-glucopyranosyl-3-(1,2-diacyl)-glycerol (α-Glc2-DAG) was detected as the LTA-anchor [3]. As the biosynthetic pathway of glycolipids is highly conserved, monoglycosylated GL in *S. suis* besides the known GL-anchor are expected. In this study, high-resolution mass spectrometry (Q-Exactive Plus coupled with a Triversa Nanomate), gas chromatography mass spectrometry (GC-MS, Agilent Technologies 6890N coupled to a 5975 inert XL MSD) and NMR spectroscopy (Bruker Avance III 700 MHz) were employed to uncover the structure of three distinct GLs originating from the well-characterized *S. suis* strains P1/7 (serotype 2), SC84 (serotype 7) and 89-1591 (serotype 25). Besides the previously described α-Glc2-DAG, two isomeric GL could be isolated: one furanosidic and one galactosidic. In addition, ion mobility tandem mass spectrometry (IMS-MSn) experiments were performed using a Cyclic IMS (Waters, Manchester, UK) to test whether these previously characterized isomeric structures can be distinguished and used as an analytical model system for shotgun lipidomics approaches.

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P 152

Deep lipidome profiling by quantum cascade laser mid-infrared imaging microscopy-guided MALDI MSI

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Question

Mass spectrometry imaging (MSI) plays a crucial role in spatial biology, facilitating the mapping of hundreds of compounds in tissue sections. However, the investigation of a ground truth for molecular identifications remains one of the main challenges in the field of MSI [1].

Methods

We investigated selective sulfatide (a class of glycosphingolipids) accumulation in arylsulfatase A-deficient (ARSA-/-) mice as a biological ground truth concept by employing ultra-fast Quantum Cascade Laser-based mid-IR imaging (QCL-IRI) to guide MSI [2]. The employed methodology is based on our concept of IRI-guided MSI [3]. QCL-IRI-guided MSI significantly reduces measurement time by focusing MSI on IRI-defined tissue areas of interest.

Results

QCL-IRI-guided magnetic resonance (MR)-MSI was performed with a mass resolution of R2~1,230,000 at *m/z* 800. The benefit of ultra-high mass resolution was evaluated by comparing sulfatide annotations at different FDR levels against *conventional* non-guided MSI with R1~77,000 at *m/z* 800. QCL-IRI-guided trapped ion mobility spectrometry (TIMS)-MSI was performed with elongated ramp times to increase the quality of ion mobility-based separation. Measured collision cross section (CCS) values were used to gain new insights in structure-CCS-relationships within the different sulfatide sub-classes with an emphasis on sulfatide hydroxylation and glycosylation. Additionally, we were able to unequivocally identify odd-chain sulfatides based on parallel reaction monitoring with on-tissue parallel accumulation and serial fragmentation (prm-PASEF) MS2.

Conclusion

QCL-IRI-guided MSI enables deep spatial profiling of lipid-classes, the evaluation and investigation of structure-CCS-relationships for e.g. the improvement of CCS prediction tools. This approach allows for MSI analysis with increased analytical depth, thus enhancing data quality for molecular identifications and reducing total measurement time up to 10-fold.

Figure 1: QCL-IRI-guided MALDI MSI. **b**, Ion images of SM4 40:1;O3 (left) and SM3 44:1;O2 (right). **b**, Number of annotations for Swiss Lipids database of sulfate-containing molecules (cyan and red) and manually curated sulfatides (gray and black) based on MALDI MSI data. **c**, Strong correlation (R^2 = 0.9988; linear fit (red); 95% confidence interval (CI)) of LC-MS-derived and MALDI-MSI-derived ion mobility data. Mean relative error = 0.5%. **d,** Ion mobility heat map with inset highlighting two SM4 isobars. **e,** Butterfly plot of prm-PASEF-derived MS2 spectra (top) and conventional on-tissue MS2 without ion mobility separation (bottom). **f,** Elucidated fragment patterns. Fragments around m/z 550 refer to the loss of the α-OH-FA.

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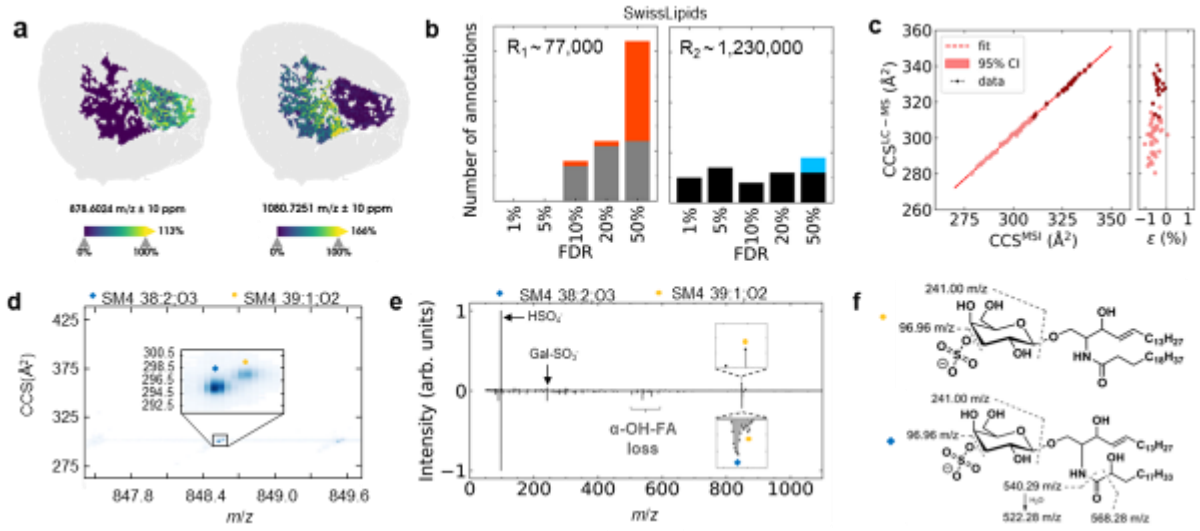
Fig. 1



P 153

Enhancing the mobility resolution for co-eluting compound classes during plasma characterisation using multi-sequence IMS n acquisitions

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Introduction

Human plasma comprises of many small molecules including amino acids, organic acids, and larger lipid molecules across many classes. To accurately characterise this biological fluid requires multiple extraction and complimentary LC-MS acquisition methodologies to deconvolve certain compounds. When analysing small polar analytes by HILIC based methods, many moderately polar and non-polar compounds like lipids are also present but co-elute in class regions and the solvent front. Coupling ion mobility with mass spectrometry has improved the separation of many of these co-eluting compounds, increase the peak capacity and improving spectral quality. But different compounds require different levels of separation to achieve the desired resolution.

Methods

Human plasma samples, obtained from healthy human donors, underwent extraction using acetonitrile and water to obtain small molecule metabolites and lipids. Extracts were analysed in both positive and negative ESI modes with the data acquired on the SELECT SERIES Cyclic IMS system using a data independent acquisition (HDMS^E) multi-sequence acquisition mode. Chromatographic separation was achieved over 10 mins using ACQUITY Premier BEH amide column and HILIC gradient elution.

Preliminary Data

Cyclic IMS allows multiple passes round the device, increasing the mobility path length and the resolution. This higher mobility resolution is achieved over a narrower mobility range when compared to the single pass acquisition. The pooled human plasma sample was initially acquired using a single pass HDMS^E acquisition mode to determine the regions of the chromatogram where compounds requiring additional passes were eluting. Due to the hydrophilic nature, those lipids remaining following the extraction procedure elute based on the polarity of the head group of the class causing them to co-elute. The single pass of the cIMS device provided sufficient resolution for many small molecules throughout the chromatographic separation, but those regions corresponding to lipids from the triacylglyceride and phosphatidylcholine classes from the beginning and middle of the gradient required additional passes to separate out these co-eluting features. A multi-sequence HDMS/MS method was created incorporating multiple HDMS/MS functions each using broad quadrupole isolation windows across specific mass ranges each corresponding to the co-eluting classes and with optimised cyclic multipass sequences. Collision induced dissociation was performed to provide high energy fragmentation spectra to assist in elucidating structural information. Employing the multi-sequence analysis for plasma metabolomics assisted in improving the depth of characterisation of the plasma matrix, enhancing the isolation of co-eluting lipid phosphatidylcholine isomers and identification using elevated collision energy spectra.

Novel Aspect

Plasma characterisation using the IMS^n acquisition mode to resolve co-eluting lipid class features.

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An interlaboratory evaluation of CCS measurements from a plasma lipid extract on a commercial SLIM platform

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Introduction

Plasma lipidomics is challenging due to the underlying biological complexity and broad dynamic range. A variety of mass spectrometry (MS) methodologies have been developed to attempt dissecting the plasma lipidome, however the precise composition remains unsettled due to insufficient resolving power to identify all isomeric lipids. We have previously shown that liquid chromatography based High-Resolution Ion Mobility (LC-HRIM) MS enables improved separation of isomers, increasing characterization depth of complex extracts. Calibration of HRIM data facilitates the determination of collision cross section (CCS) values for greater identification confidence in untargeted analyses. Here, we demonstrate the laboratory-independent reproducibility of HRIM-derived CCS values for lipids present in plasma and have developed a SLIMCCSN2 database to support adoption of HRIM into untargeted workflows.

Methods

Experiments were performed on the MOBIE HRIM platform. Four lab sites participated in the interlaboratory study for three phases of work. For the first two phases, a commercial lipid standard mixture (Avanti p/n 791500) was introduced to the system via either flow injection (FIA) or following reversed-phase (RP) LC in both polarities. In phase 3, lipids were extracted from NIST SRM 1950, and three extraction replicates were analyzed via RPLC-HRIM-MS in both polarities across five days. All data was CCS calibrated using MOBILion TuneMix and a third order polynomial fit.

Preliminary Data

Initial method development focused on optimization of the LC-HRIM-MS method, including but not limited to source settings, RPLC gradient, the HRIM trap/release and separation parameters, and optimization of the CCS calibration procedure via choice and number of calibrant ions chosen. Preliminary results from phases 1 (FIA) and 2 (RPLC) of the project displayed extraordinary CCS reproducibility, represented by the intra-lab and inter-lab %RSD values of each lipid analyzed. For phases 1 and 2, the average interlaboratory CCS %RSD was < 0.2%. In contrast, non-calibrated measurements such as arrival time (AT) and retention time (RT) had a much larger spread in values across the labs, with %RSDs < 8%. We also demonstrated that the measured CCS of the lipids was workflow agnostic, sinceCCS values generated for a given lipid using FIA were, on average, about 0.02% off from values generated using RPLC.

Having validated the general approach, we wanted to next assess the interlaboratory reproducibility of values for endogenous lipids present in a plasma extract. Initially, we benchmarked the performance via analysis of six spiked internal standards and six endogenous lipid species present in the extract. These results mirrored those from the previous phases of work, with individual labs demonstrating good reproducibility for CCS, AT, and RT. Currently, we are expanding the analyses to hundreds of lipids including isomeric and isobaric species present in the extract

P 155

Glycolipidomic changes in acute vs chronic *Fasciola hepatica* infection – insights from mass spectrometry imaging

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Fascioliasis, a foodborne trematode infection affecting livestock, results in huge economic losses.[1] This zoonotic infection also poses a threat to human health, with an estimated 2.4 million people worldwide affected.[2] Whereas the feeding activity and presence of adult *Fasciola* (*F*) *spp*. in the bile duct can cause cholangitis and biliary obstruction, the immature stage is responsible for most of the pathogenesis. During acute infection immature parasites are migrating through the liver parenchyma, causing damage initiating a host immune response. This can lead to fever, anemia, and other effects.[3] Understanding parasite-induced changes within the infested tissue on a molecular level is crucial for advancing knowledge of the underlying biology. We therefore performed (nano) liquid chromatography tandem mass spectrometry (nano-LC-MS/MS) experiments to profile the lipidome of liver from rat infected with *F. hepatica*, with a focus on glycosphingolipids (GSLs). We also performed atmospheric-pressure scanning microprobe matrix-assisted laser desorption/ionization mass spectrometry imaging (AP-SMALDI MSI) experiments to reveal local (glyco)lipidomic changes. This is crucial because an infected liver is characterized by a complex interplay of different processes, ranging from tissue damage caused by migrating immature parasites, through inflammatory processes, to liver regeneration and repair. Furthermore, nano-LC-MS/MS and AP-SMALDI MSI measurements on isolated adult *F. hepatica* were conducted to characterize GSLs, which helped to distinguish between these GSLs and GSLs involved in inflammatory processes. The combination of an orbital trapping mass spectrometer (Thermo Scientific Q Exactive HF, Thermo Fisher Scientific (Bremen) GmbH, Germany) equipped with our AP-SMALDI[5] AF ion source (TransMIT GmbH, Giessen, Germany) facilitates measurements with spatial resolution down to 5 µm.

MSI data and semi-quantitative analysis of nano-LC-MS/MS data support the hypothesis, that GSLs are involved in host-parasite interactions and tissue repair due to the upregulation of 52 GSLs during acute infection in rat liver compared to healthy controls. In addition, we provide an overview of the distribution of *F. hepatica*-specific GSL species. This reveals that the ceramide backbone of *F. hepatica* GSL is potentially a key building block in the tegument, the parasite"s surface layer. Not only are hydroxylated fatty acids widely distributed across tegumental GSL, but also ultra-long chain fatty acids were accumulated.

Acknowledgement
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Conflicts of interest
BS is a consultant of TransMIT GmbH, Giessen, Germany.

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Development and application of an in-house lipid library to enhance annotation confidence and data quality in lipidomic analysis of NIST® SRM® 1950 human plasma using high resolution mass-spectrometry (HR-MS)

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The lipidome, a complex biological subsystem, is crucial for biological and clinical research, and comprehensive studies of the lipidome at the species level require advanced analytical techniques, from which liquid-chromatography coupled to mass spectrometry (LC-MS) has become a gold standard[1]. However, within the untargeted MS-based lipidomics, data pre-processing for compound detection and lipid identification critically influences the quality and coverage of the analysis.

In response to the highly extensive and complex raw data generated, lipidomics research has relied on computational methodologies and although these solutions are indisputably valuable to support the analysis, it is a common practice in lipidomic studies to only annotate relevant lipids, introducing statistical bias arising from the conservation of unknown compounds and interpretive bias associated with the inability to contextualize lipids within the global lipidome.

Therefore, this study aims to build an in-house library-driven lipidomic workflow using UHPLC-ESI(+/-)-QTOF-MS which involves: **(i)** Apply lipid extraction and acquisition protocols that ensure lipid coverage, Fig 1A 1B, **(ii)** Build a high-mass resolution in-house library with high coverage and high confidence annotation level, Fig 1C, **(iii)** Implement an in-house library-based targeted peak detection process, Fig 1D.

Fig 1. Lipidomic workflow for the development and implementation of the in-house SRM® 1950 lipid library.

For this purpose, iterative MS/MS data from SRM® 1950 acquired in data-dependent acquisition mode was analyzed using three software-assisted annotation tools (Lipid Annotator, MS-DIAL 4 and LipidHunter). Resulted annotations were cross-matched and filtered followed by a manual MS/MS and MS1 data inspection (including RT mapping, adduct profile and isotopic pattern) resulting in an in-house lipid library containing 539 lipid species. For data pre-processing, a subsequent implementation using the *Targeted Feature Extraction* algorithm of Agilent MassHunter Profinder enabled the determination of lipid species abundances for semi-quantification.

In conclusion, our in-house library has allowed to implement in our lipidomic workflow a useful strategy with broad lipidome coverage, successfully evaluated in human plasma samples studies such as COVID-19[2] leading to a significant sample-to-result time reduction and a global lipidomic profiling.

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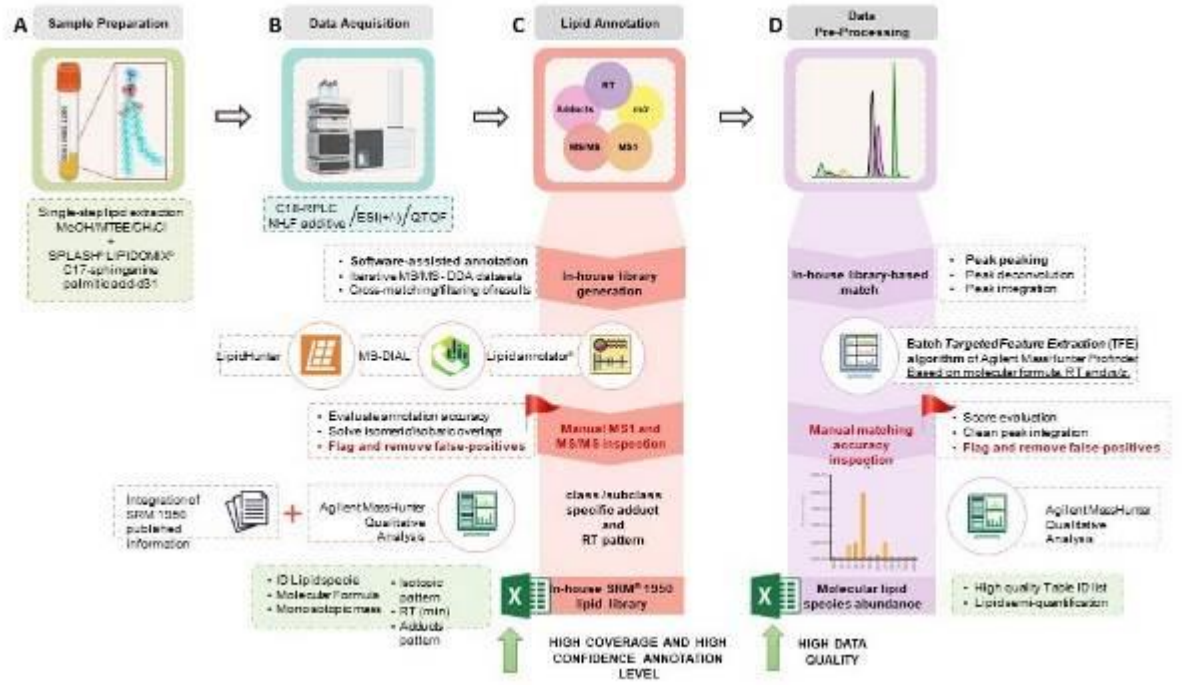
Fig. 1


A
Sample Preparation
B
Data Acquisition
C
Lipid Annotation
D
Data Pre-Processing
Single-step lipid extraction
MeOH/MTBE/CHCl3
+
SPLASH® LIPIDOMIX®
C17-sphinganine
palmitoleoic-d31
C18-RPLC
NH4F additive
/ESI(+)/QTOF
• Software-assisted annotation
• Iterative MS/MS-DDA datasets
• Cross-matching/filtering of results
In-house library generation
In-house library-based match
• Peak picking
• Peak deconvolution
• Peak integration
LipidHunter
MS-DIAL
LipidAnnotator®
Batch Targeted Feature Extraction (TFE)
algorithm of Agilent MassHunter ProFinder
Based on molecular formula, RT and/or
• Evaluate annotation accuracy
• Solve isomeric/isobaric overlaps
• Flag and remove false-positives
Manual MS1 and MS/MS inspection
Manual matching accuracy inspection
• Score evaluation
• Clean peak integration
• Flag and remove false-positives
Integration of SRM 1950 published information
+
Agilent MassHunter Qualitative Analysis
class /subclass specific adduct and RT pattern
Agilent MassHunter Qualitative Analysis
• ID Lipidspecie
• Molecular Formula
• Monoisotopic mass
• Isotopic pattern
• RT (min)
• Adducts pattern
In-house SRM® 1950 lipid library
Molecular lipid species abundance
• High quality Table ID list
• Lipidsemi-quantification
HIGH COVERAGE AND HIGH CONFIDENCE ANNOTATION LEVEL
HIGH DATA QUALITY

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C^{13} tracer studies on biosurfactant producing bacteria using HPLC-HRMS

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Biosurfactants gained interest in recent years due to its promising sustainable and environmentally friendly synthesis by microorganisms. One popular glycolipid-based biosurfactant can be produced by non-pathogenic *Pseudomonas putida* strains, which synthesize rhamnolipids consisting of one or two L-rhamnose molecules linked to up to three (unsaturated) β-hydroxy fatty acids of different chain length. As consequence, a high structural diversity arises for the class of rhamnolipids. Current research target the biosynthesis of specific rhamnolipid congeners with customized properties. However, due to limited knowledge on the biosynthetic pathways efficient synthesis and large-scale production is hampered.

In order to get a closer insight into the biosynthesis pathways of rhamnolipids, ^{13}C-based tracer studies were conducted. ^{13}C was introduced during cultivation of *Pseudomonas putida* KT2440 using isotopically labeled glucose, known to be the preferred carbon source for the rhamnose biosynthesis pathway. Furthermore, the addition of different fatty acids as well as a knock-out variant of *Pseudomonas putida* was investigated. For investigation of ^{13}C incorporation, culture supernatants were analyzed by LC-MS/MS. Chromatographic separation was carried out using reversed-phase chromatography providing efficient separation of different rhamnolipids with respect to their fatty acid chains. Rhamnolipids and related molecules were detected by electrospray ionization high resolution mass spectrometry. Furthermore, data-dependent tandem mass spectrometry fragmentation experiments were acquired.

This work presents a combination of an effective chromatographic separation and high resolution mass spectrometry for investigation on rhamnolipids. Using this method, isotopically labeled rhamnolipids can be analyzed in dependence of varying carbon sources. Therefore, this work is paving the way for profound research on the rhamnolipid biosynthesis pathway using ^{13}C-based tracer experiments.

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Culture media influence on the lipid A profile of pathological *E. coli* investigated by MALDI-MS/MS utilizing trapped ion mobility spectrometry

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Lipopolysaccharides (LPS) are an important component of the outer cell membrane of gram-negative bacteria, such as *E. coli*. These LPS can have strong immunostimulatory effects and are used in vaccines as an adjuvant. The pathological effect of LPS as an endotoxin is primarily due to its lipid anchor, known as lipid A. Lipid A consists of a diglucosamine backbone containing varying polar head groups and fatty acid residues. The composition of lipid A can impact bacterial endotoxicity and may vary between bacterial strains. However, the effect of different culture media on the lipid A profile has not been thoroughly examined.

Analyzing lipid A is analytically challenging as a result of the wide variety of species. Reversed phase-LC-MS/MS or direct analysis by MALDI-MS are commonly used to study the lipid A composition. LC-MS/MS allows for a comprehensive analysis of the entire lipid A profile, but it is more time-consuming due to the numerous extraction and purification steps required during sample preparation. In contrast, MALDI-MS is less sensitive and does not provide structural elucidation of lipid A, despite its potential for bacterial screening. Therefore, we developed a workflow based on microextraction and subsequent MALDI-MS/MS analysis utilizing trapped ion mobility spectrometry (TIMS) as an additional separation dimension. This allows for rapid and comprehensive screening of the influence of different culture media on the lipid A profile of uropathogenic *E. coli* CFT073.

The utilized TIMS dimension serves not only for additional sensitivity via noise filtering, but can also be used for the separation of isomeric or isobaric interferences. Especially constitutional isomers regarding the phosphate headgroup position can affect the endotoxicity of lipid A. Furthermore, mobility-resolved fragmentation of the identified lipid A species was achieved by parallel accumulation - serial fragmentation (PASEF). MS/MS acquisition was automated using the dataset-dependent spatial ion mobility-scheduled exhaustive fragmentation (SIMSEF) strategy in MZmine 3 via parallel reaction monitoring (prm)-PASEF.[1] For validation, the identified lipid A species, including their collisional cross sections (CCS), were further compared to LC-TIMS-MS/MS results. The developed MALDI-TIMS-MS/MS workflow yielded comparable results to LC-MS/MS, while requiring significantly less time and labor-intensive sample preparation.

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P 159

Optimization of mobile phase modifiers in ultrahigh-performance liquid chromatography-mass spectrometry for in-depth lipidome profiling of colonized OMM12 mouse model

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Liquid chromatography coupled to mass spectrometry (LC-MS) is widely employed for the comprehensive profiling of various compounds in biological samples.1 Lipidomics, as a subfield of metabolomics aims to screen lipid species in complex mixtures based on LC-MS approaches.2

In this study, we conducted a comparative analysis of three distinct column technologies while exploring various mobile phase modifiers to enhance the lipidome coverage of the Oligo-Mouse-Microbiota (OMM12) colonized mouse model—a widely used tool for functional microbiome research. Specifically, we investigated eight different buffer conditions, mainly consisting of 5 and 10-millimolar ammonium acetate or ammonium formate, both with and without 0.1% acetic or formic acid, respectively. Two C18 ethylene bridged hybrid technology (BEH) columns incorporating the hybrid surface technology (HST) and the conventional one, were compared to an HST HSS T3 C18 and a core-shell particle column (Cortecs C18), based on chromatographic performance. Our findings reveal that optimal lipidome coverage was achieved using ammonium formate in ESI (+) and ammonium acetate in ESI (-) for all column technologies. 5mM as concentration showed better results for both modes while adding formic or acetic acid did not have a big effect on lipid ionization. Notably, the HST C18 technology showed improved peak width, tailing, and asymmetry factors parameters. We demonstrated that the HST technology has higher coverage and peak shapes for different lipid classes mainly phosphatidylserine and phosphatidic acid lipid species.

We here show that optimization of the lipidome coverage requires careful selection of both mobile phase modifiers and stationary phases.

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P 160

Extraction and mass spectrometry sequencing of photo-crosslinked peptide-RNA hybrids for the analysis of protein-RNA interfaces in live cells

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Protein-RNA interactions are essential to many processes in gene expression regulation, starting from transcription over RNA splicing and modification to translation. The interface between protein and RNA carries valuable structural information about how this regulation is achieved mechanistically. Photo-crosslinking can be applied to generate covalently connected peptide-RNA hybrids at the protein-RNA interaction interface, yet, the analysis of peptide-RNA hybrids has so far been limited by their challenging isolation, liquid chromatography (LC) and sequencing via mass spectrometry (MS). Here, we present a unifying workflow for the extraction of highly pure peptide-RNA hybrids with tunable length of the RNA moiety and their analysis by LC-MS. Using a novel, high-powered irradiation device we achieve highly efficient protein-RNA crosslinking within seconds and capture RNA-protein interactions in live cells under conventional cell culture conditions. We demonstrate that previous extractions of peptide-RNA hybrids suffer from heavy contamination of non-crosslinked RNA, which ionizes especially well and suppresses the signal of peptide fragment ions, hindering their identification. Based on these findings, we optimized an extraction procedure to efficiently remove the free RNA contamination from the LC-MS sample, which allows for a higher input amount and significantly improved peptide identification because of high MS2 quality. As the RNA moiety in peptide-RNA hybrids is much more labile than the peptide moiety, previously, complete fragmentation of RNA adducts during MS2 sequencing impeded its sequencing alongside the peptide within the same MS2 scan. Therefore, we tune the collision energy to achieve well-controlled RNA and peptide fragmentation and compare various sequencing strategies of RNA and peptide in the same or separate scans. Overall, our study paves the way for the MS analysis of protein-RNA interfaces by identifying the confounding problems with sample quality of peptide-RNA hybrids and presenting a systematic optimization of the MS methodology for their sequencing.

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4D Analysis of Lipid Nanoparticles (LNP) using Elute-timsTOF Pro 2 with VIP-HESI source

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Question: As critical components of gene therapy delivery vehicles such as mRNA vaccines, lipid nanoparticles (LNPs) must be characterized in terms of composition, stability, and potential biological interactions. LNPs are composed of four main lipid species: cholesterol, phospholipids, synthetic ionizable lipids, and PEGylated lipids. Routine, in-depth characterization of LNPs necessitates the development of robust and comprehensive characterization of formulations.

Methods: Bruker´s 4D-LipidomicsTM solution combines ultra-high performance liquid chromatography (UHPLC), trapped ion mobility spectrometry (TIMS), and high-resolution mass spectrometry (HRMS): A Bruker Elute UHPLC coupled to a timsTOF Pro 2 via vacuum-insulated probe-heated electrospray ionization (VIP-HESI) can separating and characterizing LNP components by retention time (RT), collisional cross section (CCS), and mass-to-charge (*m/z*) measurements.

We analyzed a working solution of DSPC, DOTAP, DMG-PEG-2000 (Avanti Polar Lipids) and cholesterol (Sigma-Aldrich) with concentrations ranging from 1 ppb – 100 ppm of the resulting LNP component mixture and lipids extracted from NIST SRM 1950 plasma (Sigma-Aldrich) by MTBE. One aliquot was analyzed as is and the other aliquot was spiked with LNP component mixture (5 ppm cholesterol, 1 ppm DMG-PEG 2000, and 50 ppb DOTAP/DSPC). Data were processed by DataAnalysis 6.0 and MetaboScape 2023.

Results: We present the analysis of standards representing the main components of LNPs in solution and in biological matrix. Each component in the working standard solution was separated over a 15 minute reversed-phase gradient. To readily identify components in complex matrices, we employed the VIP-HESI source, achieving 5-25-fold signal enhancement over a standard ESI source. In addition to RT and m/z, CCS values were determined as an additional molecular identifier. CCS showed excellent reproducibility between the working solution and the spiked SRM 1950 sample.

In the SRM 1950 reference plasma spiked with the LNP components, the PEGylated lipids eluting at ~2.8 min showed a typical polymeric molecular weight distribution. Co-eluting, polymeric peaks were separated by TIMS. The polygon tool was used to extract +2 to +4 charge states from the remainder of the spectrum, demonstrating the utility of TIMS in separating PEGylated species. This work highlights the ability of TIMS to separate LNP components from non-endogenous species which can be used to characterize formulation purity and their fate in biological systems.

Conclusions: The Elute-timsTOF Pro 2 platform provides rapid analysis capabilities for LNP components. The VIP-HESI source was shown to enhance sensitivity for LNP lipids over conventional electrospray ionization.

The ion mobility dimension aids identification by providing CCS values for LNP components. In addition, it can isolate multiply charged, PEGylated lipids in complex plasma samples.

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Unusual Epigenetic DNA Modifications: Elucidation, Synthesis and Annotation using UHPLC-HRMS^2

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Introduction

DNA modifications have multiple functions in organisms across all domains of life, including bacteria. Especially methylation of the DNA bases cytosine at the positions 4 or 5 as well as adenine at position 6 are common modifications and therefore frequently investigated. They are associated with many cellular processes like changes in binding behaviour of the DNA to proteins, the replication and repair mechanisms or the regulation of transcription.[1] However, other modifications also exist in bacteria,as we could detect in two distinct species.

Methods

The genomic DNA of *Escherichia coli* and *Klebsiella grimontii* strains were extracted and their DNA were hydrolysed to yield the nucleobases. The composition of nucleobases and their modifications were analysed via Ultra High Performance Liquid Chromatography coupled to High Resolution Mass Spectrometry (UHPLC-HRMS) using a buffered gradient and a polar end capped column.

Possible matching DNA modifications were examined and evaluated using HRMS2 techniques, then synthesised and also hydrolysed. Finally, utilizing a comparison of retention time, HRMS1 and HRMS2 data, the modifications were identified.

Results

Investigation of the DNA modification of bacteria (*E.coli*, *K. grimontii*) showed two so far not reported DNA modifications. Additionally, 2-methylguanosine was synthesised as described by Lu *et al.* [2] and then hydrolysed to obtain the methylated nucleobase. Retention time, MS1 and MS2 data were in agreement with the *K. grimontii* sample.

Innovative aspects

- Two new DNA modifications discovered in two bacterial genera
- One new DNA modification identified

Acknowledgements

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LipidSpace: Simple Exploration, Reanalysis, and Quality Control of Large-Scale Lipidomics Studies

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Currently, lipid analysis is gaining tremendous importance. Discoveries are made, resulting in a better understanding of biological membranes and signaling functions. With new mass spectrometers moving closer to 100 Hz, lipidomics has increased speed, so lipid data acquisition is way faster than data interpretation. As a result, thousands of datasets including lipidomes (sets of lipid species from individual samples) are generated. However, they cannot be that quickly analyzed, digested, and interpreted anymore without a proper analysis tool. To overcome this bottleneck, we developed LipidSpace [1], designed to mine lipidomes structural space, fostering the rapid comparison of hundreds of lipidomes. When considering the molecular similarity of all lipids, we can better understand which classes have the potential to compensate for changed lipid classes to sustain membrane homeostasis functionally. Similarities of complete lipidomes are computed to cluster them together. An entire hierarchical relationship of the lipidomes is reported. Additionally, study variables related to the samples, such as age, body mass index, or condition, can be added to the analysis. LipidSpace offers a function to quickly determine subsets of lipids across all lipidomes that can describe these study variables well. These lipids may act as putative biomarkers, helping to understand underlying mechanisms, or serving for quality control. Four built-in tutorials give an introduction to functions and methods such as feature analysis or quality control. We used this suite to reanalyze and combine already published datasets and made additional discoveries besides the published conclusions. To summarize, it is now possible to easily compare similar or distinct lipidomes and gain quick knowledge of which lipids are shaping a distinct lipidome, which lipids can be used to separate conditions or to identify the insufficient quality of measured datasets during an early stage of a study.

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P 164

Software tools enabling real-time and post-acquisition quality control in metabolomics and lipidomics workflows

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Question: In medium-to-large scale omics studies, post-hoc data evaluation for quality control and raw data correction purposes is time-consuming, tedious, and potentially useless for preventing sample consumption in case of inadequate system performance.

In order to establish adequate system monitoring procedures, to prevent loss of valuable samples, and to increase laboratory efficiency, we present a suite of software tools enabling real-time monitoring of key quality attributes of LC-MS/MS runs including ion mobility measurements, post-acquisition review, data correction of acquired data sets, and metabolite annotation quality scoring.

Methods: A reference urine sample (NIST SRM 3672 was analyzed on a Bruker Impact VIP II Q-Tof mass spectrometer coupled to an Agilent 1290 Infinity II UHPLC. Out of 300 injections, every 10th sample was labeled a "Quality Control" (QC) sample to mimic the design of a classical metabolomics experiment.

Data quality was monitored in parallel with acquisition by inspection of endogenous metabolites previously chosen to represent the broader metabolome ("QC analytes") using the TASQ® RealTimeQC 2023b software.

Following data acquisition, MetaboScape® was used to perform post-acquisition retention time alignment, mass recalibration, and compensation for run-order effects in peak intensity measurements using a soft LOESS correction curve based on feature-specific intensities in designated QC samples.

Results: The RealTimeQC function of TASQ visualizes key attributes of acquired data from "QC runs" in real-time. It facilitates inspection of random and systematic variation across key parameters (LC peak area, peak intensity, retention time, *m/z,* and isotope pattern fidelity/mSigma) for previously defined target QC analytes. Outliers are flagged automatically. Using MetaboScape, more than 350 metabolites were automatically annotated using a Target List of metabolites described to be present in human urine (https://hmdb.ca/). Each metabolite in the dataset met the annotation quality scoring criteria of <1ppm mass accuracy and isotope pattern fidelity (mSigma) <20.

Conclusions: The data quality achieved with traditional post-acquisition QC workflows can be improved upon with real-time monitoring of QC analytes. Control over data quality is achieved by comprehensive monitoring of measurements and informed decision making while running the analysis.

Real-time QC monitoring has potential for application in early detection of instrument performance issues, prompting corrective actions and avoiding compromised results and sample loss.

Post-acquisition data visualization can direct correction of run order effects and thus further improve the data quality in LC-MS-based metabolomic and lipidomic profiling studies.

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Probing ions from deeply embedded plasmas: From simulation to realization. Investigation of a novel long distance ion transfer unit

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Efficient sampling of native ions from plasmas is crucial for the understanding and optimization of such unique environments. However, due to the complex nature of most plasma setups, it is not always feasible to sample directly from the plasma region. Our research motivated the introduction of an experimental ion optical transfer system designed to bridge a 150 cm gap between a plasma chamber and a time-of-flight mass spectrometer (TOF-MS). This setup aims to transfer plasma generated ("native") ions over large distances with minimal mass discrimination and ion loss.

Our approach uses a segmented hexapole with matching apertures. The system allows ion sampling from the plasma region, ion guiding into the MS, while also accompanying gas flow restrictions to reduce pressure and thus collisions, including a vacuum separation stage to decouple the plasma chamber from the TOF-MS.

The experimental setup consists of an entrance aperture that utilizes a lens stack to restrict flow from the plasma chamber operating at 5 Pa, while increasing the ion kinetic energy to allow efficient guidance into the hexapole. Due to geometric constraints of the plasma system, the ion transfer stage is enclosed in a 150 cm long CF40 stainless steel tube. Inventive solutions were required to bridge an unguided distance of several cm of the ion path, caused by a mandatory gate valve. To transition ions through the open gate valve, ion optical lenses were designed to create an electric field that extends into the valve body. This system was partly introduced in the simulation domain at previous conferences.

This stage was physically realized in collaboration of the ipaMS, Germany, with Fasmatech, Greece. We have begun to characterize the entire system, consisting of an RF plasma chamber (ipaMS), transfer stage (this work), and a high-resolution custom TOF MS (TOFWERK, Switzerland), in terms of sampling and transfer efficiency, and RF/DC settings. We are currently investigating possible mass discrimination and pressure dependencies, which may affect the sampling performance, as it is crucial to characterize possible fragmentation and/or ion molecule reactions within the hexapole region. RF/ DC settings are investigated to determine the optimal transfer conditions for the entire mass range. Simulation results of these parameters obtained with SIMION are promising, with ion transfer rates of up to 90%.

This contribution compares the simulation and experimental characterizations and presents the results of initial experiments using the plasma chambers. Preliminary results demonstrate successful flow restriction, optimized electric fields, and promising ion transfer rates. We believe that the insights gained through this research will contribute to the practical implementation of ion optical transfer systems at low pressures and long distances.

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Structural elucidation of a medium-heavy crude oil sample and MS^N using tandem mass spectrometry with isobaric precursor isolation in a multiple-reflection time-of-flight mass spectrometer

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A multiple-reflection time-of-flight mass spectrometer (MR-TOF-MS [1]) was developed and employed, for the first time, for ultra-high resolution tandem mass spectrometry. In addition, a new atmospheric pressure interface and multiple ion sources were built and a custom software package with advanced control and analysis techniques was developed. The software is also used to operate MR-TOF-MS devices at particle accelerators for the measurement of binding energies of exotic nuclides [2]. Isobaric precursor molecules in a medium heavy crude oil sample were isolated and dissociated for structural elucidation.

To achieve isobaric isolation, ions are injected into the TOF analyzer from a radio-frequency (RF) trap, dispersed in TOF according to their mass-to-charge ratios, and then dynamically re-trapped in the same RF trap [3]. After isolation, the ions are dissociated within the RF trap and then injected back into the TOF analyzer. This procedure can be applied repeatedly, enabling multiple-stage tandem mass spectrometry in time (MS^N), as demonstrated in an MS^4 measurement of hexamethoxyphosphazene. Isolation of precursor molecules was demonstrated with a mass separation power up to 250 000 (FWHM).

Four isobaric mass lines in a sample of medium-heavy crude oil were isolated with an isolation window of 10 mu and a suppression factor of 200. The resulting fragments were measured with a mass resolving power of 200 000 and a mass accuracy of better than 1 ppm, and their elemental compositions were identified. For two of the isobaric precursor molecules, isomeric molecular structures were identified. This structural elucidation was only possible due to the unprecedented, ultra-high mass separation power of the device, cleanly isolating the precursor molecules.

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P 167

Autonomous mass calibration of benchtop quadrupole Orbitrap™ mass spectrometers ensures continuous long-term operation and reliable drug compounds quantification

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Introduction

While Orbitrap mass spectrometers provide excellent mass accuracy, slow drifts require regular calibration of the full mass range with a mix of calibrants. Additionally, the mass drift may be corrected by using a lock mass providing sub-ppm mass accuracy. However, this lock mass correction cannot handle large mass drifts. This study introduces a self-calibration mode based on a one-point mass calibration which extends the validity of the full mass range calibration. This self-calibration enables fully autonomous operation of the mass spectrometer for over one month while maintaining the highest data quality, mass accuracy and reproducibility. This approach is evaluated for the quantification of drug compounds in plasma.

Methods

On Thermo Scientific™ Orbitrap Exploris™ mass spectrometers, after an initial mass and system calibration with Pierce™ FlexMix™ Calibration Solution, a scheduled one-point mass self-calibration is performed at regular intervals over four weeks. Mass stability and quantitation results are monitored for small molecule LC-MS analyses of three chosen pharmaceutical reference standards spiked into crashed bovine plasma. Using Thermo Scientific™ Chromeleon™ Chromatography Data System (CDS) Software for data acquisition and processing, the LLOQ values for the reference standards are determined, as well as the mass errors across the full concentration range. Mass deviation trends are monitored over several weeks.

Preliminary Data

A self-calibration mode is used to perform a scheduled one-point mass calibration on a regular basis to recalibrate the current full-range mass calibration curves using fluoranthene ions from the internal calibrant source. This integrated ion source conveniently provides both positive and negative ions, enabling to calibrate both instrument polarities without involving the Electrospray Ionization (ESI) calibration or analytical ion source.

Initial experiments are performed using three chosen pharmaceutical reference standards spiked into crashed bovine plasma to determine the linearity, dynamic range and LLOQ values for each target compound and to evaluate the mass errors across the full concentration range. Several scheduling schemes are tested to evaluate the validity of the fluoranthene based mass calibration over time and its deployment towards a fully autonomous operation of the mass spectrometer for up to four weeks.

All analytes were detected with a mass error below 3 ppm RMS and a linearity of quantification exceeding 4 orders of magnitude. Quantification limits of 25 pg/mL and 50 pg/mL (25 ppt and 50 ppt respectively) is achieved for the 3 target compounds in crashed bovine plasma. Repeatability of detection and quantification is also demonstrated for all drug compounds over several weeks.

Novel Aspect

A one-point mass self-calibration enabling the autonomous operation of quadrupole Orbitrap instruments for over one month is presented.

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On the direct detection of microplastic particles by Flowing Atmospheric-Pressure Afterglow Mass Spectrometry (FAPA-MS)

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Microplastics (MPs) are widespread pollutant particles. They can be probed with Raman and Fourier transform infrared (FTIR) spectroscopy combined with optical microscopy. In addition, pyrolysis (Py) or thermal extraction and desorption (TED) coupled to gas chromatography-mass spectrometry (GC-MS) are used for MPs characterization.[1] In the latter case, sample throughput is limited due to sample introduction and GC separation. This work presents a feasibility study for the direct and rapid analysis of MPs using high-resolution mass spectrometry and a plasma-based ambient desorption/ionization source (FAPA, flowing atmospheric-pressure afterglow).[2]

In earlier work, an in-house built ambient ionization source (modelled after the pin-to-capillary (p2c) FAPA design by Shelley *et al.*[3]) was coupled to HR-Orbitrap MS and successfully used to characterize different MPs, which were produced in-house from various plastic materials including polystyrene (PS), polypropylene (PP), low-density polyethylene (LDPE), and polycarbonate (PC). Simultaneous detection of characteristic ions of a given particle and imaging of particles on a sampling mesh was feasible. The availability of single-particle MS information is considered an advantage over GC-MS data obtained after using Py or TED for sample introduction. The detection limits (LOD) for PS MPs were 311 µm in size and 1.3 mg in mass. Also, principal component analysis (PCA) was successfully used for particle differentiation based on their composition.

This work presents an improved sample desorption/ionization approach for direct MPs sampling. This includes using higher temperatures for desorption enhancement (approximately 500 °C, achieved with economic and commercially available parts) and a tailored source housing combined with a halo-shaped (h-FAPA) source configuration.[4] The scope of the study was also expanded to include MPs from poly(ethylene terephthalate) (PET), poly(methyl methacrylate) (PMMA), and poly(vinyl chloride) (PVC) with sizes ranging from 125 to 250 µm. Data visualization and interpretation were performed using Kendrick mass defect plots and PCA. Compared to our previous results, h-FAPA-MS analyte ion signals were at least 65% higher for selected ions in all MPs. These ions were detected mainly as protonated species $[M+H]^+$. Higher thermal desorption temperatures aided in detecting all MPs, as the presence of higher molecular weight fragments added specificity to the analysis. Notably, experiments with the h-FAPA source demonstrated lower mass-based LODs for MPs compared to its earlier p2c-FAPA counterpart (*e.g.*, 14 µg vs 1.3 mg for PS, respectively).

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P 169

Utilizing the fluorescence of the MALDI-process to investigate physical and chemical properties of the matrix layer in MALDI-MS imaging

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AIM: During the MALDI-process, most molecules that serve as a matrix emit fluorescent light. This light may carry yet neglected information complementary to MS imaging (MSI) results. Previously we presented a method that allows to record optical emission spectra of matrix and analyte for each individual pixel during MALDI-MSI runs using the same laser pulse for both fluorescence excitation as well as ablation/ionization. With this method we are able to observe the emitted light of different matrices applied to different types of tissue.

METHODS: Measurements were performed on a modified transmission-MALDI (t-MALDI) setup of a dual ion source (spectroglyph) attached to a Q Exactive plus (Thermo) mass spectrometer. Fluorescent light was collected behind the sample using a microscope objective and focused into an optical fiber (400 µm diameter) of an optical spectrometer (Avaspec-ULS 2048 CI-EVO-VA-50, Avantes). MSI and spectroscopic data were processed using Python and evaluated using SCiLS lab (Bruker). Cryo-sections of mouse brain (cerebellum) were 2 mounted on a glass slide and coated with different matrices (2,5-Dihydroxobenzoic acid (DHB), 2,5-Dihydroxyacetophenone (DHAP), 4-(2-Hydroxyphenylazo) benzoic acid (HABA) or alpha-Cyano-4-hydroxycinnamic acid (CHCA)) by sublimation. As an ionization/excitation laser, a frequency tripled Nd:YLF laser (explorer OEM Nd:YLF, spectra physics) with a wavelength of 349 nm a was used. The Laser was operated between 1.8 A and 2.0 A at 100 Hz (1000 Hz for HABA) to record both an optical and a mass spectrum.

RESULTS: We collected the emission light at every pixel produced during MALDI-MSI runs of different matrices applied to mouse cerebellum. Using custom software, the resulting spatially resolved fluorescence spectra were combined with the respective mass spectra to enable parallelized data analysis. Each matrix produces characteristic fluorescence spectra. Spectra for CHCA and DHB are dominated by fluorescence between 420 nm and 560 nm, with a maximum at 460 nm (CHCA) or 486 nm (DHB) and a shoulder at 445 nm (CHCA) or 440 nm (DHB). The fluorescence signal of HABA is in total significantly weaker than that of the other employed matrices and is dominated by fluorescence between 420 nm and 650 nm with a maximum at 480 nm, a shoulder at 440 nm, and a second maximum at 565 nm. Compared to this, a more red-shifted emission band between 440 nm and 750 nm was observed with DHAP (maximum at 570 nm and a shoulder at 500 nm). For some matrices, significant differences between the fluorescence spectra on different types of tissue where observed. For DHB, spectra on the white matter are generally more intense as compared to the gray matter. Contrary, for HABA, there was no difference between white and gray matter, but the granular layer showed an overall more intense fluorescence compared to the rest of the tissue. DHAP, like CHCA, showed no difference in intensity between the different tissue types of the cerebellum.

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Automation of MS-based sample preparation for in-depth "omics: from cellular assays to 3D-model systems and protein characterization

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Motivation

Mass spectrometry (MS)-based workflows need to be highly reproducible and accommodate high sample throughput. At the same time, they should require minimal hands-on time and a fast turnaround, especially if they are to be used for larger scale studies or in an industrial/clinical setting. In order to achieve both high reproducibility and high productivity, laboratory automation is required, and new workflows should be designed with automation in mind to facilitate rapid adaption and scaling.

Here, we present a framework for automated liquid-handling in MS workflows for MALDI-TOF cell assays, protein digestion and enrichment, lipid extraction, as well as 3D-cell culture assays, using a benchtop automated liquid handling system (Cybio Felix), demonstrating the versatility and effectiveness of small-scale laboratory automation.

Results

Cell assays are essential in drug discovery, development, and quality control. Combining them with MALDI-TOF MS for fast and information-rich readout provides a versatile platform for label-free mechanistic and phenotypic cell assays. 1 Automation ensures the necessary high throughput and reproducibility. We automated crucial steps such as cell culture plate preparation, drug treatment, and MALDI MS target plate preparation including spotting of matrix chemicals.

Industrial protein characterization workflows require digestion, sample cleanup and enrichment of peptides and/or proteins. These steps often require functionalized magnetic beads or stage tips and are tedious to perform manually. Here, we developed automated workflows for protein digestion and cleanup using both SPE-stage tips and magnetic beads for antibody-based protein enrichment.

Lipidomics workflows frequently use liquid-liquid extraction as the initial enrichment step. This is tedious to perform manually, especially with the large number of samples commonly used in clinical/industrial lipidomics studies, as it often requires careful phase separation. We automated the liquid extraction steps using virtual level detection, which also allows for easy adaptions of the extraction protocol without the need for extensive re-optimizing of the automation scripts.

Assays using spheroids or organoids are powerful tools in pharmaceutical and basic research. We automated a spheroid assay using virtual level detection and dynamic tip movement during aspiration/dispensing to avoid disturbing or accidentally aspirating the spheroids. Additionally, automation drastically shortens the time the spheroids spend at room temperature outside their optimal growing conditions.2

Conclusion

Using a single benchtop liquid handling system, we were able to automate diverse MS-based workflows from lipidomics and protein characterization to cell-and spheroid-based assays.

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P 171

Versatile atmospheric pressure field desorption ion source allowing for robust operation, emitter observation, and emitter heating

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Introduction

Recently, atmospheric pressure field desorption (APFD) mass spectrometry (MS) has been introduced as a new variant of field desorption (FD) mass spectrometry [1)]. APFD aimed at combining the capabilities of FD-MS with instruments equipped with an atmospheric pressure (AP) interface. APFD has so far been demonstrated to yield both positive and negative even electron ions of highly polar or ionic compounds [1, 2)] as well as to enable the generation of positive molecular ions, $M^{+\bullet}$, of polycyclic aromatic compounds [3)]. The prototype setup for APFD was based on a nano-electrospray ionization (nanoESI) source slightly modified to allow for emitter positioning in front of the AP interface of a Fourier transform-ion cyclotron resonance (FT-ICR) mass spectrometer. The entrance electrode of the interface was set to negative or positive high voltage with respect to the emitter at ground potential, thereby permitting the formation of positive or negative ions, respectively [1)].

Methods

A new custom-built APFD source for quick and reproducible sample loading on the field emitter and its positioning at the entrance electrode of the atmospheric pressure interface of a mass spectrometer is described. The device also provides means for observation of the emitter during operation via a USB microscope camera and enables an emitter heating current (EHC) to be applied as in traditional FD-MS. To acquire APFD mass spectra, the instruments are operated as if in electrospray mode, i.e., using conventional settings.

Results

Using an EHC both speeds up the desorption of the analytes and allows for the desorption/ionization of analytes of higher molecular weight than without emitter heating, e.g., polystyrenes (Fig. 1). Using an EHC also permitted to analyze neat perfluorononanoic acid (PFNA) by negative-ion APFD. Furthermore, the signal-to-noise ratio of APFD mass spectra may be improved due to higher ion currents effected by compressing the entire process into shorter periods of spectral acquisition.

Conclusion

The new setup enables robust and reliable operation in APFD-MS and expands the range of compounds accessible by APFD. It has been designed for use on a range of instruments as it can either be used on an FT-ICR mass spectrometer (Bruker ApexQe) or in combination with a trapped ion mobility-quadrupole-time-of-flight instrument (Bruker timsTOFflex), i. e., with all current AP instruments of this manufacturer.

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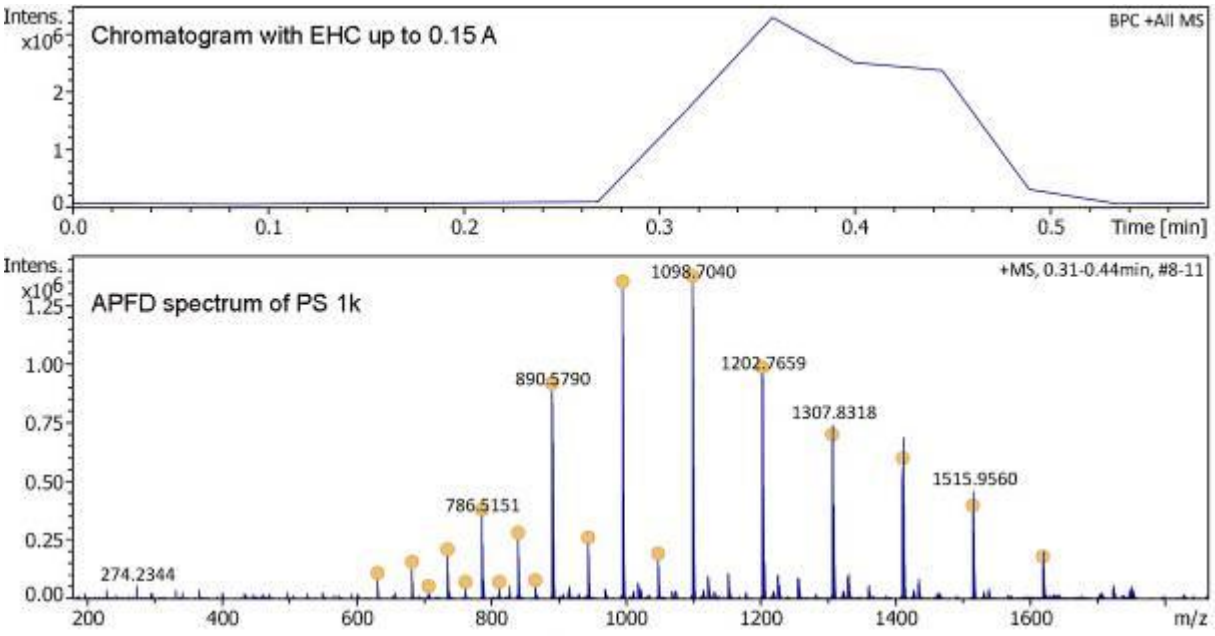
Fig. 1



Meas. m/z	Ion Formula	m/z	err [ppm]	e⁻ Conf	N-Rule
631.4306	C48H55	631.4298	-1.2	even	ok
682.4535	C52H58	682.4533	-0.3	odd	ok
707.4622	C54H59	707.4611	-1.5	even	ok
735.4943	C56H63	735.4924	-2.6	even	ok
761.5108	C58H65	761.5081	-3.6	even	ok
786.5151	C60H66	786.5159	1.0	odd	ok
811.5257	C62H67	811.5237	-2.4	even	ok
839.5566	C64H71	839.5550	-1.9	even	ok
865.5737	C66H73	865.5707	-3.4	even	ok
890.5790	C68H74	890.5785	-0.5	odd	ok
943.6190	C72H79	943.6176	-1.5	even	ok
994.6414	C76H82	994.6411	-0.3	odd	ok
1047.6838	C80H87	1047.6802	-3.4	even	ok
1098.7040	C84H90	1098.7037	-0.3	odd	ok
1202.7659	C92H98	1202.7663	0.3	odd	ok
1306.8286	C100H106	1306.8289	0.2	odd	ok
1410.8925	C108H114	1410.8915	-0.7	odd	ok
1514.9521	C116H122	1514.9541	1.3	odd	ok
1619.0221	C124H130	1619.0167	-3.3	odd	ok

Positive-ion APFD-FT-ICR spectrum of 3.5 µg polystyrene of avg. molecular weight of 1 ku when using an EHC ramp up to 0.15 A. APFD settings: ion accumulation 1.0 s per transient, shield at –4.8 kV, cap at –5.5 kV, dry gas at 1.5 l min^{-1} and 140 °C. The series of $M^{+\bullet}$ ions covered 6mer (*m/z* 682) to 16mer (*m/z* 1724). For more reliable formula assignment to the fragment ion peaks, the spectrum had been internally recalibrated based on several abundant PS molecular ion peaks as a reference. Yellow dots at the peak tops refer to peaks with formula assignment and even-electron ions are fragment ions.

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Dependency of sample introduction on the analysis of electrolyte residues in LIB recycling material via GC-MS

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The demand for lithium-ion batteries (LIBs) is witnessing a substantial increase in recent years. Meeting this growing demand requires a significant boost in the recycling of LIBs in Europe, aiming to decrease reliance on raw material suppliers. The European Union has responded to this challenge by setting regulatory targets, mandating that 65% of LIBs be recycled by 2025 and 70% by 2030.1 Despite these efforts, the recycling of LIBs lacks standardization, presenting a challenge in establishing a consistent and reliable resource.2,3

This study addresses the need for standardized recycling processes by focusing on the identification of organic electrolyte residues in black mass, a LIB recycling material. Black mass is gained from discharged LIBs, which are then shredded, dried and seperated to enhance the anode and cathode active material content. 4 The primary goal of this work is to establish a dependable methodology to maintain the quality of LIB recycling processes. Three sample introduction methods—thermodesorption, headspace, and solid-phase microextraction—are evaluated in conjunction with gas chromatography-mass spectrometry (GC-MS). In the headspace method, samples are agitated and subsequently analyzed by GC-MS, revealing the presence of linear and cyclic carbonates (e.g., dimethyl carbonate, ethyl methyl carbonate, ethylene carbonate) and aging products such as 2,5-dioxahexanedioic acid dimethyl ester (DMDOHC). The findings are then compared with those obtained through thermodesorption and solid-phase microextraction. The study further explores the possibilities and challenges associated with quantifying these residues using internal standard addition or external calibration.

In conclusion, this research showcases three effective methods for identifying and quantifying organic electrolyte residues in LIB recycling material. These methods contribute to the development of more efficient and sustainable recycling processes for LIBs, aligning with the broader goals of enhancing resource recovery and minimizing environmental impact.

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Signatures of aspirated charged droplets in single spectra intensity distributions in commercial LC-ESI-MS systems

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The investigation of the dynamics of aspirated charged droplets within commercially available MS devices has been a relevant topic in recent years. As demonstrated in the past, charged droplets are aspirated from electrospray ion sources . Despite common assumptions in the community, electrospray ionization does not lead to the full evaporation of initially formed charged droplets before they enter the instrument vacuum system. Previous research has shown that droplets can significantly contaminate the device as they pass through the inlet system and various pressure stages of the mass analyzer, which can lead to a loss of performance.

It is interesting to investigate this common observation under analytical conditions in more detail. For this, the intensity distribution within single (non-summed) spectra under constant conditions on various mass analyzers and the aspect of solvent influence is evaluated.

The utilized mass spectrometers are a Quadrupole Ion Trap (QIT) (AmaZon ETD, Bruker), a Linear Ion Trap (LIT) (LTQ XL, Thermo Scientific), a Q-TOF (6546, Agilent), UHR-TOF (maXis 4G, Bruker) and a micrOTOF (Bruker). Each of these MS has an API source supplied by the manufacturer that is coupled with an LC system (L-7200, Merck Hitachi), which includes a binary pump that delivers the injection solution with constant liquid flow. The solution contains reserpine as analyte at a concentration of 8 µM. The mobile phase is composed of a 1:1 solvent mixture of water and acetonitrile or methanol as organic solvent compound, with addition of 0.1% formic acid.

The occurrence of very intensive ion signal bursts, most likely produced by fragmented aspirated charged droplets from ESI, has been demonstrated in past experiments. This appears also to be observable by the intensity distribution of single mass spectra: In comparison to ionization methods without liquid spraying (APCI), there is a group of single spectra with extremely high total ion intensity while the median intensity is much lower with ESI than with APCI. In addition, ESI exhibits a comparably large fraction of spectra, which are entirely empty while APCI does not show such a phenomenon. In summary, this indicates that a significant fraction of the total ion signal, visible in common ESI experiments, stems in fact from aspirated droplets fragmented in the vacuum system and not from ions generated in the ion source.

The aspiration of droplets and therefore also the fraction of ions generated in the vacuum system from the droplets as well as their dynamics depend on the ion source and ESI parameters. We present a detailed analysis of the intensity distributions in dependence of such parameters and the interpretation of the experimental results in terms of droplet aspiration from ESI.

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Automated liquid handling extraction and rapid quantification of underivatized amino acids and tryptophan metabolites from human serum and plasma using dual-column U(H)PLC-MRM-MS and its application to patient samples

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Amino acids (AA) represent the building blocks of proteins and other metabolites, including nucleotides and hormones, and serve as energy source and signalling molecules. Further, AA and in particular metabolites of tryptophan (TRP) metabolism, exhibit an important role as neurotransmitters and in inflammatory response. Dysregulation of AA metabolism is often associated with pathological phenotypes and metabolic disorders, including diabetes, cancer, and phenylketonuria Alzheimer's disease. Measurements of AA levels and their metabolites in body fluids like serum and plasma are of diagnostic and clinical interest, as they provide information about the metabolic status of an individuum, insights into immune responses, neurotransmitter balance and may serve as diagnostic readouts to monitor disease progression. Quantitative analysis of AA and TRP metabolites in large sample sizes, like patient cohorts, requires reproducible and sensitive high-throughput workflows covering sample extraction, sample preparation, sample measurement and data analysis. Although LC-MS is a powerful tool for absolute quantification of small molecules, chromatographic separation of free amino acids is still challenging due to their physical-chemical properties.

For the derivatization-free absolute quantification of 20 AA and 6 TRP metabolites from serum and plasma, we developed an automatized protocol for sample extraction and preparation using an Andrew+ Pipetting Robot (Waters) and an U(H)PLC separation using a mixed mode reversed phase-anion exchange chromatography column and a reverse-phase column coupled to triple quadrupole mass spectrometry (MS) operated in multiple reaction monitoring (MRM) mode. Fast U(H)PLC-MRM-MS analysis in less than 10 minutes per sample represented excellent reproducibility (CV < 15%), linearity (R2 ≥ 0.98) and sensitivity (LLOQ ≤ 0.6 µM). Optimized protocols for automatized extraction revealed high recovery (≥ 85%) and good reproducibility (CV < 15%), for both, serum and plasma. Samples processed with the serum protocol exhibited values consistent with the literature. Analyte concentrations determined with the plasma protocol were successfully verified against certified reference values (NIST SRM 1950 plasma). Our semi-automated extraction workflow and dual-column U(H)PLC-MRM-MS method was applied to a human prostate cancer study, where we were able to discriminate between treatment regimens based on the differences in measured metabolite level.

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Improved compound identification in GC-MS analysis using an EI&CI-TOFMS

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A time of flight (TOF) mass spectrometer simultaneously operating an electron ionization (EI) and a chemical ionization (CI) source is presented for GC-MS based target, suspect and non-target analysis. By coupling a gas chromatograph (GC) via a y-splitter directly to both ionization sources, structural as well as accurate mass molecular ion information is generated in parallel. Hence, target and suspect screening analysis as well as effective non-target analysis using GC-MS is improved considerably. In this Presentation, various experiments will be discussed, illustrating the potential of the mass spectrometric setup especially for non-targeted and suspect screening approaches. The Presentation includes applications in fields such as environmental contaminants, material emissions, food flavour analysis and metabolomic research.

An Agilent 7890A GC was coupled to an ecTOF (TOFWERK, Thun, Switzerland), operating simultanesouly a 70 eV EI source and a newly developed CI ource (HRP Source, TOFWERK, Thun Switzerland) [1,2]. Various GC methods and sampling procedures were employed depending on the analytical need of the study, including liquid injection of extracted samples, headspace sampling including SPME and thermal desorption using Tenax tubes as preconcentration steps. To generate the ideal molecular ion information different reactant ions (e.g., N2H+, H3O+ and NH4+) were used for the chemical ionization process.

It is shown that the performance of the EI&CI-TOFMS is feasible for standard procedures employed by routine laboratories, e.g., target analysis for material emissions or steroid screening. Whilst standard GC-MS methods mainly focus on target analysis, especially suspect screening and non-target analysis is enabled and improved by the EI&CI-TOFMS as detector. Especially when EI library hits are only accounted as "fair" with low corresponding probability, the additional chromatographic and CI information can be used to increase compound identification confidence. False positives from an EI-only approaches can easily be identified and often correctly annotated using the additional information generated by CI. Furthermore, compounds not listed at all in libraries have a high uncertainty for identification using an EI-only approach. Using the accurate mass information on the molecular ion provided by CI, sum formula for these unknowns can be derived. Combining this molecular information with the structural information generated by EI, tentative structure elucidation becomes feasible in many cases.

[1] Bräkling, S. Anal. Chem. 2022., 94 (15): 6057-6064.

[2] Bräkling, S. et al. J. Rapid Commun. Mass Spectrom., 2023. 37:e9461, 3, 499–509.

Fig. 1

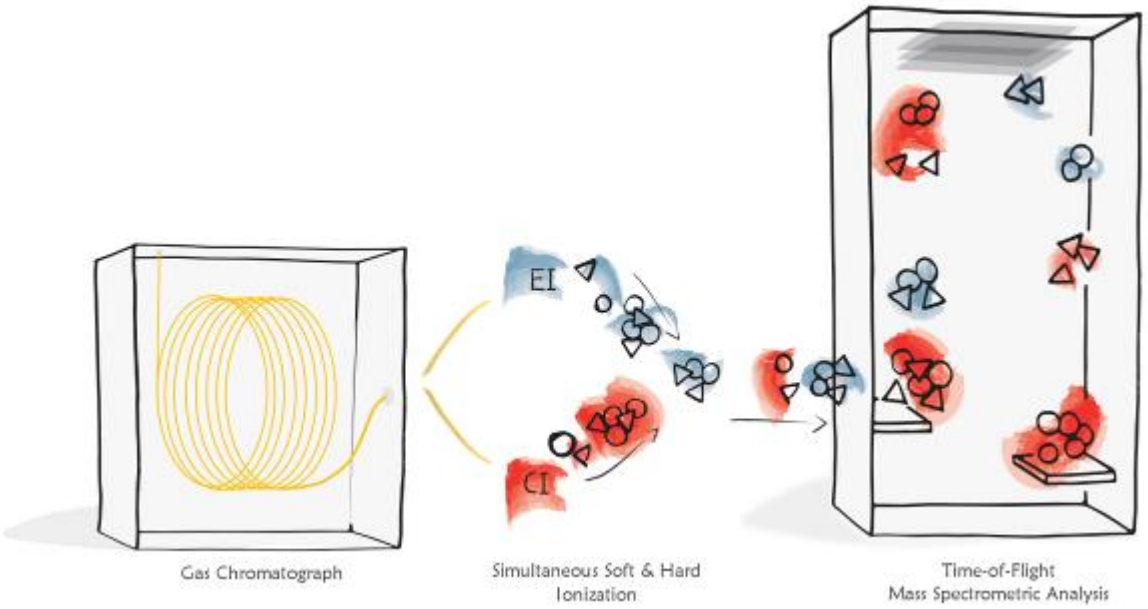
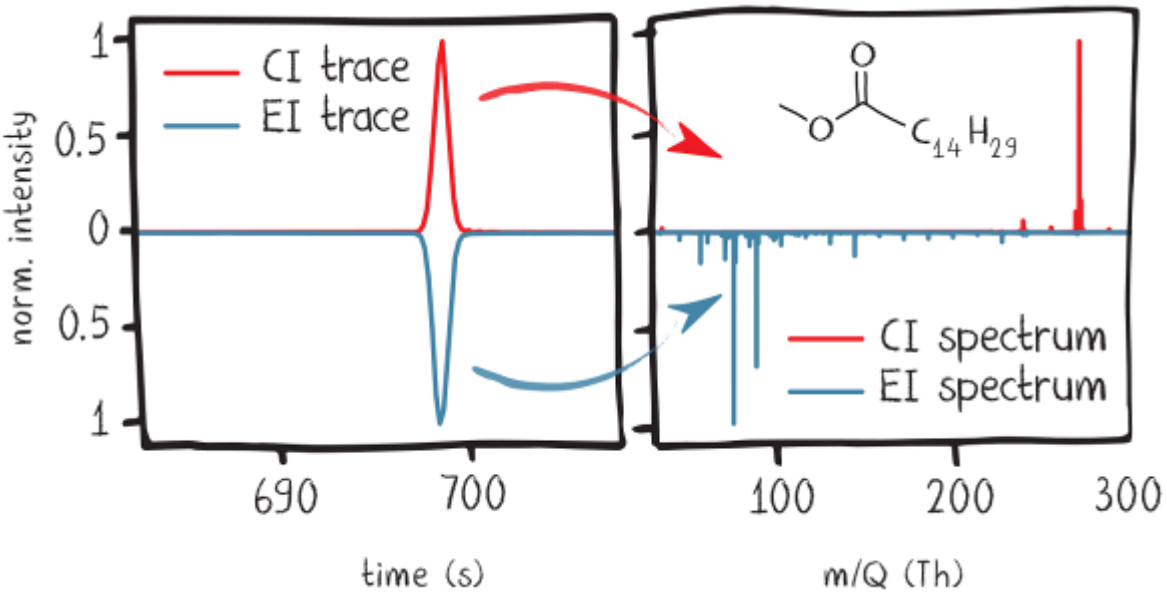

EI
CI
Gas Chromatograph
Simultaneous Soft & Hard Ionization
Time-of-Flight Mass Spectrometric Analysis

Fig. 2


CI trace
EI trace
norm. intensity
1
0.5
0
0.5
1
690
700
time (s)
$C_{14}H_{29}$
CI spectrum
EI spectrum
100
200
300
m/Q (Th)

P 177

Intercomparison study of two high-resolution TOF-MS for ultra-trace detection

L. Mokros[1], N. Pengemann[1], H. Kersten[1,2], T. Benter[1]

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[2]*Institute for Pure and Applied Mass Spectrometry, Wuppertal, Germany*

This study focuses on the detection of compounds generated by laser and/or RF generated plasmas as applied in manufacturing processes. Detection takes place at the ultra-trace (>100 ppbV mixing ratios at total pressures of 5 Pa and below, i.e., >1E- 8 mbar) level. This includes water as trace component (target >1E-8 mbar partial pressure) as well as hydrocarbons and metal hydrides (target >1E-10 mbar partial pressure). In-situ monitoring of these compounds is of paramount importance for the controlled, prolonged operation of delicate devices. Furthermore, gaining a deeper understanding of the ion-molecule chemistry prevailing in such plasmas, including the unequivocal identification as well as the temporally resolved monitoring of individual species produces as primary or secondary products, is essential. For this study, two customized high-resolution LTOF time-of-flight mass spectrometers (TOFWERK, Thun, Switzerland; m/Δm > 8000) are employed. Among other things, both instruments are equipped with a custom ion source providing a 70 eV electron ionization mode, as well as a close to 100% transmission "fly-through" ion mode. Both instruments feature identical TOF analyzers, but rather different pumping stages. Instrument one is designed in a classical split-flow turbo pump, all-aluminum, O-ring sealed housing setup. Instrument two features three dedicated turbo pumps evacuating the analyzer, the ion transfer, and the inlet/ion source compartment, respectively. Furthermore, the transfer and ion source compartments are made of conflat flange sealed stainless steel and can be heated to 120°C during operation. It was shown earlier that both heating as well as ion source and transfer stage pressures well below 1E-8 mbar are prerequisites for temporally resolved ultra-trace detection of plasma generated and/or wall desorption of water within the respective plasma recipient. The unequivocal identification of species generated by the different plasmas (e.g. NH_4^+ vs H_2O^+) is the central focus of this research. In particular, the differences between instrument 1 and instrument 2 in terms of i) background signal contributions, ii) attainable signal to noise ratios in the present gas matrix comprised mostly of hydrogen, and iii) the temporal resolution for recording full mass spectra will be highlighted.

P 178

Orbitrap exploris with simultaneously accessible ESI and LIFDI sources

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Introduction

Many highly reactive organometallic compounds are not accessible by Electro-Spray Ionization (ESI) because they are neutral molecules and do not survive protonation or other cationization processes. Instead they require an ionization method which can actively ionize the analyte. Due to the high reactivity of these analytes, it is essential that the ionization is as soft as possible. All this calls for using Liquid Injection Field Desorption Ionization (LIFDI) with an Orbitrap Exploris (Thermo Fisher Scientific) upgraded to a dual source mass analyzer.

Methods

The Orbitrap Exploris mass analyzer with standard ESI source is modified by the installation of a complementary LIFDI ion source making LIFDI and ESI accessible without downtime between sample analysis with the two quite different ionization techniques: The ESI source does operate at the front of the instrument as usual and, on demand, the additional LIFDI source can alternatively inject ions from the back end of the instrument into the HCD cell from where the LIFDI ions enter the C-trap and the Orbitrap the same way as ESI ions do.

Fig 1. Thermo Scientific Orbitrap Exploris MS platform (here Orbitrap Exploris 120 mass spectrometer) with standard ESI source and additional semi-automated LIFDI source

The spectra acquisition is identical for both ionization methods. The mass calibration of ESI is valid for LIFDI as well. Changing the method between samples is performed by using either the ESI capillary or the LIFDI capillary together with the respective set of tune parameters.

Preliminary results

LIFDI spectra of air/moisture sensitive compounds are presented with intact molecular ions like the M+• ion of Y(C9H20N3)3 with negligible fragment ion intensities. The resolving power of the Orbitrap Exploris is high enough to distinguish between the molecular ions with 13CN from those with C15N with Δm/z 6.3 mDa.

Fig 2.

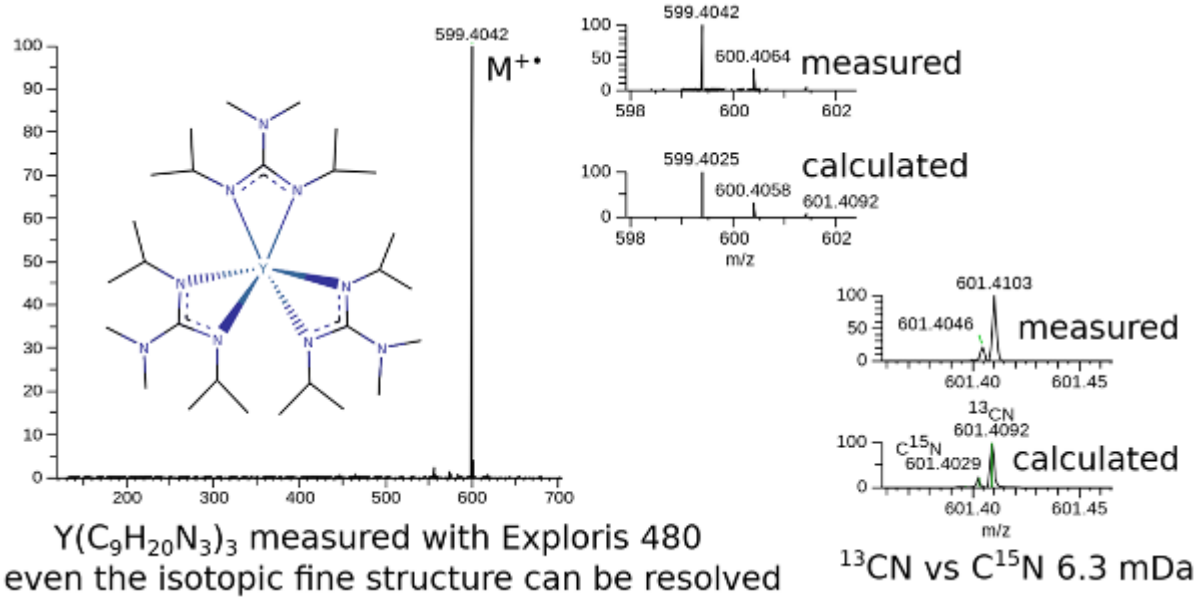
Novel aspects

The very high mass resolution of Orbitrap Exploris mass spectrometers is merged with the unmatched soft ionization properties of the LIFDI technique at anaerobic conditions without sacrificing the standard ESI capabilities at ambient conditions.

Session 13: Instrumentation and Automation
Poster Presentation

Fig. 1

Fig. 2



$Y(C_9H_{20}N_3)_3$ measured with Exploris 480
even the isotopic fine structure can be resolved

^{13}CN vs $C^{15}N$ 6.3 mDa

P 178

Intercomparison study of two high-resolution TOF-MS for ultra-trace detection

L. Mokros[1], N. Pengemann[1], H. Kersten[1,2], T. Benter[1]

[1]*Bergische Unversität Wuppertal, Wuppertal, Germany*
[2]*Institute for Pure and Applied Mass Spectrometry, Wuppertal, Germany*

This study focuses on the detection of compounds generated by laser and/or RF generated plasmas as applied in manufacturing processes. Detection takes place at the ultra-trace (>100 ppbV mixing ratios at total pressures of 5 Pa and below, i.e., >1E- 8 mbar) level. This includes water as trace component (target >1E-8 mbar partial pressure) as well as hydrocarbons and metal hydrides (target >1E-10 mbar partial pressure). In-situ monitoring of these compounds is of paramount importance for the controlled, prolonged operation of delicate devices. Furthermore, gaining a deeper understanding of the ion-molecule chemistry prevailing in such plasmas, including the unequivocal identification as well as the temporally resolved monitoring of individual species produces as primary or secondary products, is essential. For this study, two customized high-resolution LTOF time-of-flight mass spectrometers (TOFWERK, Thun, Switzerland; m/Δm > 8000) are employed. Among other things, both instruments are equipped with a custom ion source providing a 70 eV electron ionization mode, as well as a close to 100% transmission "fly-through" ion mode. Both instruments feature identical TOF analyzers, but rather different pumping stages. Instrument one is designed in a classical split-flow turbo pump, all-aluminum, O-ring sealed housing setup. Instrument two features three dedicated turbo pumps evacuating the analyzer, the ion transfer, and the inlet/ion source compartment, respectively. Furthermore, the transfer and ion source compartments are made of conflat flange sealed stainless steel and can be heated to 120°C during operation. It was shown earlier that both heating as well as ion source and transfer stage pressures well below 1E-8 mbar are prerequisites for temporally resolved ultra-trace detection of plasma generated and/or wall desorption of water within the respective plasma recipient. The unequivocal identification of species generated by the different plasmas (e.g. NH_4^+ vs H_2O^+) is the central focus of this research. In particular, the differences between instrument 1 and instrument 2 in terms of i) background signal contributions, ii) attainable signal to noise ratios in the present gas matrix comprised mostly of hydrogen, and iii) the temporal resolution for recording full mass spectra will be highlighted.

P 179

Orbitrap astral: Ensuring robustness and maintaining speed via scan sorting for DIA

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[1]*Thermo Fisher Scientific (Bremen) GmbH, Bremen, Germany*

A key development of the Orbitrap™ Exploris™ platform for extending instrument robustness was Quadrupole Configuration Switching (QCS). Here, the quadrupole rod pair, or configuration, receiving attractive resolving DC voltages is switched depending on an encoding of the isolation center which ensures each rod pair is set to attractive potentials for ~50% of isolation events. This results in a substantially equal deposition of filtered sample material on all four rods, and thereby minimizes performance-altering field perturbations and doubles the time between cleanings1. A limitation of this method is the switching time, ~6 ms on Exploris, which renders the method and the Exploris, as front-end of the new Orbitrap Astral™ instrument, incompatible "as-is" with the demands of Astral"s 200 Hz scanning in Data Independent Acquisition (DIA) experiments. In this work, we reconcile maintaining robustness to contamination and ensuring 200 Hz DIA on Astral.

The quadrupole electronic supply was redesigned to speed up settling times for RF/DC changes and QCS. These were confirmed via measurements with ions from Pierce™ FlexMix™ and codified in the control software. An optimization strategy was developed to calculate the optimal scan ordering in a DIA cycle by minimizing settling times2. Testing of these features was carried out via DIA experiments with Loop Control selection "All".

The updated quadrupole electronics halved the settling time for QCS to 3 ms. Without QCS, small increases in quadrupole RF/DC, as between DIA MS2s, required 0.6 ms, while larger changes needed ≤ 3 ms. Large decreases, as between cycles or prior to an MS1, required 4 ms. Codified in a variable settling time mechanism that applies settling times depending on the quadrupole"s prior state, DIA scanning at 200 Hz was achieved with small windows and QCS disabled. With QCS, since the prior 0.6 ms between adjacent windows increases to 3 ms when the windows are assigned different rod pairs, the scan rate is reduced to 150-170 Hz. To address this, the windows were sorted first by their assigned rod pair, and then by m/z. This reduced costly rod pair switches to 2/cycle and permitted 200 Hz scanning. It also introduced a benign peculiarity that windows adjacent in m/z are not executed in order in a cycle if assigned different rod pairs. Some DIA methods, those using larger windows or other method features with settling time penalties (*e.g.*, frequent MS1s), may not benefit by sorting scans in this manner. An optimization strategy addresses the uniqueness of a given method by analyzing the method to provide the scan ordering which minimizes overhead, defaulting to traditional ordering if sorting is not beneficial. In this work, we could balance two competing requirements: meeting the demand of high performance without compromising instrument robustness, thereby keeping Orbitrap Astral running successfully for longer.

1. Peterson, *et al*. 2019. GB2583092

2. Stewart, *et al*. 2023. GB2307690.4

P 180

Optimization of the Astral analyzer's detection system: Enabling high linear dynamic range and single-ion sensitivity for deep proteome coverage

J. Petzoldt[1], T. Shanley[1], W. Balschun[1], P. Cochems[1], I. Colonius[1], B. Hagedorn[1], A. Kreutzmann[1], A. Makarov[1], D. Mourad[1], M. Ochmann[1], R. Ostermann[1], A. C. Peterson[1], T. Phlairaharn[2], H. Stewart[1], A. Wagner[1], C. Hock[1]

[1]*Thermo Fisher Scientific, Bremen, Germany*
[2]*TUM School of Natural Sciences, Department of Bioscience, Munich, Germany*

As single-cell proteomics and other MS domains move toward studies using lower sample loads and large cohorts, researchers increasingly require instrumentation with higher acquisition speed, dynamic range, and sensitivity. The Astral™ analyzer was designed specifically with these competing demands in mind. Optimization of the ion detection system becomes therefore key to provide sensitivity without compromising dynamic range.

The detection stage is comprised of a post accelerator electrode stack, an ion detector (Fig. 1), a preamplifier, and an analog-to-digital converter (ADC). The electrode stack provides an attractive potential of 10 kV to increase the yield of secondary electrons from ions impacting the conversion dynode. The secondary electrons are then accelerated onto a scintillation crystal emitting several photons per electron, which in turn are directed to a photo-multiplier tube (PMT) with a typical gain of 105. The analog signal of the last dynode is split in the preamplifier to give two output channels with relative gain ratio of 10:1 which are further processed in the ADC. The detection system is calibrated using electrospray-ionized ions from PierceTM FlexmixTM, as well as the electronic noise band to maximize dynamic range and single-ion sensitivity. A novel method based on detuning the Astral ion mirrors [1] is used, whereby single-ions are created to measure the single-ion detector response. Furthermore, the detector response is characterized over the m/z range 42-7000 using Flexmix and ammonium hexafluorophosphate.

The in-spectrum linear dynamic range was determined to be better than 1:1000, allowing the parallel detection of single-ion events and peaks with several thousand ions in the Astral analyzer. This is achieved by the combination of the preamplifier"s dual channel setup, optimization of the ADC"s input range, and the linear detector response. To optimize the individual PMT gain and determine the number of charges in a peak, the single-ion response of the detector was calibrated with m/z 525 as a function of PMT voltage. The set voltage guarantees a consistent single-ion detection efficiency of >80% within the m/z range 42-1000 (Fig. 2) and a single-ion S/N >2. The m/z-dependent detector response (Fig. 3) over m/z 42-7000 shows a maximum at m/z ~100. The intensity drops slightly to 80% at m/z 42 while the curve drops towards higher m/z to 20% at m/z 7000 due to lower conversion efficiency.

The detection system of the Astral analyzer was characterized over a wide m/z range, showing high linear dynamic range and single-ion sensitivity suitable for deep proteome coverage. Furthermore, the number of charges and S/N for each peak in a mass spectrum is obtained by calibrating the detector.

Fig. 1: Model of the in-vacuum components of the Astral detection system

Fig. 2: Single ion detection efficiency over m/z range.

Fig. 3: Single-ion detector response vs function of m/z.

[1] Stewart, et al. 2022. US20220367165A1

Fig. 1

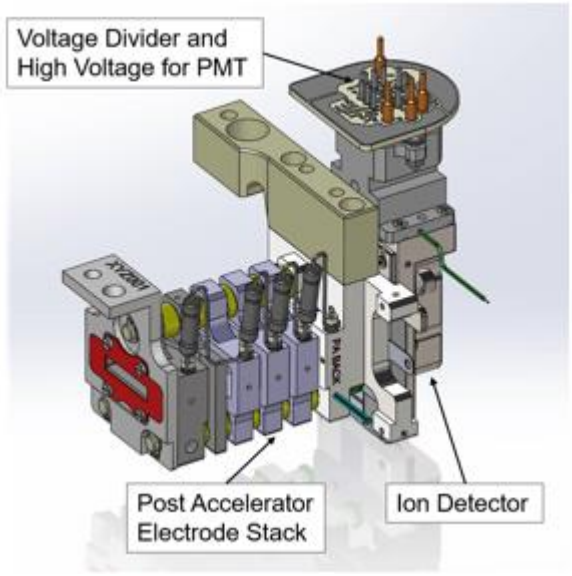

Voltage Divider and
High Voltage for PMT
Post Accelerator
Electrode Stack
Ion Detector

Fig. 2

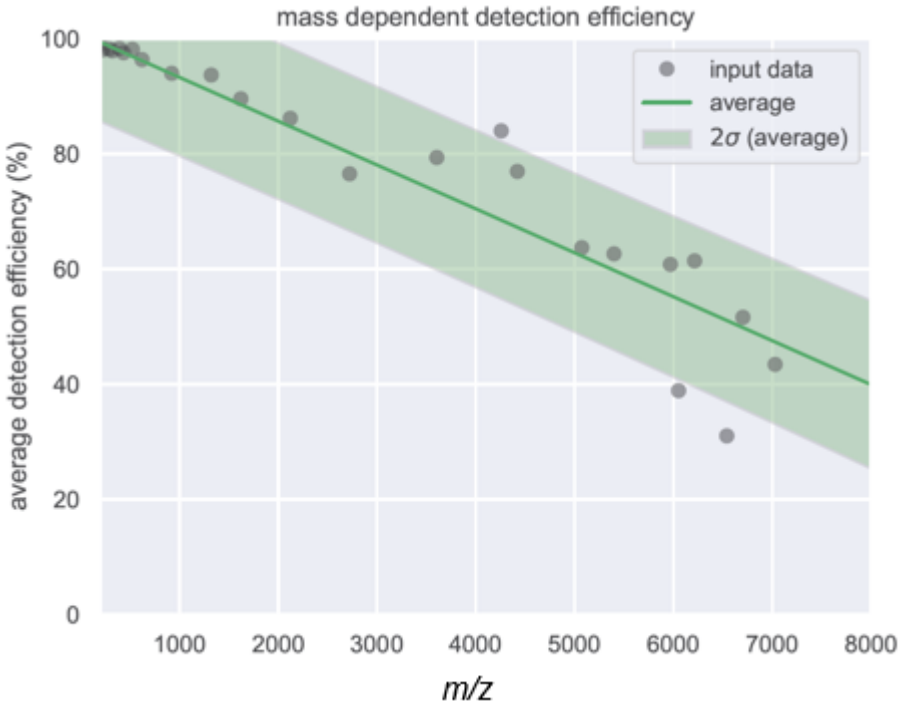
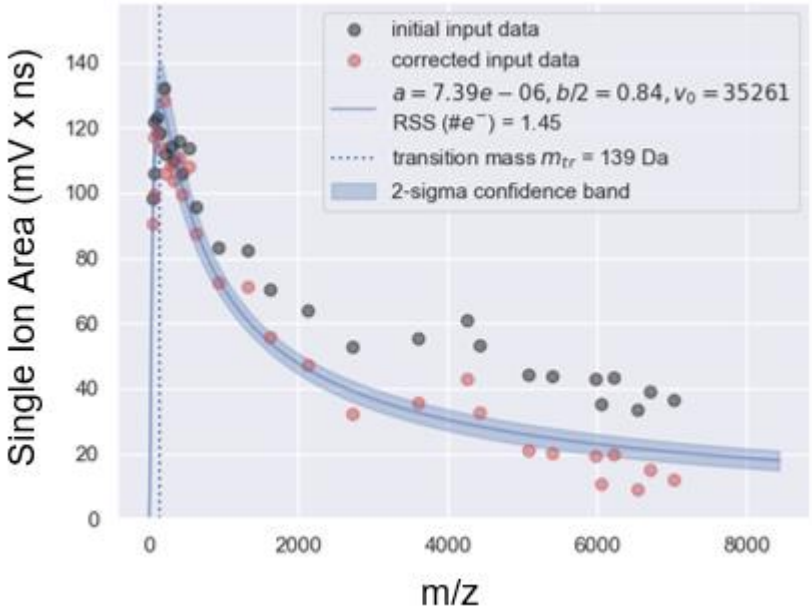

mass dependent detection efficiency
input data
average
2σ (average)
average detection efficiency (%)
100
80
60
40
20
0
1000
2000
3000
4000
5000
6000
7000
8000
m/z

Fig. 3


initial input data
corrected input data
$a = 7.39e-06, b/2 = 0.84, v_0 = 35261$
RSS ($\#e^-$) = 1.45
transition mass m_{tr} = 139 Da
2-sigma confidence band
Single Ion Area (mV x ns)
0
20
40
60
80
100
120
140
0
2000
4000
6000
8000
m/z

P 181

Data acquisition system for single particle inductively coupled plasma mass spectrometry (spICP-MS) with nanosecond time resolution

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[2]*Federal Institute for Materials Research and Testing (BAM), Berlin, Germany*

This study presents our data acquisition system prototype for spICP-MS with nanosecond time resolution (nanoDAQ) and a matching data processing approach for nanosecond resolved single particle spICP-MS data. The system continuously samples the secondary electron multiplier (SEM) detector signal with a dwell time of approximately 2 ns and enables the detection of gold nanoparticles (AuNP) as small as 7.5 nm[1] with a commercial single quadrupole ICP-MS instrument.

The acquired transient data is processed based on the temporal distance between detector events and the event density. It was shown that the inverse logarithm of the distance between detector events is proportional to the particle size and that the number of detector events corresponding to a particle signal distribution can be used to calibrate and determine the particle number concentration (PNC) of a nanoparticle dispersion.[1] The high data acquisition frequency of the systems allows recording of a statistically significant number of data points in 60 s or less, which leads to the main time limitation for analyses being merely the sample uptake time and rinsing step between analyte solutions.

[1] A. Schardt, J. Schmitt and C. Engelhard, Single particle inductively coupled plasma mass spectrometry with nanosecond time resolution, *J. Anal. At. Spectrom.*, 2024, Advance Article, https://doi.org/10.1039/D3JA00373F

P 182

Assay for analyzing adsorption properties of surfaces (APS) uncovers loss of molecules during sample handling for LC-MS/MS

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[2]*Eppendorf SE, Hamburg, Germany*

The omics field is evolving in the direction of single cell analysis, with sample sizes decreasing to the limit of detection of current mass spectrometry systems. An often-neglected reason for reduced sample and result quality is the usage of common consumables for sample preparation such as vials, tubes, and pipette tips. In most analytical applications the loss of molecules is compensated using internal standards. However, in omics thousands of analytes are quantified at a time. Here, the application of an internal standard for each analyte is not possible. The problem of adsorption of molecules to surfaces can be minimized by choosing parameters, such as solvents, additives, and pH. This is most effective when the nature of interactions with the surface is known.

To quantify and characterize the loss of analytes caused by adsorption, we developed an assay to analyze the adsorption properties of surfaces (APS). APS is based on a commercially available standard of tryptic HeLa peptides, covering a broad range of chemical properties. The tryptic peptides are analyzed with liquid-chromatography coupled to tandem mass spectrometry (LC-MS/MS) and quantified with programs used for differential quantitative proteomics. The subsequent identification and characterization of adsorbed peptides is performed with our automated script-based analysis. APS can be adapted to all kinds of surfaces used during sample preparation and sample storage. Moreover, APS does not require knowledge of the chemistry of surfaces, which most often is not available, e.g. for patent reasons.

In summary, APS is a LC-MS-based tool for precisely describing adsorption characteristics of sample handling surfaces, thereby offering the possibility of minimizing loss of analytes through a rational selection of parameters. This is particularly helpful in cases where internal standards are not applicable, like in omics studies and in cases of very small sample sizes.

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What happens when we reduce the flow rates in atmospheric pressure ion sources?

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Richard Feynman`s talk "There"s Plenty of Room at the Bottom" from 1959 is today seen as the frontier to a world of nanotechnology. He prognosticated the enhancement of scientific approaches by means of miniaturization. Nowadays, the downsizing of analytical systems is driven by economic factors (e.g., rapidity through reduced sample preparation) and ecological factors (e.g., sustainability through reduced sample and reagent amounts). Miniaturization has long found its way into mass spectrometry. Regarding low analyte concentrations, especially in environmental samples for ultratrace analyses, instruments must exhibit high sensitivity. Here, an essential factor is the choice of an appropriate ionization source.

In 1994, M. Wilm and M. Mann miniaturized the conventional electrospray ionization (ESI) developed by John Fenn into nano ESI benefiting from an overall increase in performance at flow rates of some nanoliters. While the overall signal intensity drops, an increased signal-to-noise ratio is observed, leading to a better sensitivity at reduced flow. ESI is limited to the analysis of rather polar analytes. In contrast, Atmospheric Pressure Photoionization (APPI) is most suitable for non- to medium polar compounds with free electron pairs or π-electrons, such as polycyclic aromatic hydrocarbons (PAHs) and heterocycles (PAXHs). However, high sample consumption, contamination of the mass spectrometer as well as low signal-to-noise due to chemical noises from the dopant or solvent are ever-present issues to be dealt with.

This study focuses on the limits of analyte concentrations and flow rates with APPI to increase sensitivity. All mass spectrometric measurements were performed on a LTQ mass spectrometer (Thermo Fisher Scientific, San Jose, CA, USA) using a Kr-VUV lamp at 10.0 and 10.6 eV for ionization with different PAHs/PAXHs as reference analytes. APPI typically runs at flow rates around 100 µl min^{-1}; past experience shows that 20 µl min^{-1} can enhance measurement sensitivity. The standard flow rates are a factor of 10 higher than normal ESI flow rates. Recently, we realize the reduction in flow rate in APPI down to 1 µl min^{-1} and show even a better sensitivity at enhanced flow rates than with electrospray/nanospray. To understand the behaviour at those low flow rates, signal-to-noise rates were calculated and limits of detection were measured. At lower flow rates of 1 µl min^{-1} APPI allows a much better sensitivity and reduced the limits of detection by a factor of 100. This study demonstrates that there is much more potential in APPI, and by reducing the flow rate noise signals are less disruptive during the detection of APPI-affine standards.

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Evidence that the Isc Iron-Sulfur Cluster biogenesis machinery delivers iron for [NiFe]-Cofactor biosynthesis in *Escherichia coli* studied by native mass spectrometry

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Prokaryotes are the simplest organisms living on our planet. Some bacteria can use one of the simplest molecules at all to gain energy for living. This molecule is hydrogen. Moreover, a variety of microorganisms, such as photosynthetic algae or anaerobic bacteria, can produce hydrogen. For example, *Escherichia coli*, grow by a process known as fermentation in the absence of oxygen. This initially results in formic acid production, converted into carbondioxide and hydrogen gas. This process requires the interplay enzyme complex called formate hydrogen lyase (FHL) and [NiFe]-hydrogenases (Hyd)[1].

[NiFe]-hydrogenases have a bimetallic active-site cofactor $NiFe(CN)_2CO$), in which the iron ion carries a CO and two CN^- as diatomic ligands ($Fe(CN)_2CO$). While biosynthesis of the CN^- ligands from carbamoyl phosphate is clear, biosynthesis of the CO ligand remains unresolved. Six Hyp proteins (HypA-F) are involved in cofactor biosynthesis, but only the FeS-cluster-containing HypD protein is redox-active. HypD is required to transfer the CN^- ligands from the HypE-HypF proteins to the iron, and circumstantial evidence also suggests that the CO ligand is generated by HypD from endogenous CO_2 already bound to an iron ion on a HypCD complex [2]. Native mass spectrometric (native MS) analysis of HypCD complexes defined the [4Fe-4S] cluster associated with HypD and identified adducts indicative for cofactor synthesis required intermediates (26 Da = CN^- , 28 Da = CO and 136 Da = $Fe(CN)_2CO$) specifically associated with HypC. A $HypC_{C2A}$ variant dissociated from its complex with native HypD lacked all previously observed intermediate modifications. HypCD complexes isolated from *E. coli* strains deleted for the iscS or iscU genes, encoding core components of the Isc iron-sulfur cluster biogenesis machinery [3], lacked the +136 Da modification indicative for the $Fe(CN)_2CO$ cofactor; however, it was retained on HypC isolated from suf mutants. The presence or absence of the +136 Da modification on the HypCD complex correlates with the hydrogenase enzyme activity profiles of the respective *E. coli* mutant. Notably, the [4Fe-4S] cluster on HypD was identified in all HypCD complexes independent of the mutation background. These results suggest that the iron of the $Fe(CN)_2CO$ group on HypCD derives from the Isc machinery, while either the Isc or the Suf machinery can deliver the [4Fe-4S] cluster to HypD.

[1] G. Metcalfe, F. Sargent, M Hippler, Microbiology, 2022, 168(3):001167

[2] M. Lacasse & D. Zamble, Biochemistry,2016, 55,1689-1701

[3] C. Pinske & G. Sawers, PLoS ONE, 2012, 7, e31755

P 186

Characterization of the intrinsically disordered N terminus of neuropeptide Y2 receptor by cross-linking mass spectrometry

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[2]Martin-Luther-University Halle-Wittenberg, Center for Structural Mass Spectrometry, Halle, Germany
[3]University of Leipzig Medical Center, Department of Anaesthesiology and Intensive Care, Leipzig, Germany
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The neuropeptide Y (NPY) receptors comprise a family of rhodopsin-like G-protein coupled receptors (GPCRs) that are involved in controlling food intake, memory retention, and circadian rhythm. They play an important role in mood disorders and epilepsy making them attractive drug targets. The multireceptor/multiligand nature of NPY receptors, such as the Y2R, requires a detailed understanding of their interactions with their natural ligands. Recent studies imply different conformational states involved upon binding of the ligand NPY to Y2R. However, traditional structural biology approaches, such as X-ray crystallography or cryo-electron microscopy, have not been able to capture the ensemble of dynamic conformers. To overcome these challenges, the characterization of transient states and binding modes of Y2R and its ligand are currently being investigated by cross-linking mass spectrometry (XL-MS).

We used a triply photo-leucine substituted NPY ligand variant to map Y2R/NPY interactions. The photo-reactive amino acids undergo a cross-linking reaction upon activation by UV-A irradiation (365 nm). The inherent sensitivity of mass spectrometry, the fast reaction of diazirine photo-amino acids, and the distance restraints determined by the cross-links, together with computational modelling, allowed studying Y2R/NPY interactions in an efficient manner.

Here, we present initial XL-MS characterization of Y2R/NPY interaction, reconstituted in phospholipid bicelles. Specific interactions were captured between photo-leucine labeled NPY and the intrinsically disordered N terminus of Y2R. The N terminus might provide structural flexibility necessary to direct NPY to the high-affinity binding site, located between the extracellular loop 2 and the transmembrane regions 4, 5, and 6. The cross-linking results could complement the proposed multistage Y2R/NPY binding model where NPY is first accumulated at the membrane, re-oriented, recognized by the flexible N terminus of Y receptor, and guided to the binding site.

Our cross-linking results provide first insights into the interactions within the Y2R/NPY system and will serve as basis to capture dynamic conformational states of the Y2R"s N terminus with peptide ligands in HEK293 cells. Studying the Y2R/NPY interactions in their native environment allows developing novel targeted and sensitive mass spectrometry-based methods to derive the molecular mechanisms underlying Y2R/NPY interactions.

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Analysing lipid binding of Synaptobrevin-2 and Complexin-1: a preference for negatively charged lipids

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The SNARE (soluble N-ethylmaleimide-sensitive factor attachment protein receptor) complex mediates the fusion of synaptic vesicles with the presynaptic membrane. The vesicular Synaptobrevin-2 is a key player in this process and undergoes a disorder-to-order transition upon interactions with SNAP25 and Syntaxin-1A, both anchored to the presynaptic membrane, forming the SNARE complex. This assembly is a highly regulated process involving several regulatory proteins such as the cytosolic protein Complexin-1, which inhibits and facilitates membrane fusion through an unknown mechanism. In the absence of interaction partners, both, Synaptobrevin-2 and Complexin-1, are largely unstructured. For Synaptobrevin-2 contrary structural content of the cytosolic domain in the presence of lipids was reported; accordingly, a helical nucleation site was observed in micelles but was absent in liposomes. Furthermore, a patch of positively charged residues was proposed to be critical for lipid binding of the juxtamembrane domain, while negatively charged residues at the N-terminal half of the SNARE motif were described to induce electrostatic repulsion between the protein and the membrane. Complexin-1, on the other hand, contains a C-terminal lipid binding motif composed of two consecutive amphipathic helices that form a membrane curvature sensing peptide followed by a patch of negatively charged residues as well as a N-terminal amphipathic helix.

In this study, we investigate the interactions of Synaptobrevin-2 and Complexin-1 with varying phospholipids including phosphatidylserine, phosphatidylethanolamine, phosphatidylcholine and phosphatidylglycerol. For this, we use native mass spectrometry to explore binding of the two proteins to detergent-lipid-micelles. Both, Synaptobrevin-2 and Complexin-1, bound to various lipids with comparable affinity; although a higher binding capacity was observed for Complexin-1. Importantly, a specificity for lipids was not observed. To evaluate lipid binding observed by native mass spectrometry, we further employed liposomes that simulate a physiological membrane with a curvature comparable to synaptic vesicles. Following incubation of the proteins with the liposomes, lipid binding was detected by gel electrophoresis after flotation on a sucrose gradient. Binding of Synaptobrevin-2 was only observed for liposomes composed of phosphatidylserine and phosphatidylglycerol, while Complexin-1 additionally bound to phosphatidylcholine liposomes. Lipid overlay assays confirmed binding of the two proteins to negatively charged lipids containing easily accessible phosphate groups, for instance, phosphatic acid, cardiolipin and phosphatidylinositol phosphates. We conclude that the analysis of protein-lipid interactions by native mass spectrometry involving the transfer of lipids from detergent-lipid micelles is strongly affected by the proteins properties.

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Site-specific structural changes in long-term stressed monoclonal antibody revealed with DEPC covalent-labeling and quantitative mass spectrometry

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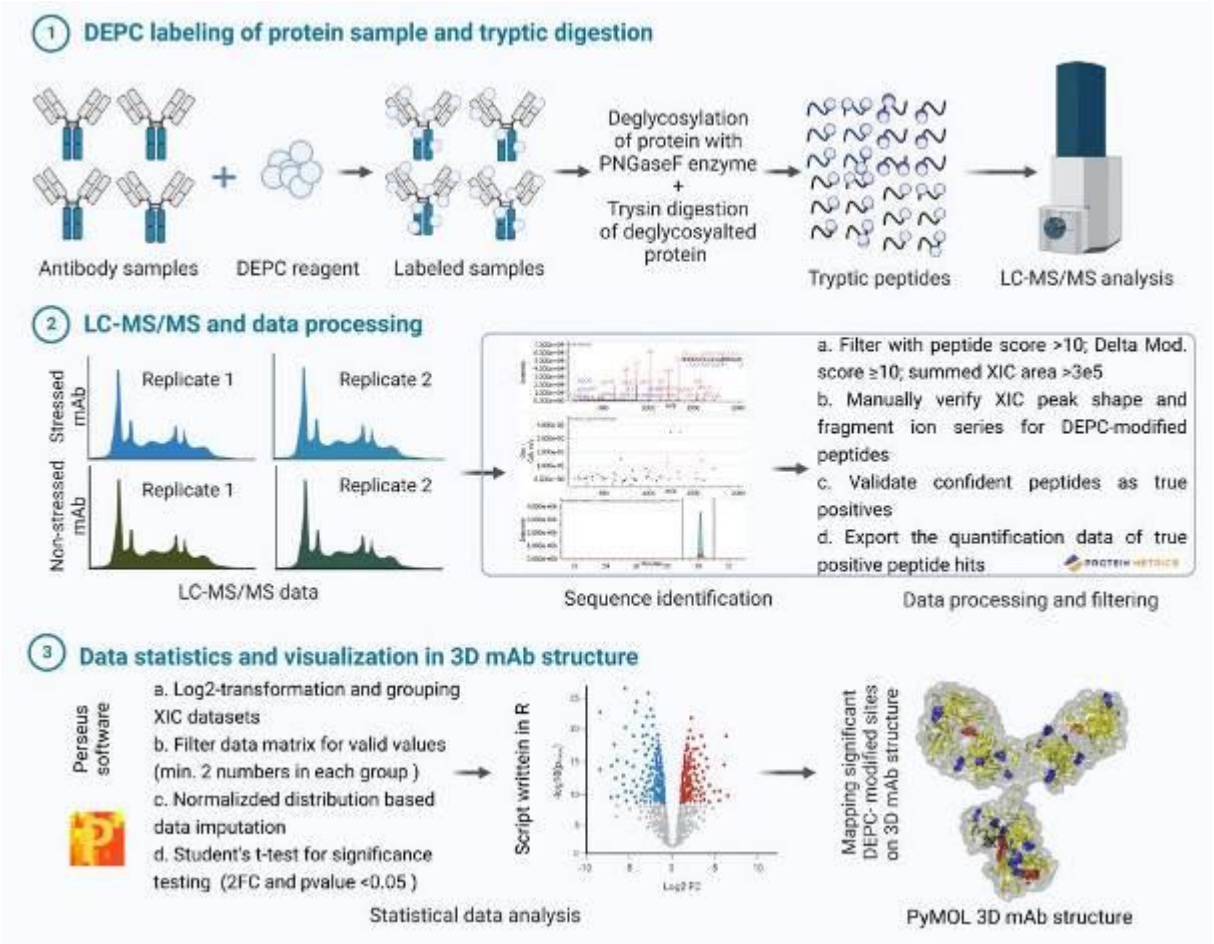
Questions: Studies of structural changes in mAb under forced stress and storage conditions are essential for the quality assessment of therapeutic proteins. Due to manufacturing or storage-related stress conditions, the degradation of proteins can happen at defined hotspots in the protein which are of significance during biologicals development. Compared to methods like Xray and NMR, MS based methods are less time and material consuming. However, none of the established covalent labeling mass spectrometry (CL-MS) methods proved to be the gold standard. Herein, we explored diethyl pyrocarbonate (DEPC) for assessing structural changes in a model mAb (SILuMAb) as a robust approach to answer multiple structure -related questions. Structural changes resulted in an altered reactivity towards DEPC at specific amino acid positions seen as a mass tag in the fragment ion spectrum. Label-free quantification enabled us to distinguish between significantly and non-significantly changed sites. In brief, unfolding should increase the incorporation of DEPC, whereas aggregation should reduce it, but also fragmentation could come into play as a third path.

Methods: 50 µg SILuMAb with or without treatment were labeled with 0.04 mM DEPC for 5 min at 37 °C. Deglycosylation was performed with 1 µL of PNGaseF (1000 U) at 37 °C for 1h. For peptide-based MS, 40 µg were denatured in 8 M urea, disulfide bonds were reduced with 0.79 mM TCEP at 37 °C for 30 min and alkylated with 15 mM iodoacetamide for 30 min at RT in the dark. Trypsin digestion was conducted in 1 M urea in NH4HCO3. LC-MS/MS was performed on an Agilent 1260 capillary HPLC coupled to a Bruker Impact II QTOF MS. Peptide identification was carried out with Byos Software Version: v4.5 (Protein Metrics). Intact protein analysis of 10 µg was achieved using SEC-UV on a 1050 series HPLC from HP. A TSKgel SuperSW3000 column (4 µm particle size, ID 4.6 mm, 30 cm length) was used (Tosoh Bioscience, Griesheim). Analysis was conducted with 0.2 mL/min for 30min using 100 mM monobasic Na3PO4 and 100 mM NaCl at pH 7.0, detection at 280 nm.

Results: Realizing a minor effect of deglycosylation on mAb structure, we focused on heat stress and identified an aggregation-prone area in the CDR region of SILuMAb. Strikingly, long term storage for up to two years at 2-8 °C significantly increased the vulnerability towards heat stress and resulted in both, increased and decreased DEPC incorporation at many positions. A complementary SEC-UV indicated fragmentation of SILuMAb. DEPC incorporation at free Cys also pinpointed structural disturbances of disulfide bonds.

Conclusions: Taken together, our data suggests that DEPC CL-MS can complement technically more challenging methods in the evaluation of structural stability of mAbs.

Fig. 1


1 DEPC labeling of protein sample and tryptic digestion
Antibody samples
DEPC reagent
Labeled samples
Deglycosylation of protein with PNGaseF enzyme + Trysin digestion of deglycosyalted protein
Tryptic peptides
LC-MS/MS analysis
2 LC-MS/MS and data processing
Stressed mAb
Non-stressed mAb
Replicate 1
Replicate 2
Replicate 1
Replicate 2
LC-MS/MS data
a. Filter with peptide score >10; Delta Mod. score ≥10; summed XIC area >3e5
b. Manually verify XIC peak shape and fragment ion series for DEPC-modified peptides
c. Validate confident peptides as true positives
d. Export the quantification data of true positive peptide hits
Sequence identification
Data processing and filtering
3 Data statistics and visualization in 3D mAb structure
Perseus software
a. Log2-transformation and grouping XIC datasets
b. Filter data matrix for valid values (min. 2 numbers in each group)
c. Normalizded distribution based data imputation
d. Student's t-test for significance testing (2FC and pvalue <0.05)
Script written in R
Mapping significant DEPC- modified sites on 3D mAb structure
Statistical data analysis
PyMOL 3D mAb structure

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Studying the molecular mechanisms of liquid-liquid phase separation of α-Synuclein by mass spectrometry

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Liquid-liquid phase separation (LLPS) has recently been recognized as major principle for the formation of "membrane-less organelles" and other functions in cells [1]. Proteins, such as tau or α-Synuclein, involved in Alzheimer"s and Parkinson"s Disease (PD), undergo LLPS prior to aggregation. Aggregation of α-synuclein results in the formation of Lewy Bodies, which are a hallmark of PD [2, 3]. As LLPS might be a triggering factor for the formation of Lewy Bodies, a molecular understanding of the mechanisms underlying LLPS of α-Synuclein is required for developing potential novel therapies to treat PD [2]. Most proteins that are able to undergo phase separation consist of IDRs [1]. α-Synuclein (14.5 kDa) is an intrinsically disordered protein (IDP) that consists of a membrane-binding, N-terminal region, a non-amyloid component (NAC) region, and a C-terminal acidic region [3]. In addition to α-Synuclein, Synapsin-1 is another high-abundant protein that is present in synaptic vesicles (SVs) [4]. Fluorescence microscopy has shown a colocalization of α-synuclein and synapsin-1 in LLPS, giving hints on a functional interaction between both proteins [5]. The heat shock protein (HSP) 70-1 is one of the major chaperones that is frequently found to co-separate with α-Synuclein and Synapsin-1. It has been suggested that HSP70-1 is recruited in stress granules to induce protein disassembly and to prevent protein aggregation [6, 7]. Using three complementary techniques of structural mass spectrometry (MS), cross-linking mass spectrometry (XL-MS), hydrogen-deuterium exchange MS (HDX-MS),and native MS, we aim to study the composition and topology of the complexes formed between α-Synuclein, Synapsin-1, and HSP70-1 upon LLPS. Upon native MS studies, we already observed complexes of α-Synuclein and Synapsin-1 as well as α-Synuclein and HSP70-1.

[1] Alberti, S. et al. (2019) [2] Ubbiali, D. (2022) [3] Rey, S. et al. (2020) [4] Milovanovic, D. et al. (2021) [5] Hoffmann, C. (2021) [6] Murphey, M. (2013) [7] Li, Y. et al. (2022)

P 190

Distinguishing animal-derived feed ingredients based on their processing status

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Processing food and feed serves several purposes including easier digestibility, increased nutrient availability, and longer shelf life. It furthermore sets off reaction networks (Maillard, oxidation, lipid peroxidation) whose traces such as post-translational modification (PTM) and backbone cleavage on proteins may serve as processing markers. For example, differentiating the processing status of milk is possible using e.g. lysinoalanine as well as lactosyl and carboxymethyl lysine. [1-3]

Monitoring the processing status of animal-derived feed ingredients is required by European Commission Regulation 999/2001. Different feeding rules apply for typically spray-dried blood products and materials requiring steam-pressure sterilisation (blood and meat-and-bone meals). However, official feed control relies on microscopy and qPCR which are not processing-sensitive.

In order to identify processing marker candidates, 18, 28, and 14 commercial samples from cattle, pig, and poultry, respectively, were analysed by standard bottom-up proteomic LC-MS/MS. Peptide identification by database search comprised in total 53 PTMs retrieved from literature and priorly proven to be the most relevant PTMs by error-tolerant search of a comparable dataset. Semi-tryptic peptides were identified in a database search with limited dynamic modifications.

Hence, more than 17,000 peptides (> 2,000 proteins) were identified in each species" dataset, a large part of which was post-translationally modified (60%) or had a non-tryptic cleavage site (10%). All 53 PTMs screened for were identified at least once, with deamidation, oxidation, carboxymethlyation, and formylation occurring most frequently.

Filtering for potential processing markers relied on peptide peak areas normalized to the summed peak areas of all peptides assigned to this corresponding protein. Thus, marker selection was ensured to be based on processing status rather than varying protein abundance. In total, 15, 29, and 19 marker candidates were singled out for bovine, porcine and poultry materials, respectively. As expected, their majority (35 out of 63) comprised processing-induced changes. Their identity was proven with synthetic authentic standards. Targeted methods are currently established. Comprehensive marker validation covering an even larger sample set is in preparation.

In conclusion, distinguishing animal-derived feed ingredients based on their processing status is feasible with site-specific processing markers. The sensitivity of the identified marker candidates corresponds to the required lower end of processing degrees covering none, spray-drying and steam-pressure sterilisation.

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Characterization of N-terminally elongated Aβ peptides by immunoprecipitation-mass spectrometry

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The aggregation and deposition of Aβ peptides in the brain is thought to be the initial driver in the pathogenesis of Alzheimer"s disease (AD). Aside from full-length Aβ peptides starting with an aspartate residue in position 1, both N-terminally truncated Aβ4–x and N-terminally elongated Aβ−3–x peptides can be produced from the amyloid precursor protein (APP). While the ratio of Aβ−3–40/Aβ1–42 can predict cerebral amyloid-β pathology with high accuracy in blood plasma (Nakamura et al., 2018), it is still unclear whether Aβ−3–x peptides mainly originate from the periphery or the central nervous system, and how they are generated. Here, we investigated whether Aβ−3–x peptides are also present in cerebrospinal fluid (CSF) and whether they can be generated by ADAMTS4, a secreted metalloprotease known to produce the Aβ4–x peptides, which are among the most abundant Aβ species found in human AD brains. Using antibodies raised against the N-termini of Aβ1–x (mAb 6E10; epitope Aβ3–8) or Aβ−3–x (mAb 101-1-1; Klafki et al., 2020) in magnetic bead immunoprecipitation followed by MALDI-TOF-MS (IP-MS), we detected Aβ−3–x peptides in conditioned cell culture supernatants of SH-SY5Y cells overexpressing human wildtype APP695 and in CSF samples (Klafki et al., 2022). To approach a potential role of ADAMTS4 in the proteolytic generation of N-terminally elongated Aβ−3–x peptides at first in vitro, we incubated the synthetic substrate peptide Aβ−23–16 with recombinant ADAMTS4 in the presence or absence of a broad-spectrum metalloprotease inhibitor and analyzed the cleavage products by MALDI-TOF-MS. Apart from the expected presence of the peptide fragments Aβ4–16 and Aβ−23–3, we also detected Aβ−23–(–4) and Aβ−3–16, indicating that ADAMTS4 cleaves APP also between residues Glu−(−4) and Val−(−3) to generate Aβ−3–x peptides. To corroborate these findings in a cell-based model, we employed previously generated HEK293 cell lines with stable overexpression of APP695wt and inducible expression of ADAMTS4 (Walter et al., 2019). By IP-MS, increased signals for Aβ4–x and Aβ−3–x peptides were found upon induction of ADAMTS4 expression, demonstrating that ADAMTS4 is involved in the generation of these Aβ species in vivo and thereby potentially implicated in the amyloid pathology in AD.

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P 192

Investigating legionella's enzymatic arsenal: Unmasking posttranslational modifications with mass spectrometry

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Legionella pneumophila, the causative agent of Legionnaires' disease, is an intracellular bacterial pathogen. It secretes more than 300 effector proteins into the host cell cytosol to create a replication-friendly niche, known as the Legionella-containing vacuole, within the host cell. A subset of these effectors engages in posttranslational modifications of host proteins. We combine mass spectrometry and structural biology to analyse the dynamic interactions between Legionella effectors and host target proteins.

The Legionella effector AnkX utilises CDP-choline to phosphocholinate the host small GTPase Rab1. The bacterial effector Lem3 can reverse the modification through hydrolytic cleavage of the bond. The functional impact of the posttranslational modification on Rab1 has been extensively studied. However, structural insights into the interactions between the bacterial enzymes and their host target Rab1 remained elusive due to the transient character of their interaction.

To stabilise the transient interactions between the bacterial enzymes AnkX and Lem3 and their human target Rab1, covalently linked enzyme-substrate complexes were obtained by employing regioselective chemistry. This minimally invasive coupling strategy aimed to preserve the natural interaction interface between the proteins and enabled the successful elucidation of the molecular interactions of AnkX and Lem3 with Rab1 by X-ray crystallography. Intact mass spectrometry was used to validate the effector:substrate structures by tracking the modification and demodification reactions in a time-resolved manner. This approach provided highly accurate enzyme kinetic data.

In addition to the well-studied target of AnkX, Rab1, we identified a so far unknown host target protein, the metabolic enzyme IMPDH, using tandem mass spectrometry. Due to the labile nature of the posttranslational modification, we developed a method combining ETD and HCD to verify the nature of the PTM and determine the modification site within IMPDH. The posttranslational modification inhibits the filament formation, which is crucial for the regulation of the enzymatic activity of IMPDH *in vivo*. In contrast to the modification of Rab1, Lem3 cannot reverse the phosphocholination of IMPDH.

This combined tool kit of structural biology and mass spectrometry helps us to comprehensively characterise PTMs in the context of Legionella infection.

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Recent HDX-MS studies reveal dramatic changes of the structural dynamics of wild-type full-length tumour suppressor protein p53 upon binding of DNA

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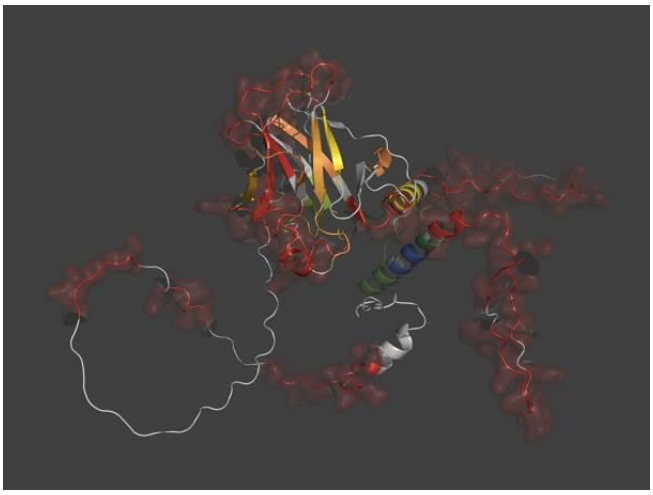
Over the past decades hydrogen deuterium exchange mass spectrometry (HDX-MS) has been more and more accepted as a routine technique for the analysis of protein dynamics. While NMR spectroscopy, the "gold standard" technique in this field, gives a more detailed picture, HDX-MS can overcome some of the limitations of NMR. Sensitivity is one of these limitations. Sample amounts and concentrations, which are needed for a NMR experiment, are not available or achievable for all kind of proteins. One example are intrinsically disordered proteins (IDPs), which are very much prone to aggregate at higher concentrations and are difficult to analyse by NMR.

The tumour suppressor protein p53 is such an IDP. It shows extremely high protein dynamics in about 40% of the polypeptide chain in the native state. These intrinsically disordered regions (IDRs) do not display any detectable structure in an NMR experiment, but still have a very important influence on the function of the protein.

p53 builds homotetramers and acts as transcription factor and tumour suppressor. For more than 30 years now, p53 has been considered as an exceptional target for the development of cancer treatments. For that reason, there is a high interest in decoding the structural dynamics of p53. A problem is, that the wild-type full-length p53 aggregates rapidly and irreversibly. It is hard to produce in amounts, which are necessary for NMR studies. Many studies try to circumvent the aggregation problem by removing some or all IDRs. Nevertheless, there is a risk, that the structure of the non-full-length constructs is different form the full-length wild-type protein. The IDR parts might play an important, if not the essential role for the correct function of the protein. This is a hypothesized principle, which might apply to all IDPs. In the case of p53, it is known that there is a complex interplay between the folded DNA binding domain (DBD) and the intrinsically disordered C-terminal domain (CTD) upon DNA binding. There are suggested models, but the binding process is still not fully understood.

We tried to investigate the structural dynamics of wild-type full-length p53 tetramers and structural changes upon DNA-binding by HDX-MS for the first time. At room temperature the full-length protein seems to be highly flexible, even in the folded DBD (see Fig.1). Studies with isolated DBD don"t display such high flexibility. Therefore we postulate that the existence of IDRs prevents the folded domains of p53 from further compaction. Interestingly, upon binding to DNA the p53 tetramer undergoes a strong compaction, so that even in the disordered CTD the HDX is limited by stable hydrogen bonds. This could be a hint for a disorder to order transition in the CTD, which has not been reported so far.

Fig. 1



P 194

Strategies for the identification of metabolites of peptide-derived drugs prohibited in sports

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Research on the metabolism of banned substances in the context of anti-doping has substantially contributed to enhance analytical sensitivity and, thereby, prolong detection windows. Moreover, specific metabolic patterns can offer information about the time of administration, dose or origin (exogenous/ endogenous) of the detected substance. While standard protocols for *in vitro* metabolism experiments are well established for low molecular mass molecules, alternative approaches are needed to investigate the metabolism of peptide and protein-derived drugs, as the mechanisms of biotransformation differ significantly from those of non-peptidic drugs.

Here, we studied the *in vitro* metabolism of various peptides and proteins prohibited in professional sports by the World Anti-Doping Agency (WADA). The experiments were conducted in serum, urine, liver S9- and skin S9-fractions by incubating the compounds of interest over 2 – 24 h, to account for the potentially characteristic degradation following subcutaneous administration.

A combination of liquid chromatography and high-resolution/high-accuracy mass spectrometry on an orbitrap system acquiring full scan and all-ion-fragmentation (AIF) data enabled the identification of numerous peptide metabolites, followed by subsequent structure elucidation by additional MS^2 experiments. In particular, the application of an isotope-labeled reporter ion screening (IRIS) simplified the detection of peptide degradation products of human growth hormone (hGH, 22 kDa) and insulin-like growth factor 1 (IGF-1, 7.9 kDa), which could serve as promising markers to uncover the subcutaneous administration of the recombinant peptide hormones for doping purposes.

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Refining protein citrullination identification in large-scale proteomics data

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Introduction:

Post-translational modifications (PTMs) regulate protein functions. Citrullination is a crucial, yet underexplored PTM linked to autoimmunity and inflammation. Despite its pathological relevance, identifying citrullination sites remains challenging due to limited enrichment tools. While direct identification of citrullination sites from deep proteomics profiling offers insights, challenges such as shared mass increases with deamidation of Gln and Asn pose potential errors. Manual inspection is required [1] for confident site identification but hampers throughput in large-scale studies. This study aims to propose an optimized data analysis pipeline for high-precision, large-scale citrullination proteomics.

Methods:

An evaluation data set was created by spiking in ~200 synthetic citrullinated peptides into cell lysates to assess citrullination identification precision. Public proteomics data sets of human tissues (PXD010154) and Arabidopsis tissues (PXD013868) were obtained from PRIDE. Citrullinated peptide identification was performed using Andromeda (MaxQuant v2.2.0.0) and MSFragger (FragPipe v19.0) search algorithms. The search results were Prosit-rescored [2], and IonFinder [3] was employed for automated neutral loss (HNCO) detection.

Results:

Citrullination identification was assessed in the evaluation dataset, where true and false positives were defined by the identification of synthetic citrullinated peptides. MSFragger without MSBooster performs better precision without post-processing than MaxQuant. With Prosit-rescoring, this approach achieved the highest precision, particularly at lower citrullinated peptide spike-in levels. Neutral loss filtering using IonFinder increased precision but led to a 25% decrease in total identifications in the lower spike-in range. Applying our pipeline to reanalyze the top 10 human tissue proteomes which most citrullinated sites reported [1] showed the known citrullination sites were successfully retrieved, and up to a two-fold increase in citrullinated sites was identified. In Arabidopsis, over 1,000 citrullination sites were identified across 30 tissues, with the highest levels observed in reproductive organs, suggesting a broader significance of citrullination in Arabidopsis.

Conclusions:

We introduced an optimized data analysis pipeline, enhancing accuracy and throughput for large-scale citrullination identification. This approach facilitates a systematic understanding of citrullination using existing datasets.

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Analysis of lipid transport mechanisms in Gram negative bacteria via native mass spectrometry

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Introduction

Lipid and lipopolysaccharide (LPS) transport plays a crucial part in maintaining the asymmetry and therefore the barrier function of the membrane hull in Gram negative bacteria, one main reason for multi drug resistance. Responsible for the transport of lipids and LPS in *E. coli* are two systems. While the lipopolysaccharide transport (Lpt) complex transports LPS to the outer membrane, the Mla system has been shown to transport lipids from the outer membrane to the inner. Our aim is to investigate the mechanisms responsible for this transport via native mass spectrometry (MS).

Methods

The analysis of membrane proteins is often challenging because of their hydrophobic nature and the need for detergents for solubilisation. Laser Induced Liquid Bead Ion Desorption (LILBID)-MS is an ionization method suited for such proteins. An infrared laser irradiates aqueous sample droplets, which leads to an explosive expansion of the droplet. This sets solubilized ions free and strips them of unwanted components such as detergents. Dependent on the laser intensity non covalent complexes can either be analysed intact or dissociated into subcomplexes or their subunits, allowing for complex analysis, assembly studies or binding experiments.

Preliminary data

We have shown, that LILBID-MS is a well suited ionisation method to characterize these transport mechanisms. It can detect bound lipids or LPS, as tightly bound lipids remain attached to the complex during the laser desorption process. To understand the underlying principles of the transport machineries in Gram-negative bacteria we want to[1] investigate and compare the Lpt- and the Mla system of E. coli. We aim to characterise the interactions between the subunits of all complexes and between the protein and their loaded lipids or LPS. The results are likely to vary, as the Lpt system forms stable complexes[2] , which form a bridge that spans from the inner to the outer membrane, while the Mla system has more short lived interactions, because its behaviour is more shuttle like.

First experiments on the Lpt-system were conducted. For example, the inner membrane bound ABC transporter consisting of LptB, LptF, LptG as well as LptC was characterized with regard to stoichiometries via LILBID and ESI-MS. Laser dissociation allowed to reveal next neighbour relationships between these proteins. Furthermore, we were able to load the complex *in vitro* with LPS and also dissociate it by adding ATP and Magnesiumchloride. We are currently working on expanding our experiments to the whole system, by first assembling it with the bridge protein LptA [3] and at a later time with the outer membrane proteins LptD and LptE.

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Deciphering solution and gas-phase interactions between peptides and lipids by native mass spectrometry

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Many biological processes depend on the interactions between proteins and lipids. Accordingly, the analysis of protein-lipid complexes has become increasingly important. Native mass spectrometry is often used to identify and characterise specific protein-lipid interactions. However, it requires the transfer of the analytes into the gas phase, where electrostatic interactions are enhanced and hydrophobic interactions do not exist. Accordingly, the question remains whether interactions that are observed in the gas-phase accurately reflect interactions that are formed in solution. Here, we systematically explore non-covalent interactions between the antimicrobial peptide LL-37 and glycerophospholipids containing different head groups or varying in fatty acyl chain length. We observed differences in peak intensities for different peptide-lipid complexes as well as their relative binding strength in the gas-phase. Accordingly, ion intensities and gas-phase stability correlate well for complexes formed by electrostatic interactions. Probing hydrophobic interactions by varying the length of fatty acyl chains, we detected differences in ion intensities based on hydrophobic interactions formed in solution. The relative binding strength of these peptide-lipid complexes revealed only minor differences presumably originating from van der Waals interactions induced by fatty acyl chains. In summary, our results demonstrate that hydrophobic interactions are reflected by ion intensities, while electrostatic interactions, including van der Waals interactions, determine the gas-phase stability of complexes.

In a new set of experiments, we are now exploring the impact of the peptide's properties on the formation of peptide-lipid complexes. For this, we designed two LL-37 variants differing in the amino acid composition of the helical region, resulting in a positively charged and a negatively charged variant of LL-37. We are currently comparing the interactions of wild-type LL-37 and LL-37 variants with glycerophospholipids containing different head groups. Lipid preferences determined by native mass spectrometry will further be compared with interactions of the LL-37 variants with lipid monolayers using a film balance. These experiments will highlight differences between the LL-37 variants in their solution behaviour and the complexes observed in the gas-phase.

P 198

Diving into phosphoproteomes through a prediction-based deconvolution algorithms

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Background

The analysis of phoshopeptides requires accurate identification and correct localization of the post-translational modification within the peptide. Here, we extend the capabilities of our deep-learning framework to accurately predict physicochemical properties of phosphorylated peptides and demonstrate the usefulness of these predictions in our search algorithm enabling the analysis of such analytes.

Methods

We curated a large training dataset with >1.6 million spectra of phosphorylated peptides to develop an accurate model for predicting their physicochemical properties. Our CHIMERYS™ algorithm utilizes these predictions to score experimental MS2 spectra. All relevant peptides in each MS2 isolation window are considered simultaneously, aiming to explain as much experimental intensity as possible with as few peptides as possible and yielding fractional contributions of peptides to the experimental spectrum. FDR control is executed with Mokapot, and post-processing employs Thermo Scientific™ Proteome Discoverer™ 3.1 software.

Results

In single-shot DDA data from IMAC-based phosphopeptide enrichment, we observe a 1.4-fold increase in identifiable and localizable phosphorylation sites. Next, we introduce our prediction-based localization strategy of scoring all possible positional phosphoisomers of a given peptide precursor against established localization tools like phosphoRS using synthetic peptide standards. The precision-recall curve shows the value of fragment ion intensities, notably improving localization in complex DIA datasets. Finally, we benchmark our algorithm in convoluted DIA spectra from phosphopeptides, deriving error rates for these workflows.

Conclusions

AI-driven, intensity-based identification and localization of phosphopeptides enables more sensitive and reliable data analysis, unlocking biological insight through added sensitivity.

Conflict of Interest Disclosure

M.F., D.P.Z, T.S., S.G., are shareholders and employees of MSAID GmbH. F.S., M.G., S.B.F., P.S., M.S, L.E., V.S. are employees of MSAID GmbH. P.N., K.F., Y.C.H, F.B., C.P, B.D., C.H., are employees of Thermo Fisher Scientific (Bremen) GmbH. D.H. is an employee of Thermo Fisher Scientific Inc.

P 199

Absolute quantification of fluorescently-labelled proteins by mass spectrometry

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Objectives: Fusions with fluorescent proteins (FPs) and fluorescence labelling are widely used for visualization of protein molecules in a variety of biological contexts. While microscopy monitors the localization, dynamics and interactions of fluorescently-labelled proteins, their molar abundance remains unknown. Proteins fluorescence is affected by bleaching, slow chromophore maturation, autofluorescence or protein folding and is sensitive to experimental conditions. To overcome these problems we developed fluorescence-independent method for the absolute (molar) quantification of a variety of FPs using LC-MS/MS and a single protein chimera standard.

Method: We selected and validated quantitypic peptides from the sequences of seven popular FPs (mScarlet-1, mKate2, mCherry, EGFP, mNeonGreen and Dendra2) and from two self-labelling proteins HaloTag and Snap-Tag. The gene encoding for concatenated peptide sequences together with for five peptides from reference protein (BSA) was designed in-silico. Corresponding chimera protein named "FP21" was expressed in *ΔargA ΔlysA* auxotrophic strain of *E.coli* in the media supplemented with 13C6,15N4-L-arginine and 13C6-L-lysine. An aliquot of *E.coli* lysate containing isotopically labelled chimera standard was either co-digested with the analysed material, or digested separately with known amount of BSA, and then spiked into the sample lysate and analysed by LC-MS/MS [1]. Absolute quantification was performed based on the extracted abundance (FragPipe, SkyLine software) of peptides derived from chimeric standard protein and corresponding FPs (Fig. 1): first, chimeric standard is quantified by BSA, then the amount of FP is calculated using corresponding isotopically labelled peptide proxies. The amount of FP-fused protein is equimolar to the FP.

Results: We demonstrated that 30 proxy peptides included into chimeric protein standard enabled targeted quantification of over 70 known FPs [2], their fusions and proteins labelled with organic dyes (e.g. Alexa) via Snap-Tag and HaloTag at the low femtomole to attomole level. Each FP was independently quantified with 3 to 5 peptides with inter-peptide CV < 20%. Several FPs could be quantified simultaneously in a single experiment directly from whole cell or tissue lysate down to single cell level. If required, FP can be also quantified by GeLC-MS/MS. Since the concentration of chimera is exactly known, it can be used as generic internal standard for absolute quantification of any protein detectable at MS1 spectra. We employed this method to monitor the expression of FPs and FP-labelled proteins in bacterial and eukaryotic cells, and in cell-free in-vitro translation-transcription systems. When combined with fluorescence readout, it delineated the kinetics of protein expression and maturation of the fluorescent chromophore.

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Fig. 1

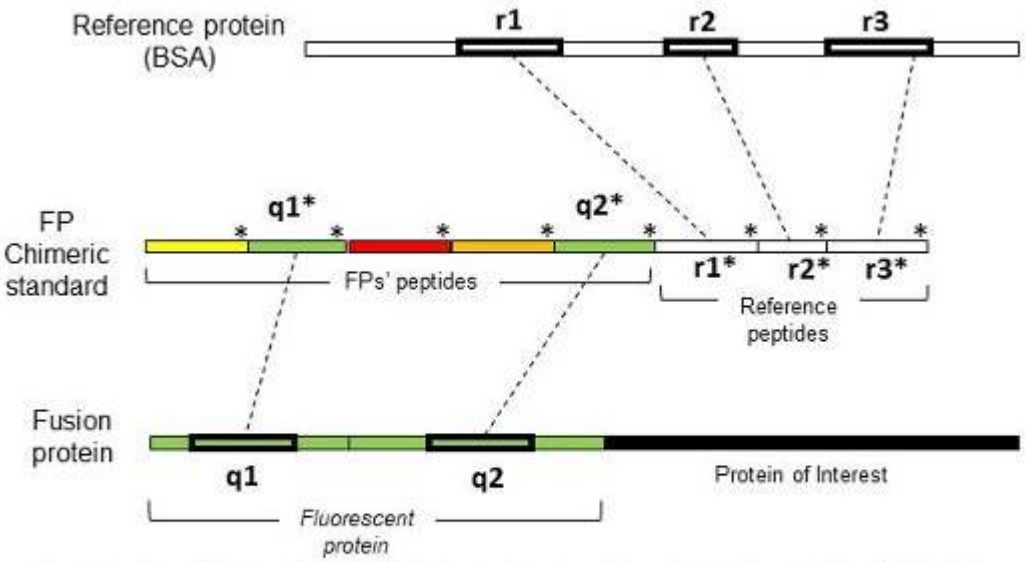

Figure 1. Quantification of FP-labelled protein using chimeric protein standard "FP21p". Surrogate peptides for individual fluorescent proteins and BSA reference peptides are shown in different colours. "*" stays for peptides comprising $^{13}C_6$,$^{15}N_4$-L-arginine and $^{13}C_6$-L-lysine

P 200

Malodorants-derived modifications of human plasma proteins: Identification of novel disulfide-adducts for verification of exposure

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Malodorants are mixtures containing compounds like mercaptans, i.e. alkyl thiols, which also cause the unpleasant smell of the defensive secretion of skunks [1,2]. They can be used to expel individuals in military and civilian defense scenarios and thus, mercaptans are used as personal protection devices or riot control agents [1,3,4]. Since exposure to malodorous formulations might result in headache, nausea, unconsciousness and coma [5,6], we describe a reliable mass spectrometry (MS) -based procedure for the verification of exposure to malodorants. Human plasma was incubated *in vitro* with the thiol *n*-butyl mercaptan (S^nBu) to form covalent protein adducts. After proteolysis of plasma proteins, such modifications were detected as peptide-adducts using micro-liquid chromatography (µLC) coupled to high-resolution tandem-mass spectrometry (MS/HR MS). We identified the side chain of cysteine 34 (Cys^{34}) of human serum albumin (HSA) as reaction partner with mercaptans. S^nBu-adducts were found as disulfides with the tripeptide cysteine-proline-phenylalanine, Cys(-S^nBu)ProPhe, after proteolysis with proteinase K (EC 3.4.21.14). Such peptide-adducts might be used as biomarkers of exposure and are expected to be formed with other malodorous thiols as well.

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P 201

Structural characterization of the interaction between full-length tumor suppressor p53 and DNA by native MS, Hydrogen-Deuterium Exchange-MS and Cross-linking MS

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The tumor suppressor p53, also known as the "guardian of the genome", plays a central role in cellular stress response. As a transcription factor, p53 targets a wide variety of genes which are involved in controlling cell cycle, DNA-repair, and apoptosis [1]. Mutations in p53 are reported in more than 50% of all tumor cases classifying p53 as one of the most important targets in cancer [2]. p53 binds to specific DNA sequences known as p53 response element DNA (RE-DNA) to exert its functions. Although p53/DNA-binding has been studied extensively, the knowledge on a molecular level is mostly limited to the structured DNA-binding domain. The other 40% of intrinsically disordered regions (IDRs) of the protein remain rather elusive. Previously, it has been shown that low-resolution, structural mass spectrometric approaches, such as native mass spectrometry (native MS) and cross-linking mass spectrometry (XL-MS) are well suited to investigate the structure of full-length human wild type p53 in the absence of DNA and upon binding to a short (26 base pairs) RE-DNA [3, 4].

The aim of this study is to shed light on the conformational changes and the structural dynamics of the p53/DNA-binding processes with a special focus on the *N*- and *C*-terminal IDRs by utilizing the full-length human wild type p53 tetramer as well as two p53 tetramerization mutants (L344P, monomer; L344A, dimer) [6]. For DNA-binding experiments, we constructed a RE-DNA library based on the endogenous p21 RE-DNA. This library contains RE-DNA varying in length, complexity, and specificity. Identification of p53/DNA-complexes is carried out by Electrophoretic Mobility Shift Assays (EMSA) and verified by native MS. The identified p53/DNA-complexes are further investigated by XL-MS and hydrogen-deuterium exchange mass spectrometry (HDX-MS) to delineate molecular details of their interaction.

Initial EMSA results show that the p53-L344P monomeric-variant binds to all RE-DNAs of the library. These findings are currently being verified by native MS, XL-MS and HDX-MS. For the p53-L344A dimeric-variant and the p53 wild type (tetramer), different stoichiometries of p53/DNA-complexes were observed in EMSA and native MS. The p53/DNA-complexes are further investigated by cross-linking mass spectrometry (XL-MS) and hydrogen-deuterium exchange mass spectrometry (HDX-MS). These datasets imply different DNA-binding modes, yielding new structural insight into DNA-recognition, DNA-binding, and transcriptional activation of p53.

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P 202

A novel proteolytic column for hydrogen/deuterium exchange mass spectrometry based on nepenthesin from monkey cups

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All proteases used in hydrogen/deuterium exchange mass spectrometry (HDX-MS) belong to the family of aspartic proteases as these work best under acidic conditions, the most common by far being pepsin. Recently, proteases have emerged as an alternative to pepsin, like protease type XIII and type XVIII. Another promising protease system are nepenthesins, a mixture of proteases derived from the carnivorous *Nepenthes spec.* plants (also referred as pitcher plants or monkey cups). In this abstract, nepenthesin is used as synonym of proteases from *Nepenthes spec.* The proteolytic activity of nepenthesin can be over a thousand times higher than pepsin [1]. The high proteolytic activity is especially useful for HDX-MS as the time after quenching must be kept as short as possible to minimize back-exchange and therefor deuterium loss. Columns containing nepenthesin are already commercially available, but they contain recombinant enzyme. They do not offer the same advantages as nepenthesin extracted from *Nepenthes* fluid, such as the enormous activity. Recombinant nepenthesin has only about the same activity as pepsin.

We cultivated several *Nepenthes spec.* plants in a greenhouse, to simulate their natural habitat. The fluid inside the pitchers of the plants contains nepenthesin, but only in a concentration ranging between 1-5 µg/ml. The naturally high activity is necessary as the pitcher fluid must be able to digest flies and other insects. According to the low concentration, high volumes of nepenthesin are needed to extract relevant amounts of nepenthesin. To gather enough material, we collected the plant fluid over two years. Afterwards, we optimized extraction and purification methods, to obtain sufficient nepenthesin for constructing a column.

We are currently optimizing the conditions for coupling nepenthesin to POROS-AL material (thermo scientific). We have already shown that by covalent binding of nepenthesin to the column material it not only keeps its high proteolytic activity, but it also significantly increases its resistance towards chaotropic denaturants, such as guanidine hydrochloride.

Finally, we are planning to construct a column and to investigate its performance in HDX-MS experiments compared with commercially available pepsin columns. We also seek to answer the question whether combining nepenthesin and pepsin with their different cleavage specificities in one column will help in increasing the sequence coverage in HDX-MS experiments.

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P 204

A metabolomics approach using Simultaneous Quantitation and Discovery (SQUAD) on high resolution accurate mass full MS1 level

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Introduction

There are different analytical strategies for metabolomics purposes which are split into two main sections: Untargeted and targeted approaches. Untargeted setups are used for hypothesis-generating purposes which need identification of compounds of interest as a starting point. Targeted approaches are used for validation and translational purposes since they do offer accurate quantification but usually include few prioritized metabolites only and require known compounds. The presented "Simultaneous Quantitation and Discovery" (SQUAD) workflow shows a combination of identification, accurate quantitation of preselected metabolites and the possibility of retrospective data mining while making use of the high-resolution accurate mass abilities of orbitrap based systems.

Methods

Metabolite Reference Standard NIST SRM 1950 plasma sample and isotope-labeled amino acids and organic acids were purchased from Sigma and CIL, respectively. Plasma was spiked with a dilution series of the labeled compounds and extracted with an excess of methanol. Reversed phase chromatography was applied as the technique of choice for pre-separation of the metabolites. Detection and data acquisition was performed on a Thermo Scientific™ Orbitrap Exploris™ MX system, an Orbitrap based mass spectrometer with Full MS1 capabilities, while switching polarity in an alternating scan-to-scan fashion. Thermo Scientific™ TraceFinder and Compound Discoverer software utilizing mzCloud™ spectral database were used for data processing, analytes quantitation, and unknown annotation.

Preliminary Data

The "Simultaneous Quantitation and Discovery" (SQUAD) workflow putatively identifies a high number of metabolites in combination with accurate and precise quantitation of known compounds from the same sample injection. Regarding the quantitative part of the assay, we observed an absolute sensitivity with a LOQ in the low fmol range on column for the plasma-spiked amino acids and organic acids. In addition, we assessed the identification capabilities of the approach. Unlike MS2 based setups, the identification through full scan MS1 data relies on accurate mass detection and the mapping of isotopic clusters only. Through excellent mass accuracy, mass stability and isotope fidelity, 2105 compounds out of the 2561 unique compounds detected could be assigned to a chemical formula, and 427 compounds could be putatively identified as a specific chemical compound. This equals a rate of 20 % detected unique compounds with a putative identification.

Overall, we developed an approach which enhances productivity by making use of a single injection for both unknown identification of potentially biological significant compounds as well as accurate and precise quantitation of already known features combined in one HRAM based, future-proof assay.

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Analysis of polar metabolites in dried blood spot (DBS) using HILIC-LC-MS/MS

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Human health can be profiled through different biological samples and different analytical and "-omics"-approaches, such as proteomics or metabolomics. Blood microsampling is a suitable alternative to venous blood, plasma, or other invasive sampling methods to profile human health, especially in vulnerable groups such as infants and elderly. Dried blood microsampling such as dried blood spot (DBS) can be used in targeted and untargeted metabolomics for screening and diagnosis, despite they have been originally developed for the diagnosis of genetic diseases, mostly in newborn or children. Other fields of application of DBS are screening of exogenous metabolites for antidoping, toxycology analysis or therapetic drugs monitoring. In most cases, untargeted metabolomics for DBS is performed by reversed-phase liquid chromatography coupled to mass spectrometry (RPLC-MS). However, this method misses the analysis of polar metabolites such as amino acids, nucleotides and many others. Here we developed and optimized extraction procedure and a HILIC-LC-MS/MS method for untargeted analysis of DBS. 63 standard molecules, including amino acids, CoA, carnitines, acids and sugars, and five different extraction solvents were tested for the method development. Analysis was performed on a Sciex ZenoTOF 7600. Data treatment and annotation were performed using MS convert[1], SLAW[2] and in-house R[3] scripts, and data was evaluated by the number of detected features, reproducibility and annotation rates.

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P 206

Analysis of mycotoxins in plant-based milk alternatives via UPLC-MS/MS

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Plant-based milk alternatives are an important commodity, increasingly favored by consumers as a substitute for animal milk. Despite their sustainability, these products face a substantial challenge, primarily associated with the frequent contamination of their raw materials by molds, which produce a diverse array of mycotoxins.

In order to comprehensively assess the risk associated with this product category, we developed and validated a QuEChERS-based workup followed by UPLC-MS/MS analysis to quantify 28 different mycotoxins produced by *Alternaria*, *Aspergillus* and *Fusarium* species.

For the mycotoxins with labelled internal standards quantification was conducted via SIDA. Those mycotoxins are AFB1, AFB2, AFG1, AFG2, STC, OTA, DON, 3-AcDON, T-2, HT-2, Enniatin A1, AOH, AME, and TeA. Enniatin A, B, B1 and Beauvericin were quantified using structurally related derivatives as internal standards. Moreover, NIV, FusX, ZEN, AOH-3-G, AOH-3-S, AME-3-S, ATX I, ALTP, and TEN were quantified through matrix calibration and DON-3-G through matrix-correlated response.

Using the validated method, the toxin contamination of 94 plant-based milk alternatives was quantitatively determined. This selection includes oat, almond, soy, rice, cashew, spelt, pea, hazelnut, buckwheat, pistachio, millet, sunflower, hemp, tiger nut, chestnut, macadamia, chia, barley, and walnut drinks. Particular emphasis has been placed on oat, almond, soy and rice drinks, which are most popular among consumers. Despite the high dilution of plant-based milk alternatives, mycotoxins were detected in all samples, with only two exceptions. In general, however, it was observed that plant-based milk alternatives do not represent a significant health risk in terms of the investigated mycotoxin contamination due to their relatively low levels. Nevertheless, elevated levels of specific toxins were observed in individual cases.

In the case of consuming an analyzed oat milk, the TDI for Type A Trichothecenes would be exceeded by double. In a risk assessment for AFB1, MOEs up to 298 were determined in tiger nut milk, and even up to 60 in the case of (young) children. Aflatoxins were also frequently detected in almond milk with MOEs up to 1043. Among the genotoxic *Alternaria* toxins, notably higher concentrations of AOH and its derivatives were found in hazelnut milk, walnut milk, and buckwheat milk, with their highest concentrations exceeding the TTC value by up to more than seven times. The hazelnut milk samples tested were also contaminated with TeA at levels of up to 85.94 µg/L, although the TTC value was not exceeded for adults, it was for (young) children.

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Development of a global metabo-lipid-prote-omics workflow to compare healthy proximal and distal colonic epithelium in mice

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A mass spectrometry-based metabo-lipid-prote-omics workflow was developed to characterize the molecular interplay within the proximal (PC) and distal (DC) colonic epithelium of healthy mice from a single tissue biopsy. This multi-omics dataset lays the foundation to better understand the interplay of metabolites, lipids, and proteins and to study the regional differences in healthy mouse colon tissue. First, a methyl *tert*-butyl ether-based extraction method was optimized so that from a single tissue biopsy, above 350 reference-matched metabolites, above 1850 reference-matched lipids, and above 4500 proteins were detected by using targeted and untargeted metabolomics, untargeted lipidomics, and shotgun proteomics. The targeted assay quantified 122 metabolites, including amino acids, acylcarnitines, nucleotides, free fatty acids, short-chain fatty acids, and organic acids. Next, each omics dataset was statistically analyzed by itself. The principle component analysis of each omics technique showed a clear separation between the two tissue types. Around 170 significantly changed metabolites, 200 significantly changed lipids, and 420 significantly changed proteins have been detected in the distal and proximal region of the colon. By using the joint pathway analysis (1), BioPAN (2), and Pathview (3), the datasets were analyzed together to generate a deep understanding of the underlying complex regulatory network within the colon. For example, differences in mucin formation were detected on the substrate level as well as on the enzyme level, and altered lipid metabolism by the detection of phospholipases hydrolyzing sphingomyelins to ceramides. In conclusion, combining the three mass spectrometry-based omics techniques can better entangle the functional and regional differences between PC and DC tissue compared to each single omics technique.

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P 208

Data independent analysis evolution exploring the use of high resolving power multi-reflecting time-of-flight mass spectrometry selectivity for metabolite identification

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A hybrid quadrupole multi-reflecting time of flight system (Q-MRT) has been used to perform LC-MS non-targeted screening to identify pharmaceutical drug xenobiotics in the urine of a healthy volunteer patient. Using a system resolving power > 200,000 FWHM small molecule xenobiotics are mass resolved from endogenous biological matrix interferences to facilitate LCMS ppb mass accuracy, the impact upon xenobiotic and corresponding biotransformation identification using 10 Hz LCMS negative ion electrospray has been assessed.

A metabolite identification workflow has been used to perform a human urine drug screen of a healthy volunteer to identify administered pharmaceutical compounds and their biotransformation products. Repeat analysis has been undertaken for human urine samples (time course points 0 hours, 2 hours, 4 hours, and 6 hours), over a twenty-four-hour period.

The therapeutic drugs administered (acetaminophen, naproxen and carbamazepine, ionise preferentially using ES+. However, using ES-, additional metabolite confirmation and discovery can be attained where ES+ and ES- metabolite screening assays are performed. Data processing tolerances of tr (0.1 min) and mass accuracy (+/-2ppm), product ion count ≥1 and expected/theoretical fragment ion tolerance 0.2 mDa were applied to reduce false detections.

An RMS error of 761 part per billion (ppb) has been obtained for 1813 metabolite detections, which are comprised of small molecules between m/z 150 and m/z 427, along with ppb mass accuracy for MSE data independent analysis (DIA) fragment ions. In the case of acetaminophen, for repeat analysis of the human urine time course point samples an mass measurement error (RMS) for acetaminophen sulfate (882 ppb), acetylcysteine acetaminophen (740 ppb) and acetaminophen glucuronide (773 ppb) have been attained over a 24-hour period.

For the fragment ions obtained of [acetaminophen sulfate - H]- a mass measurement error of 735 ppb (m/z 107), -58 ppb (m/z 150) and 28 ppb (m/z 230) have been acquired, with mass resolution >150,000 (m/z 150) at 10 Hz. Alongside precursor/fragment ion ppb mass accuracy, the [M-H]- A+1 and A+2 fine isotope of acetaminophen sulfate structure has provided an additional identification criterion. Using a method that employs enhanced mass scale resolving power, >300,000 FWHM, further investigations into the use of FIS as an identification criterion have been performed.

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Novel Aspect: Broadband DIA for drug xenobiotics in biological matrices using a high mass resolving power quadrupole multi-reflecting time of flight system.

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The impact of ppb mass accuracy upon biotransformation product identification using negative Ion non-targeted urinary screening multi-reflecting time-of-flight LCMS

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Introduction

A hybrid quadrupole multi-reflecting time of flight system (Q-MRT) has been used to perform LC-MS non-targeted screening to identify pharmaceutical drug xenobiotics in the urine of a healthy volunteer patient. Using a system resolving power > 200,000 FWHM small molecule xenobiotics are mass resolved from endogenous biological matrix interferences to facilitate LCMS ppb mass accuracy, the impact upon xenobiotic and corresponding biotransformation identification using 10 Hz LCMS negative ion electrospray has been assessed.

Methods

Negative ion electrospray using LCMSE data independent acquisition (DIA) precursor/fragment ion data acquisition was performed using a multi-reflecting quadrupole time-of-flight mass spectrometer (system resolving power >200,000 FWHM). Human urine samples (time course points 0 h, 2 h, 4 h & 6 h post dose) were analysed. 5 µL injection volumes of urine samples 1:10 diluted (H2O) were utilised for RP-LC with a 12-min gradient.

Preliminary Data

A metabolite identification workflow has been used to perform a human urine drug screen of a healthy volunteer to identify administered pharmaceutical compounds and their biotransformation products. Repeat analysis has been undertaken for human urine samples (0 h, 2 h, 4 h & 6 h), over a twenty-four-hour period.

The therapeutic drugs administered (acetaminophen, naproxen and carbamazepine, ionise preferentially using ES+. However, using ES-, additional metabolite confirmation and discovery can be attained where ES+ and ES- metabolite screening assays are performed. Data processing tolerances of tr (0.1 min) and mass accuracy (+/-2ppm), product ion count ≥1 and expected/theoretical fragment ion tolerance 0.2 mDa were applied to reduce false detections.

An RMS error of 761 part per billion (ppb) has been obtained for 1813 metabolite detections, which are comprised of small molecules between *m/z* 150 and *m/z* 427, along with ppb mass accuracy for MSE (DIA) fragment ions. In the case of acetaminophen, for repeat analysis of the human urine time course point samples a mass measurement error (RMS) for acetaminophen sulfate (882 ppb), acetylcysteine acetaminophen (740 ppb) and acetaminophen glucuronide (773 ppb) have been attained over a 24-hour period.

For the fragment ions obtained of [acetaminophen sulfate - H]- a mass measurement error of 735 ppb (*m/z* 107), -58 ppb (*m/z* 150) and 28 ppb (*m/z* 230) have been acquired, with mass resolution >150,000 (*m/z* 150) at 10 Hz. Alongside precursor/fragment ion ppb mass accuracy, the [M-H]- A+1 and A+2 fine isotope of acetaminophen sulfate structure has provided an additional identification criterion. Using a method that employs enhanced mass scale resolving power, >300,000 FWHM, further investigations into the use of FIS as an identification criterion have been performed.

Novel Aspect

Broadband DIA for drug xenobiotics in biological matrices using a high mass resolving power quadrupole multi-reflecting time of flight system.

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Determination of the metabolic pathways of different Rycals in *in-vitro* samples for doping control purposes

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The continuous development of new drugs is associated with an increase of potential drugs of abuse in sports. This evolution necessitates the continuous improvement of existing test methods and the implementation of new substances to routine doping controls. The new substance class of ryanodine channel complex stabilizers (Rycals), that is assigned to the category S0 for non-approved substances of the Prohibited List by the World Anti-Doping Agency (WADA) is of particular interest for sports drug testing proposes. Due to their stabilizing properties regarding the Ca2+-channels under intense physical activity, Rycals are suspected to have a major impact on the endurance performance of athletes.[1] To date, the compounds S107, JTV-519, ARM 036 and ARM 210 have been discussed to show this stabilizing effect onto the Ca^{2+}-channel.[1-4] While the metabolic behavior of S107 was already investigated, little is known on the metabolism of JTV-519, ARM 036 and ARM 210.

In this study we investigate the *in-vitro* metabolic pathways of the Rycals S107, JTV-519, ARM 036 and ARM 210. To obtain metabolic transformation, the substances were incubated using human liver microsomes (HLM). All samples were analyzed using liquid chromatography high resolution mass spectrometry (LC-HRMS). Except for ARM 036, where only Phase-I metabolites were observed, each substance showed the occurrence of Phase-I as well as Phase-II metabolites. For S107, a new class of metabolic products, showing C-oxidation was identified. The formation of O-demethylated metabolites appears to be valid for all substances investigated in this study. Same applies for the formation of oxidized metabolites. However, at this stage no distinction can be made between N-oxidized, S-oxidized and hydroxylated metabolites. Information gained from this study may be applicable to routine doping control in order to improve the existing methods.

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Quantification of the Colibactin Precursor *N*-Myristoyl-D-Asparagine in *Escherichia coli* by TFC-LC-MS/MS

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Numerous species of human gut microbiota have been linked with the development of colorectal cancer (CRC). However, it remains unknown if bacteria have a direct influence on oncogenic mutations. Certain strains of *Escherichia coli* in the human intestinal microbiome, including those from CRC patients, harbor the pathogenicity island *pks*, which encodes for a set of enzymes that synthesize the small-molecule genotoxin Colibactin. Colibactin induces DNA double-strand breaks in eukaryotic cells, which can lead to cell cycle arrest and inflammatory conditions that may promote CRC carcinogenesis. Due to the high chemical reactivity, Colibactin remains still non-isolable. Therefore, quantification approaches focus on the more stable cleavage product *N*-Myristoyl-D-Asparagine, which is released at the end of the Colibactin formation pathway.

For analysis, reversed-phase high-performance liquid chromatography (RP-HPLC) was used. Method development involved the evaluation of various stationary phases. To lower sample preparation times and extraction steps, turbulent flow chromatography (TFC) was utilized for an online-sample cleanup prior to the LC analysis. Furthermore, a new custom-synthesized ^{13}C-labelled internal standard was used. Hyphenation with electrospray ionization-mass spectrometry (MS) enabled sensitive and selective quantification through MS/MS-experiments in parallel reaction monitoring mode.

The developed online-TFC-LC-MS/MS method allows sample cleanup and reproducible analysis of diluted culture supernatants within 12 min. Thereby different genetically modified *Escherichia coli* M1/5 strains were analyzed to scrutinize their ability to produce Colibactin.

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Investigation structures of *In Vitro* and *In Vivo* metabolites of a novel 20-Keto-Steroid S42 by LC/GC-MS analysis and organic synthesis

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The synthesis of S42, a new 20-keto-steroid, was published in 2006 by *Uyanik et al*.[1] Later, S42 was found to be a selective androgen receptor modulators (SARMs) candidate.[2] Effectively, S42 shows not only anabolic function but also displays tissue-specific transcriptional activity, which can minimize side effects in the prostate and cardiovascular system.[3] Therefore, S42 was proposed as a cure for muscle-related diseases.[4] For these reasons, *Thevis et al.* expressed concerns on the potential of S42 for illicit sports doping.[5] For the control and investigation of S42 abuse, *in vitro* and *in vivo* metabolism experiments were conducted and excretion products were analyzed with LC- and GC-MS-orbitrap instrumentation.

S42 *in vitro* experiments gave access to S42-*mono, bis and tris*-OH phase I metabolites which were identified by GC-MS-orbitrap analysis, while sulfate and glucuronide derivatives from phase II metabolism were investigated by LC-MS. Additionally, urine samples of rats treated with S42 were analyzed by either direct injection with LC-MS or after workup and derivatization with GC-MS. To clarify the structure of the radical cationic molecular ion $[M]^{+\bullet}$ at *m/z* 540.2918 from the sulfate fraction of rat urine samples, GC-MS/MS experiments were conducted. Abundant product ions resulting from extensive fragmentation of the respective molecular ion were found at *m/z* 143.0887, 157.1043, and 243.1204, respectively (see Fig. 1). These significant fragment ions were also discovered in the GC-MS spectrum of TMS-S42-*mono*-OH yielded from the *in vitro* phase I metabolism experiment. Close inspection of the spectra and comparison with MS data of synthetic reference materials evidence that the ion at *m/z* 540.2918 originates from the TMS-S42-*bis*-OH-4H derivative of S42. We furthermore assume that the S42 steroid rings A or B and C are reduced and exhibit two double bonds. The interpretation of MS data of S42 phase I and phase II metabolites will be presented.

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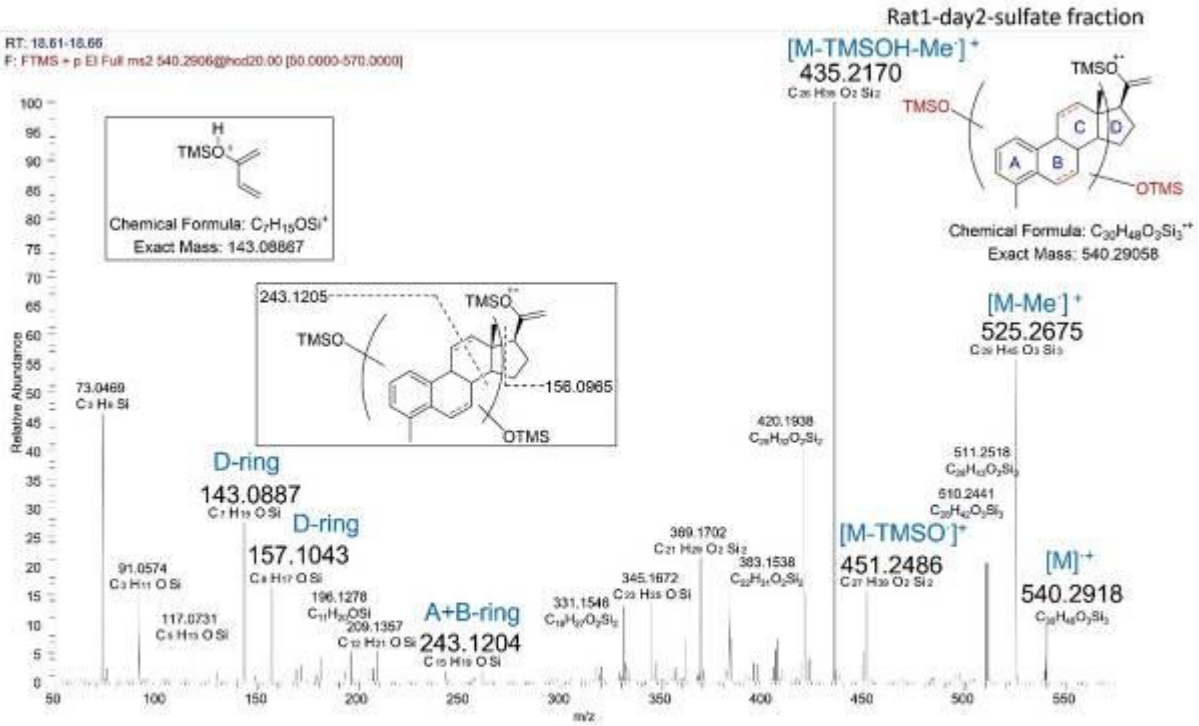
Fig. 1



GC-EI-MS/MS spectrum of the molecular ion of TMS-S42-bis-OH-4H at *m/z* 540 isolated from the sulfate fraction from a rat urine sample on day 2.