NEDERLANDSE VERENIGING VOOR MASSASPECTROMETRIE

PROGRAM FALL MEETING

Morning

09.00-09.30	Welcome and registration
09.30-09.35	Introduction
09.35-10.20	Michiel Vermeulen, RUN ***Keynote lecture:*** *Deciphering lineage specification during early embryogenesis in gastruloids using multilayered proteomics*
10.20-10.40	Kevin Jooβ, VU *Multi-laboratory benchmarking of capillary electrophoresis – mass spectrometry (CE-MS) approaches for intact proteoform analysis in top-down proteomics*
10.40-11.00	Coffee / posters
11.00-11.20	Jessica Del Castillo-Alferez, Sanquin *Proteolytic signatures of coagulation identified and monitored by plasma peptidomics*
11.20-11.40	Marek Noga, MUMC *Development and validation of fluxomics methods for diagnostics of inborn errors of metabolism*
11.40-12.00	Alienke van Pijkeren, RUG *Reducing the impact of contamination to produce reliable single cell proteomics data*
12.00-12.15	ALV
12.15-13.30	Lunch / posters

Afternoon

13.30-14.15	Marko Mank, Danone *Robust and high resolution all ion fragmentation LC-ESI-IMS-MS analysis for in-depth characterization or profiling of up to 200 Human Milk Oligosaccharides (HMOs)*
14.15-14.35	Manuel Peris-Diaz, UU *Asymmetric N-glycosylation occupancy in the tailpiece of recombinant IgA1 revealed by combining glycoproteomics and native electron-capture charge reduction mass spectrometry*
14.35-14.55	Mariyana Savova, LACDR *Effect of Synbiotic Treatment on Fecal Metabolome in Infants with Cow's Milk Allergy*
14.55-15.15	Coffee /posters
15.15-15.35	Wouter de Bruijn, WUR *Separation and characterization of isomers in food by cyclic ion mobility spectrometry – mass spectrometry (cIMS-MS)*
15.35-15.55	Dinko Šoić, TUD *NovoGlyco: mapping protein glycosylation in prokaryotes*
15.55-16.30	Panel discussion 'AI and ethics in science and research' *Panel members: Magnus Palmblad (LUMC) Tess Afanasyeva (Sanquin), Sarah Carter (TUD)*
16.30-16.40	Closure
16.40-17.30	Drinks

BOOK OF ABSTRACTS

LIST OF ORAL PRESENTATIONS

BOOK OF ABSTRACTS

LIST OF POSTER PRESENTATIONS

P016 Hendrik Krolle, VU/MS Vision
Benchmarking Novel and Established Native Mass Spectrometry Methods for High-Mass Applications

P017 Alex Mommer, MU
An optimized method for the quantification of leukotrienes in human plasma using liquid chromatography high-resolution mass spectrometry (LC-HRMS)

P018 Jochem Simonsz, Prinses Maxima Centrum
Development and Validation of a High-Sensitivity LC-MS/MS Assay for Monitoring Asparaginase Activity in Pediatric ALL Treatment

P019 Pavel Sinitcyn, UU
Fast and deep phosphoproteome analysis with the Orbitrap Astral mass spectrometer

P020 Laura Steinbusch, Maastricht UMC+
Targeted urine analysis using UPLC-QTOF-MS as a fast and novel assay for Inborn Metabolic Diseases leading to kidney stones

P021 Iuliia Stroganova, VU
Unravelling the dynamics of the aggregation process of the Ac-PHF6-NH2 tau segment P020

P022 Dennis van den Heuvel, Shimadzu
Oxygen Attachment Dissociation (OAD) MS/MS for the structural identification of double-bond positions in potential lipid biomarkers of pancreatic ductal adenocarcinoma

P023 Remko van Loon, Agilent
Quantitating Over 200 Pesticides in Black Tea Using GC/MS/MS with Steady Performance and Maximized Uptime

P024 Julia Vreugdenhil, UU
De novo sequencing of human milk oligosaccharides using IMS-MS

P025 Linus Wollenweber, UU
The odd one out – Mass spectrometric detection of the ever-changing IgG4 landscape

P026 Agnieszka Smolinksa, MU
Optimization of Faecal Headspace Sampling with High-Capacity Sorptive Extraction Probes (HiSorb) and GC-MS for Volatolomics Applications

P027 Drosos Katsavelis, RUG/Quantall
Insight in the mechanism of action of Semaglutide in Alzheimer's disease as assessed by shotgun proteomics

ORAL PRESENTATIONS

O001

Deciphering lineage specification during early embryogenesis in gastruloids using multilayered proteomics

Michiel Vermeulen[1,2]

1. *Department of Molecular Biology, Faculty of Science, Radboud Institute for Molecular Life Sciences, Oncode Institute, Radboud University Nijmegen, Nijmegen, The Netherlands*
2. *Division of Molecular Genetics, The Netherlands Cancer Institute, Amsterdam, The Netherlands*

The development of an adult human being from a single fertilized egg is accompanied by the generation of ~200 functionally distinct cell types. Each of these cell types expresses only a subset of the 20.000 genes that the human genome encodes for. Cell-type specific gene expression patterns thus ensure the generation of hundreds of phenotypes based on a single genotype. Transcription factors play an important role in driving cell-type specific gene expression, but epigenetic modifications of DNA and core histones also regulate changes in gene expression and phenotype during development and during adult life. Our lab is using quantitative mass-spectrometry based (interaction) proteomics and next generation DNA sequencing technology to decipher (epi)genetic regulation of gene expression in (differentiated) stem cells. In recent years, our lab has implemented and developed various proximity biotinylation workflows to characterize the proximal proteome for chromatin associated protein complexes and epigenetic modifications. In my lecture I will provide an update of these approaches and the results they have generated.

O002

Multi-laboratory benchmarking of capillary electrophoresis – mass spectrometry (CE-MS) approaches for intact proteoform analysis in top-down proteomics

Kevin Jooß[1,2], Noah Gould[3], Alexander R. Ivanov[3], Qianjie Wang[4], Liangliang Sun[4]

1 *Division of Bioanalytical Chemistry, Department of Chemistry and Pharmaceutical Sciences, Amsterdam Institute of Molecular and Life Sciences, Vrije Universiteit Amsterdam, Amsterdam, The Netherlands.*
2 *Centre for Analytical Sciences Amsterdam (CASA), The Netherlands*
3 *Barnett Institute of Chemical and Biological Analysis and Department of Chemistry & Chemical Biology, Northeastern University, Boston, MA, USA.*
4 *Department of Chemistry, Michigan State University, East Lansing, MI, US*

Proteoforms are protein variants produced from a single gene, which can acquire distinct functional properties through mutations, polymorphisms, alternative splicing, and post-translational modifications (PTMs). Gaining insight into these proteoforms is crucial for understanding their roles in biological processes, thereby enhancing our knowledge of human health and disease. In recent years, the technique of "top-down" mass spectrometry (TDMS) –which involves analyzing intact proteoforms followed by fragmentation – has gained popularity, facilitating the characterization of complex proteomes in biological samples.

Capillary zone electrophoresis (CZE), developed in the 1980s, is a key method for separating biomolecules, particularly proteoforms. Separation in CZE relies on differences in electrophoretic mobility (µep), related to a proteoform's net charge and hydrodynamic radius. The integration of CZE with top-down mass spectrometry (TDMS) has proven to be an effective strategy for analyzing complex proteomes. Despite its advantages, there is a prevailing belief in some segments of the analytical community that CZE lacks the robustness, reproducibility, and sensitivity required for effective proteoform characterization. Furthermore, the interfacing of CE with MS is often regarded as technically more challenging than liquid chromatography (LC)-MS, primarily due to the need to apply an electric field across the separation capillary.

In light of these advancements, we launched a global cross-laboratory study, supported by the Top-Down Consortium (https://ctdp.org/initiatives/ce-ms-initiative/), to assess and showcase the performance of CZE-MS for Top-Down Proteomics (TDP). Our objective is to create a comprehensive repository of data from 11 laboratories using various CZE-MS platforms, including capillary- and microfluidic chip-based setups. The study incorporates a range of CE-MS interfaces (5) and mass spectrometry systems (11) utilized by participants, encompassing many commercially available CE systems and mass spectrometers suitable for TDP applications.

Participants analyzed two types of samples: (**i**) a commercially available standard mixture containing six proteins ranging from approximately 9 kDa to 68 kDa, and (**ii**) an intact protein yeast extract. For the protein mixture, groups employing similar CE conditions exhibited a remarkably consistent migration order. After internal standard correction, the relative standard deviation (RSD) values for migration times were consistently below 1% ($n \geq 3$). The capabilities of CZE-TDMS were further demonstrated by achieving baseline separation of IgF-1 LR3 and its deamidated variant, along with the ability to localize the position of this PTM within the amino acid sequence. In the more complex yeast extract samples, most participants identified over 1,000 proteoforms across triplicate runs, regardless of setup and methodological differences. Additionally, a high degree of run-to-run reproducibility in terms of observed migration times was observed across all participants. In conclusion, our findings demonstrate that CZE-TDMS is a flexible, powerful, and reasonably reproducible technique for analyzing intact proteoforms in diverse laboratory environ

O003

Proteolytic signatures of coagulation identified and monitored by plasma peptidomics

Jessica Del Castillo [1], Alexander B. Meijer[1], Herm Jan M Brinkman[1,2] , Arie J. Hoogendijk[1], Joost C.M. Meijers[2], Maartje van den Biggelaar[1,*], Tirsa T. van Duijl[1,*]

1 *Department of Molecular Hematology, Sanquin Research, Amsterdam, The Netherlands.*
2 *Department of Experimental Vascular Medicine, Amsterdam University Medical Centers, University of Amsterdam, The Netherlands*

BACKGROUND

Coagulation comprises a network of proteolytic processes initiated by tissue factor (TF) that regulate the function of plasma proteins and result in the formation of a fibrin clot. Altered proteolysis of coagulation proteins is implicated in hemostatic and fibrinolytic disorders. Here, we employed plasma peptidomics to identify the proteolytic products of coagulation and subsequently monitor the impact of tissue factor concentrations and thrombin inhibition in proteolytic processing.

METHODS

Pooled citrated plasma from healthy donors was clotted in vitro by recalcification and addition of increasing concentrations of tissue factor (TF), in the absence or presence of a thrombin inhibitor hirudin. Endogenous peptides were enriched by solid phase extraction (SPE), analysed by LC-MS/MS in DDA mode (Orbitrap Fusion Lumos) and identified using PEAKS X. Thrombin generation in clotting plasma was measured using the Calibrated Automated Thrombogram (CAT) assay with a microtiter plate reading fluorometer (Fluoroskan Ascent, ThermoLab systems) and Thrombinoscope® software (Thrombinoscope BV).

RESULTS

Plasma coagulation revealed a distinct and richer endogenous peptidome in which fibrinopeptides A and B were most abundant. Unexpected proteolytic hotspots were identified in the C-terminal region of fibrinogen α chain. The activation peptides of coagulation factors prothrombin ($_{316}$TAT-DGR$_{363}$) and FXIIIA ($_{6}$TAF-VPR$_{38}$) were recognized as well as their proteolytic degradation products. Peptides derived from protease inhibitors mapping within their internal bait (e.g. $_{705}$VGF-MGR$_{715}$ of alpha 2 macroglobulin) or C-terminal regions (e.g. $_{426}$SLN-CVK$_{464}$ of antithrombin) were observed exclusively upon coagulation and were sensitive to increasing TF concentrations. Thrombin inhibition blocked the formation of coagulation activation peptides and the proteolytic processing of hemostatic regulators, but not inflammatory proteins such as acute phase SAA4. Finally, quantitative monitoring of coagulation with calibrated automated thrombograms showed rapid cleavage of fibrinopeptides correlated with initial thrombin generation followed by prothrombin, FXIIIA, and protease inhibitors.

CONCLUSION

Plasma peptidomics exposed a distinct endogenous peptidome upon clotting, enriched with activation peptides from zymogen-to-enzyme transitions and proteolytic products of protease inhibitors. Monitoring changes in the peptidome under different coagulating conditions provided insights into thrombin and tissue factor sensitive proteolytic processing in plasma. This peptidomics strategy can be leveraged to study proteolytic processing of protease networks in bleeding or thrombosis.

O004

Development and validation of fluxomics methods for diagnostics of inborn errors of metabolism

Marek J. Noga[1], Sandra Coenen[1], Huub Waterval[1], M. Estela Rubio Gozalbo[2], Dirk J. Lefeber[3], Jörgen Bierau[4] and Laura K.M. Steinbush[1]

[1] *Department of Clinical Genetics, Maastricht UMC+, Maastricht, the Netherlands*
[2] *Department of Pediatrics, Maastricht UMC+, Maastricht, the Netherlands*
[3] *Translational Metabolic Laboratory, Radboudumc, Nijmegen, the Netherlands*
[4] *Department of Clinical Genetics, Erasmus MC, Rotterdam, the Netherlands*

With the growing discovery of new gene variants through high-throughput DNA sequencing, there is an increasing need to assess their pathogenicity. We demonstrate that metabolic labelling with stable isotopes in cell culture, combined with mass spectrometry (cellular fluxomics), can uncover and verify the molecular phenotypes of variants of unknown significance.

We optimized targeted LC-MS/MS methods to detect ^{13}C and ^{15}N incorporation into intermediates of the pyrimidine de novo biosynthesis pathway and sugar metabolism. *In silico* MS/MS fragmentation was used to predict fragment ion masses for selective detection of isotopomers.

We evaluated the diagnostic performance of cellular fluxomics in 45 fibroblast cell lines: 15 patient-derived (4 with a known variant in the pyrimidine de novo biosynthesis pathway and 11 with congenital disorders of glycosylation (CDG)) and 20 controls. Cells were grown to near confluence and then shifted to medium supplemented with $^{13}C_6$-glucose, $^{13}C_5$-glutamine, or ^{15}N-glutamine. Label incorporation was tracked for up to 24 hours. Control cell lines were cultured up to three times to assess the reproducibility of the experiments.

Cells with pathogenic variants in CAD or UMPS showed significantly reduced incorporation of ^{13}C and ^{15}N into the products and intermediates of the pyrimidine biosynthesis pathway. One UMPS line showed elevated orotate levels, suggesting that the variant increased the K_m for orotate. Validation experiments revealed that the labelled UMP ratios varied depending on the batch of culture medium. This effect was stronger with $^{13}C_5$-glutamine than with ^{15}N-glutamine but did not eliminate the differences between affected and control cells. Due to these batch effects, establishing absolute reference intervals is currently not possible, and label incorporation in patient cells is evaluated relative to a parallel-cultured control line.

In the analysis of sugar metabolism, we independently detected ^{13}C incorporation into the sugar, ribose, and nucleobase components of nucleotide sugars. Patient cell lines showed reduced ^{13}C incorporation into intermediates of the UDP-galactose and GDP-mannose biosynthesis pathways, consistent with pathological variants.

We validated a fluxomics method for functionally classifying variants of unknown significance in pyrimidine biosynthesis genes and demonstrated the potential of clinical fluxomics for studying metabolic genes related to CDG. MS/MS also proved effective at distinguishing positional isotopomers of sugar metabolism intermediates, enabling the deconvolution of metabolic labelling through alternative pathways.

O004

Reducing the impact of contamination to produce reliable single cell proteomics data

Alienke van Pijkeren[a], Kristoffer Basse[a], Surina Chuckaree[a], Jing Zheng[a], Mohammed Hanzala Kaniyar[b], Omar Rosas Bringas[b], John LaCava[b,c], Peter Horvatovich[a], Guinevere Lageveen-Kammeijer[a]

[a] *Analytical Biochemistry, Groningen Institute of Pharmacy, University of Groningen, Groningen, the Netherlands*
[b] *European Research Institute for the Biology of Ageing, University Medical Centre Groningen, Groningen, the Netherlands*
[c] *Laboratory of Cellular & Structural Biology, The Rockefeller University, New York, USA*

INTRODUCTION

Single cell proteomics (SCP) is an emerging analytical technique advancing our understanding of cellular protein function and heterogeneity. Hitherto, the community is pushed to report on the highest number of detected and quantified proteins, but it is important to consider that the protein content is in the few-hundred picogram range and even a small amount of contamination can lead to a large bias in the measured data. In this project, we aim to assess and reduce the amount of contamination by investigating several experimental parameters during cell isolation and sample preparation. Furthermore, as broadly discussed community guidelines for SCP data analysis are still missing, various data analysis software packages and methods are used to further explore the reliability of SCP data analysis.

METHODS

A549 and HeLa cells were isolated and processed by the CellenONE platform either in a 96-well plate or Cellenion's EVO96 proteoCHIP. To assess and reduce the amount of contamination, experiments were performed using the commonly used sample preparation workflow or using an optimized one, which includes extensive cleaning of the CellenONE and the use of a laminar flow cabinet. Next to single cells, each experiment included 50 cell samples and "0 cell" samples (undergoing the same workflow but without cell dispension) for contamination control. All samples were analyzed using a nanoElute2 LC system coupled to a timsTOF SCP-MS. Data was analyzed either with Spectronaut V19 or DIANN 1.9. In Spectronaut, different analysis procedures were investigated using the single cell data; individually (method evaluation workflow), together (matching), or together with 50 cell samples (boosting).

RESULTS

Performing the regular sample preparation workflow in a 96-well plate with HeLa cells resulted in a striking high number (1234 ± 203) of identified protein groups (PGs) in the "0 cell" samples using the method evaluation workflow. A total of 72.2% of the identified PGs in the single cell samples (1708 ± 266 PGs) were found back in the "0 cell" samples, indicating a high level of contamination. This was reduced to 0.1% (zero cells: 1 ± 1 PGs, single cells: 839 ± 381 PGs) when the optimized CellenOne protocol and the EVO96 proteoCHIP was used. Previous data was analyzed by Spectronaut and by analyzing the same dataset in DIANN 1.9 no PGs could be quantified in the "0 cell" samples.
Next, the different data analysis procedures were evaluated using single A549 cells (n=64). The method evaluation, matching, or boosting workflows resulted in 785 ± 289, 1508 ± 150, and 1748 ± 482 PGs, respectively. Additionally, a filtering step was performed which excluded identified PGs that contained less than two peptides and PGs without unique peptides. Filtering decreased the number of IDs to 496 ± 238, 1129 ± 149, and 1086 ± 373 for the method evaluation, matching, and boosting workflows respectively. These results suggest a less reliable protein identification in the boosted data analysis method.

CONCLUSIONS

In conclusion, as SCP is still under methodological development, we hope that our results raise awareness and can form the basis of general recommendations to the SCP community on the importance of assessing and minimizing contamination in analytical workflows. Minimizing contamination and performing appropriate data analysis is crucial for obtaining reliable SCP data.

O004

Robust and high resolution all ion fragmentation LC-ESI-IMS-MS analysis for in-depth characterization or profiling of up to 200 Human Milk Oligosaccharides (HMOs)

Marko Mank[1], John Gonsalves[1], Julia Bauzá-Martinez, Kelly A. Dingess[1], Bernd Stahl[1]

1 *Danone Research & Innovation*
2 *Skid Visual Science*

BACKGROUND

Human milk oligosaccharides (HMOs), the third largest component of human milk (HM), are crucial for infant health and development. HMOs are complex and diverse molecules with frequent occurrence of isomers. Their abundances and diversity are influenced by maternal genotype and lactational stages. Analyzing the diversity of HMO structures remains challenging for analytical techniques such as liquid chromatography coupled to mass spectrometry (LC-MS). Albeit some novel approaches combining ion mobility (IMS) with LC-MS have improved structural resolution, they often focus on a limited subset of HMOs, lacking the throughput necessary to obtain fundamental insights into HMO composition.

METHODS

We introduce an LC-ESI-IMS-MS/MS method combining high-resolution RT information, IMS drift time, CID fragmentation, and accurate spectral measurements to simultaneously identify and quantify up to 200 HMOs ranging from DP 2-13. The method was optimized on HM standard reference material 1953 (SRM1953). Two HMO fractions isolated from pooled HM, containing either neutral or acidic HMOs, were used to optimize LC separation and achieve relative quantitation of HMOs. RT validation was done using commercially available HMO standards. The method was applied to HM samples from donors, under written informed consent. Prior to LC-ESI-IMS-MS/MS characterization, all samples underwent thawing, treatment with formic acid, lipid separation and cleanup by mixed cationic exchange (MCX). Data acquisition and processing were done using UNIFI software and R scripts.

RESULTS

Hydrophilic Interaction Liquid Chromatography (HILIC) was chosen for its reproducible separation of complex HMO mixtures. Compared to Porous Graphitized Carbon (PGC), HILIC offered better predictable elution of HMOs based on degree of polymerization (DP) and better stability over time (Fig1A-B). Optimization of ion source and fragmentation strategy led to an optimal tridimensional strategy for HMO isolation and peak refinement capable of distinguishing co-eluting isomers (e.g., Fig1C). Two gradients were optimized for deep characterization or high-throughput analysis, detecting up to 200 and 132 HMOs respectively (Fig1D). The high-throughput gradient effectively identified milk types I-IV and provided insights into HMO composition throughout lactation (Fig1E-F).

CONCLUSION

Our LC-IMS-MS method allows comprehensive monitoring and confident identification of HMOs. Facilitating screening of an unparalleled broad array of HMOs up to DP13, identifying functional relevant features, including novel isomers. With its throughput and depth, this approach will advance the study of HMOs in a result-driven approach, by improving our understanding of these crucial milk components and ultimately accelerate tailorizing breast milk substitutes closer to HM.

FUNDING SOURCE(S)

Danone Research & Innovation

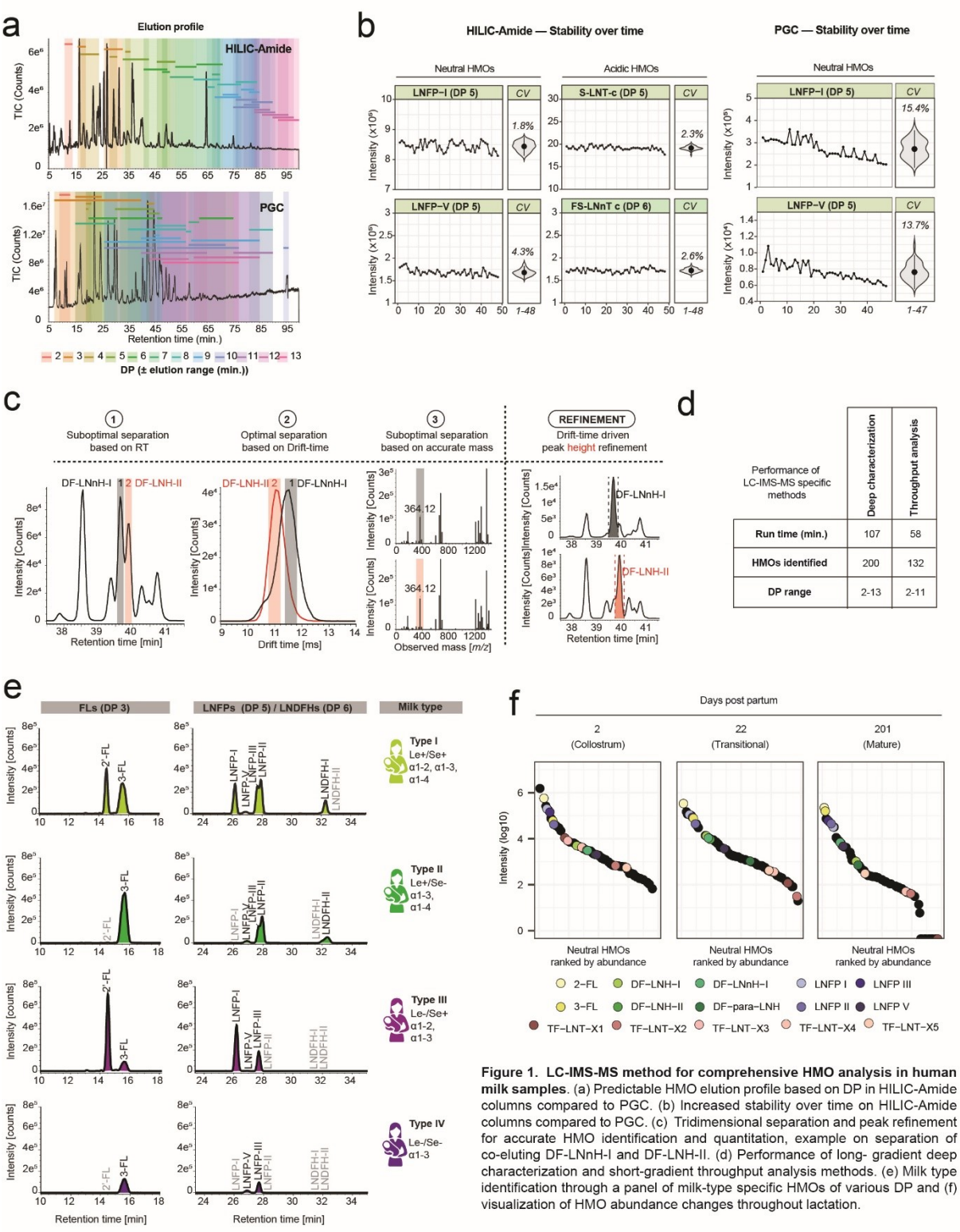


Figure 1. LC-IMS-MS method for comprehensive HMO analysis in human milk samples. (a) Predictable HMO elution profile based on DP in HILIC-Amide columns compared to PGC. (b) Increased stability over time on HILIC-Amide columns compared to PGC. (c) Tridimensional separation and peak refinement for accurate HMO identification and quantitation, example on separation of co-eluting DF-LNnH-I and DF-LNH-II. (d) Performance of long- gradient deep characterization and short-gradient throughput analysis methods. (e) Milk type identification through a panel of milk-type specific HMOs of various DP and (f) visualization of HMO abundance changes throughout lactation.

O005

Asymmetric *N*-glycosylation occupancy in the tailpiece of recombinant IgA1 revealed by combining glycoproteomics and native electron-capture charge reduction mass spectrometry

Manuel David Peris-Díaz [a], Evolène Deslignière[a], Shelley Jager[a], Albert Bondt[a], Nadia Mokiem[a],Arjan Barendregt[a], Albert J. R. Heck [a]

[a] Biomolecular Mass Spectrometry and Proteomics, Bijvoet Center for Biomolecular Research and Utrecht Institute for Pharmaceutical Sciences, Utrecht University, Utrecht 3584 CH, The Netherlands

Ensuring high-quality biopharmaceuticals, particularly monoclonal antibodies (mAbs), requires robust manufacturing processes that guarantee consistent glycosylation, a critical quality attribute essential for efficacy and safety. Here, we employed a variety of mass spectrometry (MS)-based approaches, both (glyco)peptide- and protein-centric, to resolve the complex glycoproteoform landscape of recombinant IgA1 produced in HEK293 cells. These key immunoglobulins harbour several *N*- and *O*-glycosylation sites, making them considerably more heterogeneous than their IgG counterparts. We provide quantitative data on the occupancy and glycan composition for each IgA1 glycosylation site. Combining all data, we revealed that IgA1 molecules consist of at least three distinct populations with varying *N*-glycosylation site occupancies at the C-terminal tailpiece, namely one with both glycosylation sites occupied, another with both glycosylation sites unoccupied, and a third asymmetric population with one glycosylation site occupied and the other unoccupied, challenging the prevailing acceptance that IgA1 *N*-glycosylation is symmetrical. This finding is significant, given that the tailpiece is involved in interactions with the J-chain and the Polymeric Immunoglobulin Receptor, and in general as antibody glycosylation is a quality attribute that needs to be carefully monitored, as the presence and nature of these modification can affect the antibody's efficacy, lifetime, stability and binding and/or neutralizing capacities. Optimizing strategies to produce recombinant IgA1 requires efficient and specific quality control analytical strategies as presented here, which is essential for therapeutic IgA1-based antibody development. We expect that the integrated MS-based strategy presented here may be beneficial to comprehensively characterize the glycoproteoform profiles of IgA1-based therapeutics, thereby improving their production and optimization processes and facilitating the pathway to bring more IgA1-based therapeutics into clinical applications.

O008

Effect of Synbiotic Treatment on Fecal Metabolome in Infants with Cow's Milk Allergy

Mariyana V. Savova[a], Pingping Zhu[a], Alida Kindt[a], the PRESTO study team, Harm Wopereis[b], Clara Belzer[c], Amy C. Harms[a], Thomas Hankemeier[a]

[a] *Metabolomics and Analytics Centre, Leiden Academic Centre of Drug Research, Leiden University, Einsteinweg 55, 2333CC, the Netherlands*
[b] *Danone Research & Innovation, Uppsalalaan 12, 3584 CT Utrecht, the Netherlands*
[c] *Laboratory of Microbiology, Wageningen University, Stippeneng 4, 6708 WE Wageningen, the Netherlands*

SCOPE

Cow's milk allergy (CMA) is one the most prevalent food allergies in early childhood often treated via elimination diets including amino acid-based formula (AAF) consumption. Targeting the gut microbiome by supplementing AAF with synbiotics (AAF-S) is a promising additional nutritional strategy. Although AAF-S has been found to alleviate allergic symptoms and modify the gut microbiome, its impact on the metabolome remains underexplored. This work aimed to assess the effect of cow's milk tolerance acquisition and of synbiotic (inulin, oligofructose, *Bifidobacterium breve* M-16V) supplementation on the fecal metabolome in infants with IgE-mediated CMA.

METHODS AND RESULTS

The 39 CMA-allergic infants received AAF or AAF-S for a year during which fecal samples were collected at three timepoints. The samples were subjected to GC-FIC and LC-MS metabolomics analyses covering gut microbial metabolites including short chain fatty acids (SCFAs), tryptophan metabolites, and bile acids. Longitudinal analysis was performed using linear mixed models and repeated measures analysis of variance simultaneous component analysis (RM-ASCA+). The results suggested amino acids, bile acids, and branched SCFAs alterations in infants who outgrew CMA during the intervention. Whereas synbiotic supplementation significantly modified the fecal metabolome after six months of intervention, including altered purine, bile acid, and unsaturated fatty acid levels, and increased metabolites of infant-type *Bifidobacterium* species: indolelactic acid and 4-hydroxyphenyllactic acid.

CONCLUSION

This study offers no clear conclusion on the impact of CM-tolerance acquisition on the fecal metabolome. However, our results show that six months of synbiotic supplementation successfully altered the fecal metabolome and suggest induced bifidobacteria activity, which declined at 12 months of intervention.

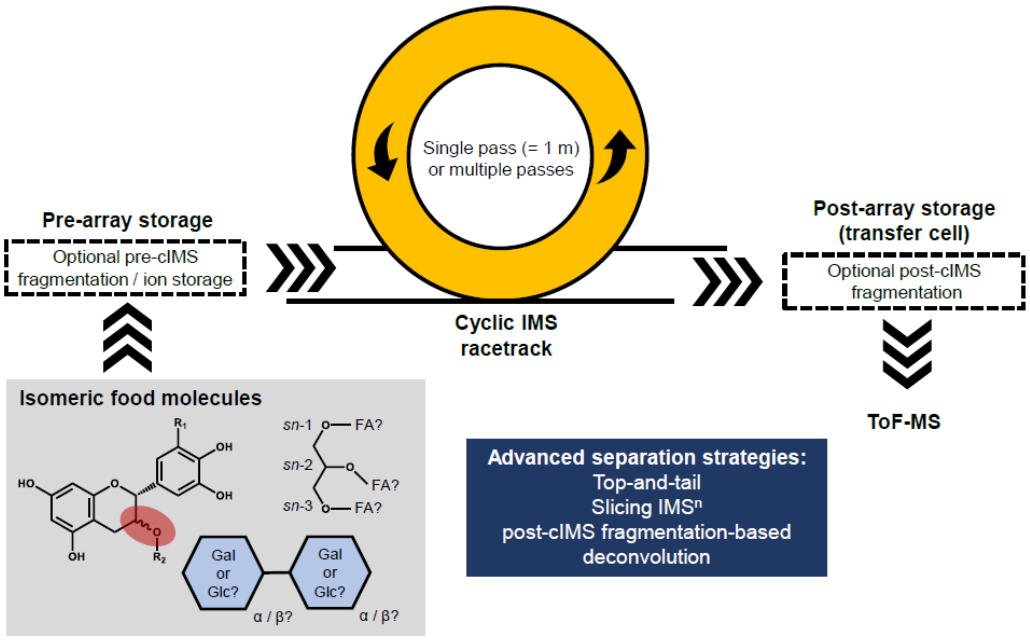
O009

Separation and characterization of isomers in food by cyclic ion mobility spectrometry – mass spectrometry (cIMS-MS)

Bram van de Put*, Carlo R. de Bruin*, and **Wouter J.C. de Bruijn**

**Equal contribution*
Laboratory of Food Chemistry, Wageningen University, Wageningen, the Netherlands

Analytical techniques such as LC-MS or NMR are widely used for analysis of complex mixtures of food molecules but are lacking in separation of isomers. Separation of isomers is important for determining food compositions and their effects on human health, and for monitoring reactions of food molecules. Thus, we have been exploring the use of cyclic ion mobility spectrometry - mass spectrometry (cIMS-MS) as a tool for the separation of isomeric food molecules. Here we would like to discuss our findings thus far, with examples to illustrate the possibilities as well as the challenges associated with cIMS-MS. The travelling wave-based cyclic racetrack in combination with pre- and post-array storages provided great flexibility for separation and fragmentation of isomeric flavonoids (e.g. catechin and epicatechin), saccharides (e.g. glucose, galactose, and their anomers), and triacylglycerols (e.g. *sn*-positional isomers). In most cases multiple passes of the ions in the racetrack were necessary. Still, many of these separations are extremely fast (<100 ms up to a few hundred ms). For specific cases (e.g. certain triacylglycerol isomers), much longer cIMS separation times (>1000 ms) could be used to achieve separation. Furthermore, advanced strategies (e.g. top-and-tail, slicing) and deconvolution based on post-cIMS fragmentation proved essential in analysis of complex mixtures of multiple isomers. Formation of charge site isomers often posed a challenge for protonated or deprotonated ions, but could usually be prevented by using metal-ion adducts. Remaining challenges with cIMS-MS include CCS calibration to accurately and reproducibly determine experimental CCS values from multiple passes. Additionally, reliable *in silico* modelling of large or flexible gas phase ions for prediction of CCS to rationalize observed separations or identifications is currently a hurdle. Despite the remaining challenges, our results demonstrate the potential of cIMS for separation and characterization of isomeric food molecules.



O010

NovoGlyco: mapping protein glycosylation in prokaryotes

Dinko Soic[1, 2] and Martin Pabst[1]

[1] *Delft University of Technology, Department of Biotechnology, The Netherlands*
[2] *Faculty of Pharmacy and Biochemistry, University of Zagreb, Zagreb, Croatia*
contacts: d.s.soic@tudelft.nl or m.pabst@tudelft.nl

Protein glycosylation is a widespread post-translational modification that plays a critical role in all domains of life. In prokaryotes, protein glycosylation is known to be involved in disease-causing processes, aiding immune evasion and promoting infections[1]. Additionally, in contexts such as the human microbiome, microbial glycoproteins present promising opportunities for developing diagnostic markers and targets for molecular intervention[2].

The extensive diversity of monosaccharides used by microbes to produce glycomolecules, along with structural variations between strains, makes their identification extremely challenging. Consequently, methods developed for eukaryotic glycoproteins are often not applicable, and most recent open database search approaches still require laborious curation of large datasets[3,4].

Here we present NovoGlyco, a novel microbial glycoproteomics platform for identifying and characterizing microbial protein glycosylation. NovoGlyco employs untargeted oxonium ion discovery, sequence tag matching, and parent-ion offset binning to identify glycoproteins from large-scale proteomics data. The identified glycoproteins can be explored in an interactive dashboard which ultimately provides insight into novel microbial glycan structures. We demonstrate our newly developed tool by analysing proteomics data from common human pathogens, environmental bacteria, and Asgard archaea, the closest known prokaryotic relatives to eukaryotes. Finally, we demonstrate the application to environmental enrichments, paving the way for exploring glycoproteins in metaproteomic studies.

REFERENCES

1. *Szymanski CM, Wren BW. Protein glycosylation in bacterial mucosal pathogens. Nat Rev Microbiol. 2005 Mar;3(3):225–37.*
2. *Aminov R, Aminova L. The role of the glycome in symbiotic host-microbe interactions. Glycobiology. 2023 Dec 1;33(12):1106–16.*
3. *Ahmad Izaham AR, Scott NE. Open Database Searching Enables the Identification and Comparison of Bacterial Glycoproteomes without Defining Glycan Compositions Prior to Searching. Mol Cell Proteomics MCP. 2020 Nov 25;19(9):1561–74.*
4. *Pabst M, Grouzdev DS, Lawson CE, Kleikamp HBC, de Ram C, Louwen R, et al. A general approach to explore prokaryotic protein glycosylation reveals the unique surface layer modulation of an anammox bacterium. ISME J. 2022 Feb;16(2):346–57.*

POSTER PRESENTATIONS

P001

Hyphenation of ion mobility mass spectrometry and infrared action spectroscopy to probe high order peptide oligomers (and other systems)

S. Bakels[1], S. Daly[2], Melissa Baerenfaenger[1], J. Commandeur[2], A. M. Rijs[1]

[1] *Division of BioAnalytical Chemistry, Vrije Universiteit Amsterdam, the Netherlands*
[2] *MSVision, Televisieweg 40, 1322 AM Almere, The Netherlands*

Peptide and protein aggregation, a critical process in the human body, is directly linked to age-related neurodegenerative diseases such as Alzheimer's and Parkinson's. Understanding the early, neurotoxic stages of peptide aggregation is crucial for developing strategies to control and prevent these conditions. However, this understanding is complicated by a cascade of molecular events occurring across various time and energy scales, resulting in complex, heterogeneous mixtures of peptide aggregates.

In this study, we present a novel approach that combines multiple analytical techniques in a single experiment. First, ion mobility slicing is employed to examine the structures of oligomers with identical m/z ratios. Next, infrared spectroscopy is used to investigate these mass- and mobility-selected oligomers to further characterize their structural properties.

To facilitate this, we customized a Synapt G2 (Waters) to allow for optical access, ion trapping, and ion mobility slicing, leading to the development of the **Photo-Synapt**. By integrating small, table-top infrared and UV lasers with the modified mass spectrometer, we are able to perform comprehensive structural investigations of a wide variety of molecular systems.

We will demonstrate the use of the Photo-Synapt in studying the early stages of peptide aggregation, focusing on the self-assembly of peptide segments from the hexapeptide VEALYL in insulin. VEALYL is known to form amyloid fibrils, which can lead to loss of activity, immune responses, or amyloidosis. Our mass- and mobility-selective IR spectroscopy sheds light on key structural interactions, the presence of beta-sheet signatures, and the competition between intramolecular and intermolecular interactions, which drive the formation of higher-order aggregates.

Additionally, we will highlight the versatility of the instrument by demonstrating its use in various applications, including the identification of glycan fragments from intact glycoproteins, oligonucleotide analysis, and the study of interactions between herbicides and metals.

P002

Optimization of a Gas Chromatography–Mass Spectrometry (GCMS) Method for Detecting 28 Allergens in Various Personal Care Products

Adnan S. AL-Mussallam, **Abdullah T. Bawazir,** Rawan S. Alshathri, Omniyah Alharthi and Fahad S. Aldawsari *

BACKGROUND:

Fragrance substances are widely used in different cosmetics products that emit and diffuse a pleasant and fragrant odor. A single cosmetic product with fragrance may contain between 10 and 300 ingredients. Typically, these ingredients include a blend of alcohol, oils, and other aromatic components. Importantly, essential oils, which are present in the majority of personal care products, from deodorants to facial moisturizers, are common allergens. allergen-causative chemicals exist among cosmetic products, such as preservatives, emulsifiers, UV absorbers, and natural plant ingredients. Owing to a broad range of potential allergenic fragrance cosmetic products, the analytical capability to detect and measure a given allergen chemical poses a multifactorial challenge. From a safety perspective, cosmetic products containing fragrance substances might pose a health risk.

OBJECTIVE:

The purpose of the method is to inform an individual consumer to avoid that ingredients in case of allergic history. In addition to the 26 allergens classified as potentially allergenic substances (PASs).

METHODS:

The allergen kit concentration was 400 g/mL, while the stock standard was prepared by taking 625 L and transferring it to 5 mL of methanol to obtain 50 g/mL; then, the solution was stored below 0 C. Fresh working standards were prepared for every analysis, along with 10 g/mL of bromobenzene as an internal standard (IS). For calibration curve plotting, the solutions were prepared by diluting a known volume.

RESULTS:

The optimized analytical method was adopted to separate and identify different allergens using a non-polar GC column stationary phase based on the standard method (EN16274, 2012). The GC oven program was initially set at 80 _C to 280 _C at 10 _C/min. The results demonstrated that d-limonene, eucalyptol, and benzyl alcohol were co-eluted and that the selectivity was unsatisfactory to meet the validation requirements.

P003

Dissecting maternal and infant antibody repertoires using clonal profiling

Albert Bondt, Kelly A. Dingess, Jing Zhu, Guanbo Wang, Minjie Tan, Albert J.R. Heck

The development of infant immunity starts during gestation and extends into early life. Until postnatal antigen exposure, however, germinal center formation is suppressed, and antibodies in the fetus/infant are at that stage predominantly maternally derived via placental transport. This neonatal Fc-receptor mediated transport is heavily skewed to immunoglobulin (Ig)G1, and no IgA is found in the infant at birth. The extend to which the infant starts producing their own repertoires has been extremely difficult to study due to the high level of maternal antibodies.

We recently developed mass spectrometry-based methods to analyze the IgG1 and IgA1 antibody repertoires with clonal resolution. Here, we applied these methods on samples from four mother-child pairs. We analyzed maternal serum, cord blood, and colostrum at time point (T) 1, as well as maternal serum, infant serum, and mature milk at T2 (2-3 months post partum). No IgA clonal repertoires were observed in cord blood, and no IgG repertoires were recorded for the milk samples.

In accordance with previous findings, we observed for IgA substantial overlap between the two maternal serum samples of each donor. This was also, but less pronounced, the case for the colostrum and mature milk samples. For three out of the four donors we additionally found overlap between the serum and milk samples. No IgA was detected in cord blood, and no overlap was observed between the infant serum at T2 and maternal samples. We did observe a distinct repertoire of IgA clones in the infant serum, clearly proving infant IgA production.

For IgG, we found substantial overlap between the maternal serum samples, and even more so between maternal serum at T1 and the cord blood. At T2, the overlap between maternal serum and infant serum was much lower for most donor couples. This was most likely caused by the decrease in IgG concentration in the infants. The decrease was, however, less pronounced as would be expected considering IgG half-life. Indeed, in the infant serum we observed various clones that we did not detect in the maternal samples, indicative again of the infant's own antibody production.

In conclusion, using mass spectrometry-based Fab clonal profiling we have been able to dissect the infant antibody repertoire from the maternal repertoires, showing infant-unique IgA and IgG production within the first few months of life.

P004

Mass Spectrometry Instrumentation Dedicated to the Studies of Viral Protein Complexes and Particles

Chouzenoux, P. [1,2,3*], S. Daly[1], J. Commandeur[1], A. M. Rijs[2]

[1] *MS Vision, Televisieweg 40, 1322 AM Almere, The Netherlands.*
[2] *Division of BioAnalytical Chemistry, Vrije Universiteit Amsterdam, Department of Chemistry and Pharmaceutical Science, De Boelelaan 1108, 1081 HZ Amsterdam, The Netherlands.*
[3] *Centre for analytical Sciences Amsterdam, 1098 XH Amsterdam, The Netherlands.*

X-ray diffraction imaging is one of the most important techniques in structural biology, but has some limitations. Samples require crystallization, cryogenic preservation, and the technique is not sensitive to the dynamics of aggregation. Also, sample preparation, experimental conditions, and data analysis can be time intensive. Single Particle Imaging (SPI) appears to overcome these limitations, offering a new method to study protein complexes and viral capsids [1]. SPI relies on the coupling of the X-ray Free Electron Laser (XFEL) with the advanced mass spectrometry technology developed within the MS SPIDOC's consortium, opening up a vast array of improvements and possibilities [2]. By using X-ray photons delivered in the order of femtoseconds pulse, the technique will induce photon scattering on the studied particle, creating a diffraction figure before significant radiation damage [1].

A wide array of novel mass spectrometry technology, including ion source optics for efficient desolvation of large ions, a compact ion mobility device, a digital quadrupole and ion trap [3,4], and a compact time of flight mass spectrometer have been developed by the members of the SPIDOC's consortium. This project will use this MS technology to design, optimize and integrate these elements into a single mass spectrometer designed for native mass spectrometry of large biomolecules and biomolecular complexes.

Here, we will present two of these technologies: the digital quadrupole and the ESA-TOF. The digital quadrupole enables new abilities to explore complex biological systems, by precisely controlling and rapidly switching the waveform duty cycle [5]. For instance, the device has an unlimited mass range as both amplitude and frequency can be changed, and a higher resolution than a standard quadrupole using a sinusoidal waveform. A comparison of the performance will be performed using simulation tools and experimental measurements.

The compact mass spectrometer, which combined an electrostatic analyser (ESA) and time-of-flight, has been characterized extensively through ion optic simulation. The simulations suggest a resolution of at least 8,500. We have just replaced the TOF of a qTOF Ultima with the ESA-TOF, and testing will begin shortly. A UI for controlling voltages and visualizing the mass spectrum and allowing to interface with existing analysis programs will also be developed. The ultimate goal of this project is to design and build Q-TOF with a small footprint, high sensitivity and resolution and unlimited mass range.

REFERENCES: [1] Alan Kadek et al, Drug Discovery Today 2021; [2] T. Kierspel et al, Analytical and Bioanalytical Chemistry (2023); [3] Florian Simke et al. JINST, 202

P005

Employing ballistic gradients, vacuum jacketed columns with the Xevo MRT MS to increase lipidomic throughput whilst maintaining highly confident identifications.

Matthew E. Daly[1], Nyasha Munjoma[1], Rob S. Plumb[1], Jason Hill[2], Nick Tomczyk[1], Lee A. Gethings[1], Richard Lock[1],Ross Chawner[1] and Jan Claere[3]

[1] *Waters Corporation, Wilmslow, UK*
[2] *Waters Corporation, Milford, UK*
[3] *Waters NV, Antwerp, Belgium*

INTRODUCTION

One common theme between all omics applications is the ever-increasing size of patient cohorts, driven by the need for identification of novel disease biomarkers and increasing the power of the studies. However, as these studies scale to thousands of patient samples throughput becomes the limiting factor. It may be possible to decrease separation time, however this comes at the cost of peak capacity and feature detection. Vacuum jacketed columns (VJC) can significantly increase peak capacity and narrow peak widths, thereby allowing faster chromatography when combined with fast scanning acquisitions without information loss, or maintaining chromatography duration while increasing peak capacity. Vacuum jacketed columns were therefore applied to the analysis of the lipidome of healthy controls and patients with different cancers.

METHODS

Lipids from serum samples of healthy controls and patients with bladder, colon or kidney cancer were extracted using IPA and spiked with EquiSPLASH™ to act as an internal control. Lipids were separated on a 1mm diameter Phenyl-Hexyl column, consisting of either 100 or 50 mm length. Corresponding columns in VJC format were also applied for the separation. A standard 10-minute gradient was used with all columns before scaling down to sub-1 minute gradients. The LC eluate was connected to the Xevo MRT Mass Spectrometer, operating at 100Hz acquisition rate in a data-independent mode of acquisition. Generated data were analysed using a combination of in-house and third-party informatic tools.

PRELIMINARY DATA

Generated datasets were aligned, peak picked and normalised using Lipostar2 informatics. Lipid identifications were returned from searching against the LipidMAPS™ structure database. Initially data acquired by employing a conventional column (2.1mm diameter) using a 10-minute gradient were analysed for benchmarking purposes. Several highly abundant biomarkers could be identified using the conventional method, which related to cell proliferation and signalling pathways (including lysophosphatidylcholines and phosphatidylcholines) and relative abundance differences observed between healthy controls and cancer groups. Chromatographic methods were then scaled down to 1mm id and column length reduced to maintain the gradient time which resulted in a similar number of features being identified.

Increased flow rate and lowering of gradient time(s), reduced the peak capacity as expected and as such fewer features were detected. However, major biomarkers were still observed and samples could be separated based on abundance. Statistical analysis comprising of multi-variate statistics, resulted in similar profiles being generated via unsupervised principal component analysis (PCA), regardless of the chromatographic methodology employed. This ultimately resulted in the most statistically significant features between the patient groups being identified for 1mm and VJC-based data. Finally, methods were transferred to a 1mm vacuum jacketed column and when a 10-minute gradient was employed, peak capacity was shown to have increased when compared to conventional column formats, which facilitated a larger number of identified features. The high scan speed afforded by the prototype benchtop multi reflecting time-of-flight (MRT) instrument allowed adequate profiling of sub-1 second peaks observed during VJC separations without compromising on mass accuracy at the ppb level and resolution. Application of VJC using a reduced gradient time compared favourably to conventional columns and separation time, allowing for an 30% increase in throughput whilst maintaining equivalent chromatographic performance.

NOVEL ASPECT

High-throughput, highly confident identification and relative quantification of lipid cancer biomarkers from human sera.

P006

Conversion and integration of OMICS data using the Xevo MRT MS and third-party informatic workflows

Lee A. Gethings[1], Ian Morns[1], Pete Reay[1], Simon Jones[1], Nyasha Munjoma[1], Jayne Kirk[1], Richard Lock[1], Ross Chawner[1] and Jan Claereboudt[2]

[1] *Waters Corporation, Wilmslow, UK*
[2] *Waters NV, Antwerp, Belgium*

INTRODUCTION

OMIC studies typically generate large and complex data sets, derived from various sample types such as biofluids. As mass spectrometry (MS) technology rapidly advances and with data acquisition methods such as data independent analysis (DIA) progressing, the ability to delve deeper into the metabolome and lipidome is significantly improved, whilst at the same time generating high-dimensional datasets. In this study, we describe a data processing pipeline which converts MS data that was collected using a novel, multi-reflecting ToF into a generic file format, that can be readily processed by a variety of third-party informatic tools.

METHODS

Metabolomic and lipidomic data relating to samples from a range of sources, including plasma, serum and urine were collected using LC-MS. HILIC and reversed-phase chromatographic conditions were used for metabolite and lipid analysis respectively, with gradients consisting of standard UPLC™ (~10min) and rapid (<3min) timescales. MS data were acquired using DDA and DIA approaches with the Xevo MRT Mass Spectrometer, capable of providing high resolving power and with ppb mass accuracy. Data were collected using high acquisition rates to coincide with the rapid gradient conditions, allowing for adequate sampling and ensuring sufficient data points per chromatographic peak. Subsequent data were converted into generic mzML format via the in-house data conversion tool for processing with multiple third-party informatics.

PRELIMINARY DATA

Metabolomic data originating from urine and lipidomic data in the form of extracts from human plasma samples were collected using waters_connect™ software, prior to being converted into mzML format using the DATA Convert tool. The tool performs several steps to generate mzML outputs, firstly performing a data conditioning step to lockmass correct mass spectra before stripping lockmass spectra from the data. Specifically, for DDA data, SetMass values are lockmass corrected and MS/MS scans for the same SetMass are merged. Dependent on the intended third-party informatics, the resulting mzML was also centroided. Following data conversion, the processing pipeline for the metabolomic and lipidomic datasets consisted of using MZmine3 and MS-DIAL, whilst Skyline was used for data visualizaton. To provide ultimate flexibility with data format streams, the ability to seamlessly connect from waters_connect to commercially available software using the application program interface (API) is also demonstrated for both OMIC datasets. Lipostar2 and MARS (Mass AnalyticaTM) demonstrate this additional flexibility and were used for
interrogating the lipid and metabolite datasets respectively. The results ascertained via both routes (i.e., conversion to mzML or API), highlight the instrument capabilities in all cases for polar metabolites and lipids, exhibiting mass accuracies in the region of 200 ppb, enabling greater confidence in compound identification(s) and maintaining high resolution across the acquired mass range(s). Based on the lipidomic analyses (EquiSPLASH™ dilution series constructed in a plasma matrix), high sensitivity and dynamic range is highlighted with 5 ng/mL levels routinely achieved. In the case of rapid chromatographic separation, a 3 min metabolomic analysis yielded comparable results with equivalent samples, which were acquired using ‘standard’ chromatographic conditions. For example, based on extracted compounds; mass accuracy in the region of 200 ppb, quantitative accuracy (>25 points per peak), spectral quality and mass resolution were readily achieved for high throughput analyses.

NOVEL ASPECT

Sophisticated OMIC data conversion workflows relating to multiple acquisition formats acquired using a novel, benchtop multi-reflectron ToF to generic format(s)

P007

Unraveling the Mystery of Peptide Aggregation;

Probing hIAPP Oligomers with nanoESI-TIMS-TOF Mass Spectrometry

Ariadni Geballa-Koukoula[1,2], Anouk M. Rijs[1,2]

[1] *Division of Bioanalytical Chemistry, Department of Chemistry and Pharmaceutical Sciences, AIMMS Amsterdam Institute of Molecular and Life Sciences, Vrije Universiteit Amsterdam, 1081 HV Amsterdam, The Netherlands*

[2] *Centre for Analytical Sciences Amsterdam, 1098 XH Amsterdam, The Netherlands*

Protein misfolding and aggregation are key contributors to the pathology of many degenerative diseases. In type 2 diabetes, the human islet amyloid polypeptide (hIAPP), a 37-residue peptide hormone, aggregates and deposits in pancreatic β-cells, causing cellular dysfunction and death, which accelerates disease progression. The formation of oligomeric species, which precedes the development of insoluble amyloid fibrils, is believed to be a critical toxic event. However, the pathways of aggregation and the intermediate species involved in this transition remain unclear, underscoring the need to investigate hIAPP aggregation in detail to better understand its role in type 2 diabetes. Ion mobility-mass spectrometry (IM-MS) provides a powerful platform for examining the early stages of protein aggregation by separating and characterizing intermediate species in real-time. Coupled with nano-electrospray ionization (nanoESI), this method excels in preserving the native-like structures of fragile oligomers due to its lower flow rates, higher ionization efficiency, and minimal sample consumption. NanoESI, particularly in aqueous solutions containing volatile buffers, ensures reproducibility in analyzing aggregation phenomena under near-physiological conditions.

In our study, we focus on hIAPP and its key amyloidogenic regions, specifically the peptide fragments NFGAIL (22-27), NFGAILSS (22-29), and SNNFGAILSS (20-29), to investigate their aggregation behavior under physiological conditions. Our findings reveal distinct aggregation profiles for each peptide, identifying a range of oligomeric species over time, including both lower-order aggregates (e.g., dimers) and higher-order aggregates (e.g., up to 11-unit oligomer). IM-MS analysis allowed us to capture the conformational states of these oligomers as they evolved. By integrating these structural insights with a broader understanding of hIAPP's pathological roles, we aim to identify the toxic intermediates responsible for β-cell dysfunction. This work offers valuable perspectives on the formation of harmful oligomeric species before they progress to irreversible fibril formation.

P008

LC-MS/MS method development for the quantification of the bis(diethyldithiocarbamate)-copper (II) complex, disulfiram and its metabolites

A.B. Heijnis[1], B.W.M. van den Broek[1], J.J. Simonsz[1], M. Schmucker[2], S. Ohmura[2], T.G.P. Grünewald[2], A.D.R. Huitema[1], M.H.M. Diekstra[1]

[1] *Princess Máxima Center for Pediatric Oncology (PMC), Utrecht, The Netherlands*
[2] *German Cancer Research Center (DKFZ), Heidelberg, Germany*

INTRODUCTION

In this research we aim to investigate the possibility for repurposing disulfiram (DSF) for the treatment of Ewing Sarcoma. DSF is a well-known drug for the treatment of alcoholism and degrades rapidly in the stomach after oral administration. DSF consists of two units of the first metabolite, diethyldithiocarbamate (DDTC) (Figure 1**Error! Reference source not found.**), which is a strong chelator of heavy metals. Recent studies have demonstrated that DDTC exhibits anti-cancer properties when combined with copper by the formation of the bivalent diethyldithiocarbamate-copper (CuET) complex.

OBJECTIVES

To understand whether CuET accumulates in Ewing Sarcoma cells, we aim to develop a quantitative liquid chromatography mass spectrometric (LC-MS/MS) method to measure the CuET complex, disulfiram and its metabolites.

METHODS

We aim to measure CuET, DSF and its metabolites in one method using LC-MS/MS. DSF and CuET are both highly hydrophobic, indicating that reversed phase LC would be suitable. The metabolites will be measured as well to get an insight into what part of the CuET complex actually arrives at the tumor environment. To measure xenografts and tumor tissues, we aim to develop a solid-phase extraction (SPE) method. Using SPE, sample matrices are removed for increased sensitivity. As a deuterated reference standard of the copper complex is not readily available, we aim to synthesize our own internal standard from deuterated DDTC and copper chloride to compensate for instrumental variations. Compounds containing free thiol groups, as accounts for DDTC, are known to be highly reactive towards other compounds containing sulfides, such as proteins.

RESULTS

Currently, we were able to determine the multiple reaction monitoring (MRM) transitions of four compounds. Consequently, we were able to measure these in one measurement (**Error! Reference source not found.**) using a gradient of water and acetonitrile containing formic acid. In this chromatogram, the observed retention order is based on increasing hydrophobicity. A double peak is observed for CuET, with the first peak eluting exactly at the retention time of DSF. To determine the stability of the compounds, dilutions were made in LC-compatible organic solvents, combined with buffers in acidic, neutral and alkaline conditions. These solutions were repeatedly measured over a day to get an insight into the stability in solution.

CONCLUSION

The absence of a peak representing DDTC is probably due to the reactivity and instability of the compound. To improve this and obtain an increased instrumental response, we aim to derivatize the metabolite using an alkylating agent. The double peak indicates an equilibrium between DSF and CuET, even within the chromatographic column. The setup of a stability research is crucial to provide stable solutions for the optimization of the instrument. Eventually, by determining the intracellular concentration of CuET, DSF and its metabolites, we will be able to determine the active drug amount that reaches the tumor in Ewing sarcoma cell lines. This work has the potential to significantly impact Ewing sarcoma care and possibly improve therapeutic strategies for other tumor types as well.

P009

Glycosphingolipid Profiling in Neuroblastoma Organoids

Kevin Hooijschuur[1], Anne Borst[2], Dr. Judith Wienke[2], Dr. Bernd Stahla[3], Prof. dr. Jan Molenaar[2] and Prof. dr. Geert-Jan Boons[1]

[1] *Chemical Biology and Drug Discovery, Utrecht University, Universiteitswet 99, Utrecht, Netherlands*
[2] *Prinses Máxima Center for Pediatric Oncology, Heidelberglaan 25, Utrecht, Netherlands*
[3] *Nutricia Research, Uppsalalaan 2, Utrecht, Netherlands*

Glycosphingolipids consist of complex oligosaccharides that are conjugated to sphingolipids. Changes in glycosylation are frequently observed in disease, and are commonly associated with cancer. One example is neuroblastoma, a cancer that develops in immature nerve cells and that is predominantly found in children aged 5 years or younger. Glycosphingolipid GD2 are used as a target for monoclonal antibody therapy against neuroblastoma, but the presence of GD2 in healthy neural tissue causes severe pain as a side effect for this treatment strategy. Therefore, GD2 with O-acetylation on its sialic acids was proposed as a more specific and safe target. We previously developed an ion mobility-mass spectrometry (IM-MS) method using a reference library of standards with isomeric O-acetylated sialosides (1). This method employed ion mobility to measure ion-neutral collisional cross-section values that identify the O-acetyl position on glycans from proteins and tissues. Here, we present procedures to extract glycosphingolipids from cells for glycan analysis with IM-MS. This method aims to validate proposed treatment targets and identify novel potential targets for neuroblastoma treatment.

Glycosphingolipids were isolated from neuroblastoma organoids derived from different patients with extraction procedures that were tolerated by the base-labile O-acetyls. Enzymatic cleavage was used to detach the sphingolipid from the glycan and the free glycan was provided with a chargeable label. The glycans were analyzed by hydrophilic interaction chromatography IM-MS. In-source collision induced dissociation was employed to sequence exact glycan structures by fragment analysis with IM-MS.

First, standards of O-acetylated glycosphingolipids were subjected to the glycan isolation procedures that are to be used for the isolation of glycosphingolipids from organoids. This was done to evaluate the degree of migration and hydrolysis of the O-acetyls during the isolation of glycosphingolipids from organoids. Glycosphingolipids from four neuroblastoma organoid samples were examined for the presence of GD2 and its O-acetylated derivative. The O-acetyl position was readily determined, but expression across the different organoids was variable. As a result, the scope of analysis was expanded to other abundant glycosphingolipid structures. With in-source fragmentation, diagnostic fragments of glycosphingolipid glycans could be identified by referring to collisional cross-section values found from glycosphingolipid standards. This revealed the differences and similarities between the glycosylation expressed by the organoids, providing information about upregulated and downregulated glycan synthesis pathways. These results demonstrate that ion mobility-mass spectrometry can be applied to analyze glycosphingolipid glycosylation in biologically relevant samples. On top of that, this method can be applied for discovery of new biomarkers or therapeutic targets.

REFERENCES:

(1) G.M. Vos, K.C. Hooijschuur et al. *Nat Commun (2023),* **14**, 6795

P010

Improved multivariate quantification of plastic particles in human blood using non-targeted pyrolysis GC-MS

Kas Houthuijs[a], Wilco Nijenhuis[a], Martin Brits[a], Martin van Velzen[a], Sicco Brandsma[a], Marja Lamoree[a] and Frederic Béen[a,b]

[a] *Amsterdam Institute for Life and Environment (A-LIFE), Vrije Universiteit Amsterdam, De Boelelaan 1085, 1081 HV Amsterdam, The Netherlands*
[b] *KWR Water Research Institute, 3433 PE Nieuwegein, The Netherlands*

Micro- and nanoplastics (MNPs) have been recognized as an environmental threat and there is global concern about their presence in humans. To investigate human exposure and the toxicological effects of MNPs a method to identify and quantify these plastic particles is essential. Pyrolysis-GC-MS (Py-GC-MS) has recently emerged for the analysis of MNPs in human blood, as it provides polymer-specific MS fingerprints and semi-quantitative data. However, pyrolysis also introduces complex effects, such as secondary reactions between different polymer types and matrix compounds, which complicate quantification.

In this work, we employ a non-targeted approach to MNP characterization using double-shot Py-GC-MS in scan mode. MS features were deconvoluted from the chromatograms using PARAFAC2 and annotated based on their EI fragmentation spectra. Recovery experiments reveals that the pyrolysis of the polymers PE and PVC is unaffected by the presence of blood, while PET undergoes extensive secondary reactions with the matrix. Regression analysis on all extracted features identifies the most accurate markers for polymer quantification and by relying on multivariate models we further decrease quantification errors with respect to single-marker results.

This study highlights the importance of considering secondary effects in pyrolysis for the quantification of MNPs and provides valuable chemical insight into the pyrolysis process, which can potentially be exploited in future development of the experimental method.

P011

Novel strategies for the characterization of the early-stages of aggregate formation of a-synuclein using ion mobility mass spectrometry

Andrea Istrati[a,b], Raya Sadighi[a,b], Melissa Bärenfänger[a,b], Anouk M. Rijs [a,b]

a) Division of Bioanalytical Chemistry (MS-Laserlab), Department of Chemistry and Pharmaceutical Sciences, AIMMS Amsterdam Institute of Molecular and Life Sciences, Vrije Universiteit Amsterdam, 1081 HV Amsterdam, The Netherlands

b) Centre for Analytical Sciences Amsterdam, 1098XH Amsterdam, The Netherlands

The development of novel therapeutics for Parkinson's disease (PD) is becoming increasingly important due to the detrimental effects of this disease on individuals and due to its vastly increasing prevalence. α-synuclein (α-syn) has been identified as the primary protein involved in Parkinson's pathology and has gained significant interest as a target in PD treatment. However, due to the intrinsically disordered nature and diverse aggregation pathway of this protein, this can be very complex.

Several analytical methods can be used at different stages of the aggregation process to study this protein which have already provided key information on the late stages of aggregation. Despite significant efforts, knowledge on the early stages of α-syn aggregation is limited, while these are of particular importance due to increasing evidence that early aggregates are more toxic than late-stage fibrils. Ion Mobility - Mass spectrometry (IM-MS) is a promising method of studying these early aggregates and can provide a depth of information on the structure and aggregation of this protein. Furthermore, it can be also used to study novel therapeutics and identify how they bind and alter this aggregation process. In this project, MS was used in combination with size exclusion chromatography (SEC) and trapped ion mobility mass spectrometry (TIMS) to provide novel insights into the structure of early α-syn aggregates. In addition, MS based techniques were also used to study a novel monoclonal antibody (mAb) and single chain variable fragment (scFv) that were developed to specifically target α-syn oligomers.

This work resulted in the characterisation of α-syn oligomers up to a heptamer (and possibly beyond) using MS and conformational pathways up to a pentamer using TIMS. The novel biotherapeutics showed binding to α-syn monomer and early-stage oligomers although no binding preference could be determined at this moment.

P012

Epitope mapping of broadly neutralizing antibodies against the GII.4 variant of human norovirus using HDX-MS

Cynthia E. Kelley, Juyeon Park, Lisa C. Lindesmith, Ralph S. Baric, George Georgiou, Joost Snijder

Human norovirus causes over 700 million cases of viral acute gastroenteritis annually. Its extensive genetic diversity and limited information on broadly conserved neutralizing epitopes create significant barriers in broadly applicable vaccine development. In this study, two antibodies with exceptional cross-strain neutralization, VX1 and VX6, were identified from an individual who was immunized with an experimental oral vaccine. Using hydrogen-deuterium exchange mass spectrometry, the epitopes of these antibodies were mapped onto the protruding (P) domain of viral protein 1 of norovirus GII.4. VX1 shows a significant decrease in deuterium uptake in peptide R287-N298 within the P2 domain, whereas VX6 has decreased deuterium uptake in peptide F250-F257 in P1. The findings were biochemically confirmed through studies showing loss of neutralization potency upon mutation of the epitope sequence. Determination of these epitopes provides guidance in further studies towards the design of immunogens for broadly protective norovirus vaccines.

P013

MONOCLONAL ANTI-RHESUS D: DO THEY REALLY WORK?

Koike G[1,2,3], Leuvenink RW[2,3], Mons DJ[1], Ligthart P[4], Bondt A[2,3], Heck, AJR[2,3], Schoot EVD[1], Vidarsson G[1,2,3]

[1] *Department of Experimental Immunohematology, Sanquin Research, Amsterdam, The Netherlands*
[2] *Biomolecular Mass Spectrometry and Proteomics, Bijvoet Center for Biomolecular Research and Utrecht Institute for Pharmaceutical Sciences, University of Utrecht, Utrecht, The Netherlands*
[3] *Netherlands Proteomics Center, Utrecht, The Netherlands; [4]Erythrocyte Serology, Sanquin, Amsterdam, The Netherlands*

The main limitations of polyclonal anti-D antibodies to prevent Rh disease are the availability and costs. Attempts to produce effective monoclonal anti-D antibodies have failed, possibly due to altered Fc-glycans found in anti-D which is afucosylated. However, two monoclonal anti-D antibodies have been produced and commercialized by Bharat Serums and Vaccines Limited – namely Rhoclone, and its recombinant version, Trinbelimab – and are currently in use in some countries in Africa and Asia. The aim of the present work is to evaluate the efficacy of these monoclonals in preliminary *in vitro* assays, as well as to determine their sequences and Fc glycosylation.

Using different mass spectrometry-based methods, we determined that Rhoclone and Trinbelimab are human IgG1s that use the IGHV1-69 and IGLV2-14 germline segments, and they are identical. We also analyzed their glycopeptides, and verified that Rhoclone is more fucosylated than Trinbelimab, result that is coherent with their ADCC assay performance (afucosylated anti-D control>polyconal anti-D>Trinbelimab>Rhoclone≈fucosylated anti-D control). Future efforts are aimed at identify if these functional differences translate to clinical efficacy.

P014

Fast MALDI mass microscopy

Aljoscha Körber, Bryn Flinders, Frans Giskes, Ron M.A. Heeren, Ian G.M. Anthony

Matrix-assisted laser desorption/ionization (MALDI) mass spectrometry imaging (MSI) maps the spatial distributions of thousands of molecules in tissue. However, its low acquisition speed of approx. 40-50 pixels s^{-1} prevents its routine application in hospitals. This low throughput 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 acquisitions. Still, high throughput MSI at simultaneously low pixel sizes can be achieved with mass microscopy, in which a >200 μm^2 large area is ionized, preserved during time-of-flight analysis and magnified onto a fast spatially sensitive detector. In the past, we have shown that mass microscopy combined with a Timepix3 detector allows acquiring images at >600,000 pixels s^{-1} and 2.5 µm spatial resolving power.[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 sensitivity and mass resolution. 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 a novel optical interface with a homogenized laser beam (Explorer One, Spectra-Physics, Stahnsdorf, Germany) and a Cry 60 scintillator (Crytur, Turnov, Czech Republic). For initial testing, 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 Rust scripts.

PRELIMINARY RESULTS

To characterize the new laser interface, we imaged TEM grids at speeds of 8,000 pixels s^{-1}, and at a spatial resolving power of at least 4.3 µm independent of matrix crystal size. We found that implementing passive charge compensation results in superior image quality and 2-3 order of magnitude higher ion yield. 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.

REFERENCES

1 *Körber, A., Keelor, J. D., Claes, B. S. R., Heeren, R. M. A. & Anthony, I. G. M. Fast Mass Microscopy: Mass Spectrometry Imaging of a Gigapixel Image in 34 Minutes. Anal Chem **94**, 14652–14658 (2022).*

P015

Exploring the *O*-Glycome by CE-MS: a method development story

Karthika Korumadathil Shaji[1], Jing Zheng[1], Hector F. B. Rhault Loponte[1], Peter L. Horvatovich[1], Guinevere S.M. Lageveen-Kammeijer[1]

[1] *University of Groningen, Groningen Research Institute of Pharmacy, Analytical Biochemistry, 9700 AD Groningen, the Netherlands.*

One of the most important co- and post-translational modification of a protein is glycosylation (the attachment of a sugar moiety group, also known as glycans). This modification plays a vital role in various biological events such as cellular communications, signaling process, immune system function, protein folding and stability. Glycosylation is involved in many biological events and minor changes in the glycomic profile have been related to various diseases, e.g. cancer development and metastasis, making them an ideal diagnostic biomarker. Three main glycosylation types are known; glycoproteins, glycolipids and proteoglycans, where protein glycosylation can be either in a *N*- or *O*-linked configuration. Due to the lack of generic enzymes, their large heterogeneity and the variety in core structures, *O*-glycans are less studied compared to *N*-glycans. To further complicate their analysis, glycans exist in various isomeric structures, which cannot readily be distinguished solely by mass spectrometry (MS) and requires an additional separation technique prior to MS. Capillary electrophoresis (CE) has already been demonstrated to be an excellent technique for *N*-glycan analysis as it operates at high sensitivity and is able to separate isomers. However, the applicability of CE-MS for the analysis of *O*-glycosylation remains relatively unexplored. This is mainly related to the separation mechanism of CE, as analytes require a charge to be separated, which is often introduced by labelling the reducing end of the glycan. As common release strategies (β elimination) for *O*-glycans make the reducing end unavailable for labelling, the analytes are more challenging to be analyzed by CE-MS. With the introduction of an *O*-glycan release procedure that protects the reducing end, a range of new opportunities for chemical labeling becomes available. Notably, this method also allows for the sequential release of both *N*- and *O*-glycome from the same sample. Therefore, this study focuses on extending the glycoanalytical CE-MS portfolio by adding an additional layer (*O*-glycans) and comparing it to the already developed LC-MS workflow. Both *N*- and *O*-glycan were sequentially released from total plasma and fetuin. After the release, an internal standard (DP7) was spiked to the sample, followed by a labeling procedure with either 2-aminobenzoic acid (2AB) or Girards Reagent P (GirP) reagents for LC- or CE-MS analysis, respectively. In this study, we demonstrate a successful release of *N*- and *O*-glycans from fetuin, and the most abundant forms could be detected at using solely 30 ng as an injection amount by CE-MS. To ensure the highest sensitivity possible, further experiments will focus on optimization of the sample preparation workflow by critically evaluating each analytical step. Additionally, the workflow will be assessed on different sample types (e.g. plasma, tissue and cells). The presented approach allows to gain a deeper understanding of the glycome and allow us to assess in more details glycomic intra-tumoral heterogeneity (e.g. in head and neck squamous cell carcinoma).

P016

Benchmarking Novel and Established Native Mass Spectrometry Methods for High-Mass Applications

Krolle H.[1,2,3]*, Bocxe M.[1,2], Chouzenoux P. [1,2,3], Daly S.[1], Commandeur J.[1] and Rijs A. M.[2,3]

[1] *MS Vision, Televisieweg 40, 1322 Almere AW, The Netherlands*
[2] *Division of Bioanalytical Chemistry, Vrije Universiteit Amsterdam, De Boelelaan 1105, 1081 HV Amsterdam, The Netherlands*
[3] *Centre for Analytical Sciences Amsterdam, 1098 XH Amsterdam, The Netherlands*
* *hk@msvision.com*

The structural characterization of large protein complexes and ligand interactions has been demonstrated to be of vital importance for understanding individual protein functions. [1] Native mass spectrometry (nMS) methods combine the high resolution and sensitivity of mass spectrometry with a physiologically similar protein environment that preserves non-covalent interactions. [2] However, many proteins or protein complexes are difficult to resolve in native mass spectrometry, due to deconvolution and resolution issues of common mass spectrometers. [3] Therefore, a benchmarking study comparing different instrument and method combinations for various protein applications is vital for improving nMS technology for high-mass samples. The following approaches are currently being investigated with preliminary results:

I) Charge Reduction: The interaction of electro-sprayed ions with basic vapor molecules (e.g. Triethylamine) abstracts protons and lowers the charge state of the gas-phase protein. [4] By implementing both solution- and gas-phase additives, the charge state distribution can be lowered and the thus increased spacing between adjacent peaks aids in identification and interpretation.

II) Instrument Comparison Framework: A solid statistical framework is the basic requirement to compare different instruments. Contrary to OMICS approaches, nMS methods result in less mass spectra making a comparison challenging. [5] By utilizing biological and technical replicates, internal standards and an independent data analysis from raw files; a categorical analysis across instruments is ensured, regardless of vendor and applied nMS method.

III) Comparison of Orbitrap and qTOF instruments: nMS samples can give highly complex mass spectra which are often difficult to interpret. This can be further complicated by instrument-specific effects which can make data interpretation even more difficult. To investigate these instrument-specific effects, a Thermo Scientific Orbitrap and a Waters qTOF are compared, with the aim of identifying and minimizing the individual influences during high mass acquisitions. [5, 6] This fundamental knowledge will then be utilized to further develop established nMS methods and modify instruments to improve their high mass capabilities.

Here, the first steps towards meeting these goals will be presented, showing initial work on controlling the charge state of proteins, and preliminary results on benchmarking of unmodified instruments.

In the future, more instruments and methods, including IMS, CDMS, UVPD/IMRPD-MS, will be examined and compared for common nMS targets, to both determine advantageous method-instrument combinations and further improve on them.

REFERENCES: [1] Havugimana et al., *Cell*, 2012 [2] Tamara et al., *Chem. Rev.*, 2021; [3] Lössel et al., *J. Am. Soc. Mass Spectrom.*, 2014, [4] Touboul et al., *J. Phys. Chem.*, 2007; [5] Lermyte et al., *Nat. Methods*, 2024; [6] Snijder et al., *J. Am. Chem. Soc.*, 2014

P017

An optimized method for the quantification of leukotrienes in human plasma using liquid chromatography high-resolution mass spectrometry (LC-HRMS)

Alex Mommers[1], Agnes Boots[1], Gertjan den Hartog[1]

[1] *Department of Pharmacology and Toxicology, Institute of Nutrition and Translational Research in Metabolism (NUTRIM), Maastricht University.*

Leukotrienes (LTs) are inflammatory lipid mediators produced by leukocytes, executing functions such as chemo-attraction (LTB4) and smooth muscle contraction (LTD4). LT antagonists are increasingly used as adjunctive medication for chronic obstructive pulmonary disease (COPD) to mitigate LT-induced bronchoconstriction, with mixed results. This variability is due to LTs' diverse functions, variable production ratios depending on disease progression, and measurement challenges in biological samples. Their involvement in various chronic conditions makes LTs valuable as both diagnostic markers and therapeutic targets.

We have developed a method using solid-phase extraction and LC-HRMS to quantify LTs in human plasma. Our approach targets metabolites from the two main leukotriene pathways, i.e. the LTB4 pathway (including LTB4 and its downstream metabolites) and the cysteinyl leukotriene pathway (encompassing LTC4, LTD4, and LTE4). Utilizing a high-pH mobile phase, effective separation of ionized LTs, and significantly enhanced electrospray ionization efficiency were achieved. Additionally, by employing a novel approach to model individual mass spectral peaks, we could improve detection limits and signal-to-noise ratios (Figure 1).

Our method, leveraging the strengths of HRMS and advanced data-preprocessing techniques, enables sensitive and precise quantification of multiple compounds within the LT pathways in human plasma. It offers a powerful tool for monitoring treatments and interventions in various chronic conditions.

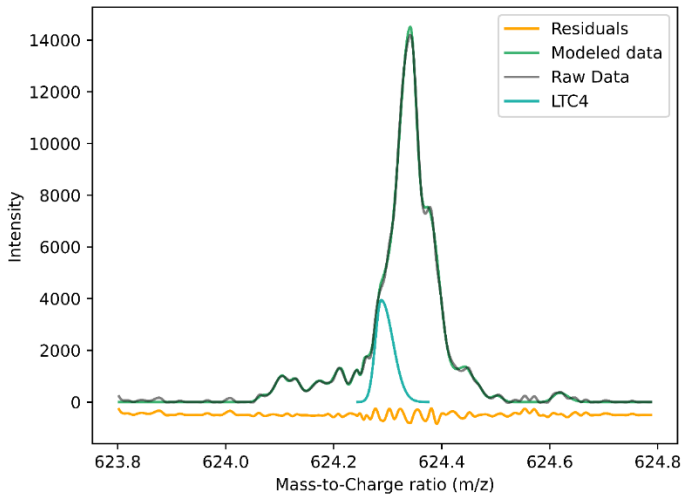


Figure 1: Part of a mass spectrum showing interpolated raw data, modeled data, their residuals and the modeled LTC4 $[M-H]^-$ ion of a COPD patient.

P018

Development and Validation of a High-Sensitivity LC-MS/MS Assay for Monitoring Asparaginase Activity in Pediatric ALL Treatment

Jochem Simonsz

INTRODUCTION

Asparaginase is essential in treating pediatric acute lymphoblastic leukemia (ALL), as it depletes L-asparagine, an amino acid crucial for leukemic cell survival, thus inhibiting their proliferation. Therapeutic Drug Monitoring (TDM) is applied to ensure that asparaginase activity exceeds 100 international units per liter (IU/L) to detect enzyme inactivation and adjust dosing. However, current enzymatic spectroscopic methods, such as the AHA-method widely used in clinical practice, lack high sensitivity and precision. Therefore, we aim to develop and validate a liquid chromatography-tandem mass spectrometry (LC-MS/MS) assay for the measurement of asparaginase activity in serum, specifically targeting pegylated asparaginase (pegASNase) and Erwinia asparaginase (Erwinase).

METHOD

The method for quantifying enzymatic activity of asparaginase in serum was developed using Hydrophilic Interaction Liquid Chromatography coupled to tandem mass spectrometry (HILIC LC-MS/MS). The reaction involved incubating serum samples with L-asparagine-β-hydroxymate for 120 minutes, followed by protein denaturation to halt the reaction. The produced L-aspartic acid was measured in the samples. Quadratic calibration curves were established using commercial pegASNase and Erwinase vials prepared in fresh frozen plasma, with activities specified by the manufacturer. Enzyme activity, expressed in IU/L, was calculated by comparing the amount of L-aspartic acid produced to the calibration curves.
The method was validated according to ICH M10 guidelines[1] for bioanalytical method validation of ligand-binding assays and chromatographic methods. Validation parameters included sensitivity, specificity, accuracy and precision. Accuracy and precision were evaluated at activity levels of 20, 100, 500, and 1000 IU/L for both enzymes, requiring values remained within 20% deviation above the lower limit of quantification (LLOQ) and 25% at LLOQ. A cross-validation was performed to determine if the LC-MS assay could reliably replace the AHA method, requiring mean activity levels from both assays to fall within ±30% of each other.

RESULTS

The validation process demonstrated that the LC-MS/MS assay was both sensitive and specific for quantifying asparaginase activity in the range of 20–1000 IU/L, with a strong quadratic correlation ($r^2 > 0.99$). Accuracy and precision requirements were met, with minor deviations observed: 5 out of 72 quality control (QC) samples for pegASNase and 3 out of 72 for Erwinase did not meet the established criteria for accuracy and precision. These deviations accounted for only 6.9% of pegASNase samples and 4.2% of Erwinase samples, indicating that overall assay performance remained valid and the clinical impact was negligible.
Cross-validation results showed high comparability between the two methods. For Erwinase, 86 out of 95 samples met the ±30% criterion with a correlation coefficient of 1.03 (SE = 0.02, $p < 0.05$). For pegASNase, 120 out of 150 samples met the same criterion with a correlation coefficient of 0.84 (SE = 0.02, $p < 0.05$).

CONCLUSION

Our developed HILIC LC-MS/MS assay is a validated, effective method for monitoring asparaginase activity in serum, providing a precise and efficient alternative to enzymatic spectroscopic tests. Although minor within-run deviations were observed, the assay's performance, supported by validation and cross-validation results, confirms its suitability for clinical application. The HILIC LC-MS/MS assay has been successfully implemented in pediatric oncology. Future research should aim to refine the assay to address sample variability and explore the inclusion of glutaminase activity measurement within the same assay.

REFERENCES: *International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use. (2019). ICH guideline M10 on bioanalytical method validation and study sample analysis.*

P019

Fast and deep phosphoproteome analysis with the Orbitrap Astral mass spectrometer

Noah M. Lancaster*, **Pavel Sinitcyn*,** Patrick Forny*, Trenton M. Peters-Clarke, Caroline Fecher, Andrew J. Smith, Evgenia Shishkova, Tabiwang N. Arrey, Anna Pashkova, Margaret Lea Robinson, Nicholas Arp, Jing Fan, Juli Hansen, Andrea Galmozzi, Lia R. Serrano, Julie Rojas, Audrey P. Gasch, Michael S. Westphall, Hamish Stewart, Christian Hock, Eugen Damoc, David J. Pagliarini, Vlad Zabrouskov & Joshua J. Coon

Owing to its roles in cellular signal transduction, protein phosphorylation plays critical roles in myriad cell processes. That said, detecting and quantifying protein phosphorylation has remained a challenge. We describe the use of a novel mass spectrometer (Orbitrap Astral) coupled with data-independent acquisition (DIA) to achieve rapid and deep analysis of human and mouse phosphoproteomes. With this method, we map approximately 30,000 unique human phosphorylation sites within a half-hour of data collection. The technology is benchmarked to other state-of-the-art MS platforms using both synthetic peptide standards and with EGF-stimulated HeLa cells. We apply this approach to generate a phosphoproteome multi-tissue atlas of the mouse. Altogether, we detect 81,120 unique phosphorylation sites within 12 hours of measurement. With this unique dataset, we examine the sequence, structural, and kinase specificity context of protein phosphorylation. Finally, we highlight the discovery potential of this resource with multiple examples of phosphorylation events relevant to mitochondrial and brain biology.

P020

Targeted urine analysis using UPLC-QTOF-MS as a fast and novel assay for Inborn Metabolic Diseases leading to kidney stones

Waterval W.A.H., Wang P., Goossens A., Beurskens A.G.C., Noga M., Körver-Keularts I.M.L.W., Habets D.D.J., **Steinbusch L.K.M.**

Maastricht University Medical Center +, Maastricht, Netherlands

Kidney stones are one of the most common disorders of the urinary tract, affecting about 10-15% of the general population in the Western world. Kidney stones are most often diagnosed in male adults between the age of 30 and 50 years but not exclusively. Kidney stones that present in childhood are often due to eight rare inherited metabolic disorders (IMD). Early diagnosis and proper management of these IMD is warranted given the associated risk of progressive renal injury.

In most biochemical laboratories, the quantitative analysis of urine metabolites for the diagnosis of inborn metabolic diseases (IMD) affecting kidney stone formation requires three separate analytical assays. The diagnostic reports often not only give results on the specific metabolites important for kidney stone formation but also about all other metabolites quantified in that assay. We developed and implemented a new, fast and simplified targeted method to consolidate these assays in one targeted UHPLC-QTOF screening replacing the previously three separate assays.

This new IMD renal screening is based on our earlier published targeted urine metabolomics screening which already contained all 9 metabolites but reported semi-quantitative results. By using an metal-free column we were able to measure oxalic acid as an aluminiumoxide-adduct ion. By adding stable isotopes as internal standards for all 9 metabolites we could easily turn our semi-quantitative TUM screening into a quantitative renal screening. Doing so we reduced instrument time, labour, urine volume needed and only quantify those metabolites that are involved in kidney stone formation. The analytical results were compared with those of the previous three separate assays and reference values for this new method were calculated. The clinical validation shows the suitability of the method to detect patients suffering from IMD leading to kidney stones.

In conclusion, we developed a fast targeted urine kidney stone analysis which replaces all of the dedicated urine analyses for efficient screening of patients suspected of an IEM leading to kidney stones.

P021

Unravelling the dynamics of the aggregation process of the Ac-PHF6-NH2 tau segment P020

Iuliia Stroganova[1,2], Zenon Toprakcioglu[3], Agathe Depraz Depland[1,2], Tuomas P. J. Knowles[3], and Anouk M. Rijs[1,2]

1 *Division of Bioanalytical Chemistry, Department of Chemistry and Pharmaceutical Sciences, AIMMS Amsterdam Institute of Molecular and Life Sciences, Vrije Universiteit Amsterdam, 1081 HV Amsterdam, The Netherlands*
2 *Centre for Analytical Sciences Amsterdam, 1098 XH Amsterdam, The Netherlands*
3 *Yusuf Hamied Department of Chemistry, University of Cambridge, Cambridge CB2 1EW, United Kingdom*

KEYWORDS: Aggregation, tau peptide, ion mobility mass spectrometry, fluorescence assay

Peptide and protein aggregation, the transition of soluble functional proteins into insoluble amyloid fibrils and plaques, is a remarkable physical process in the human body. The inevitable formation of amyloid aggregates is the key feature of age-related neurodegenerative diseases such as Alzheimer's, Parkinson's, and prion diseases[1]. In order to be able to control and direct peptide self-assembly, a complete understanding of all processes along the peptide aggregation pathways is essential. The aggregation process is a very complex reaction involving many intermediate species of different sizes and shapes, including monomers, transient low abundance soluble oligomers that are thought to have significant toxicity, and insoluble fibrils that dominate the later stages of the self-assembly. A combination of different techniques is required to study species formed at different times on the aggregation curve. Here, we use a variety of experimental techniques to study aggregation of the Ac-PHF6-NH2 peptide of the tau protein. Thioflavin T (ThT) binding fluorescence assays, transmission electron microscopy (TEM), and circular dichroism (CD) spectroscopy are employed to gain insight into the fibrillar structures, and soft nano-electrospray ionization (nESI) ion mobility mass spectrometry (IM-MS) experiments2 are used to follow the abundances of oligomeric species along the aggregation pathway. By monitoring fluorescence intensity over time and using the global fitting of kinetic data 3 , the dominant molecular mechanism of aggregation of the Ac-PHF6-NH2 peptide with heparin can be elucidated. We have found that the secondary nucleation plays an important role. The TEM technique was used to determine the fibrillar morphologies, showing both straight and twisted fibrils, as well as ribbon-like structures. Using the IMMS approach, we were able to study the abundances of oligomeric species formed during aggregation over time. IM-MS shows that the maximum number of oligomers is reached at about half time of the ThT kinetic aggregation curve, when the monomer and fibrils are assumed to be at equal concentrations. Furthermore, heparin does not appear to alter the structure of the oligomeric species compared to those formed in the absence of heparin. Taken together, this provides an interesting insight into the aggregation process of the AcPHF6-NH2 peptide and sheds the light on the molecular-level understanding of intermediate oligomeric species.

REFERENCES

[1]. Chiti, F., & Dobson, C. M. (2017). Annual review of biochemistry, 86, 27-68.
[2]. Stroganova, I., & Rijs, A. M. (2021). In Ion Mobility-Mass Spectrometry, 206-242.
[3]. Meisl, G., Kirkegaard, J. B., Arosio, P., Michaels, T. C., Vendruscolo, M., Dobson, C. M., ... & Knowles, T. P. (2016). Nature protocols, 11(2), 252-272.

P022

Oxygen Attachment Dissociation (OAD) MS/MS for the structural identification of double-bond positions in potential lipid biomarkers of pancreatic ductal adenocarcinoma

Dennis van den Heuvel[1]; Emily Armitage[2] Alan Barnes[2]; Elon Correa[3]; Sén Takeda[4]; Wen Chung[5]; Neil J. Loftus[2]

[1]Shimadzu Benelux, 's-Hertogenbosch
[2]Shimadzu Corporation, Manchester
[3]Liverpool John Moores University, Liverpool, United Kingdom
[4]Department of Anatomy, Tokyo University School of Medicine, Tokyo, Japan
[5]Leicester HPB Unit, Glenfield Hospital, Leicester, United Kingdom

OBJECTIVE(S)

Pancreatic ductal adenocarcinoma (PDAC) is an aggressive cancer with one of the lowest survival rates due to late diagnosis and high resistance to treatment. To aid early detection, potential lipid biomarkers of PDAC have been identified in human serum using untargeted LC-MS/MS with collision induced dissociation (CID). Since the architecture of lipids is directly related to biochemical function, it is essential to structurally characterise lipids to understand their biological roles. Lipid class and chain length can be identified using CID, however alternative fragmentation is required to localize double bonds. In this study, Oxygen Attachment Dissociation (OAD) has been applied to provide C=C specific fragmentation to structurally characterise lipids significant in PDAC.

MATERIAL AND METHODS

Healthy and PDAC patient serum extracts were analysed using LC-OAD-MS/MS (LCMS-9050, Shimadzu Corporation). The OAD Radical source I was used to introduce gas phase O/OH radicals into the collision cell to enable C=C specific fragmentation. Spectra were acquired with simultaneous OAD and CID-MS/MS in ESI+ and ESI- ion mode. Data were analysed using MS-DIAL, which incorporates the OAD database for automated annotation of data acquired using OAD-MS/MS. The research project was approved by the Pancreatic Cancer Research Fund Tissue Bank (PCRFTB) Access Committee.

RESULTS

Untargeted LC-MS/MS analysis of serum samples from PDAC patients and healthy controls revealed significant alterations in lipids putatively identified as PC(18:1_18:2), PC(18:2_18:2), PC(18:2_20:4), LPC(18:2) and LPE(18:2). OAD-MS/MS spectra revealed that all these potential lipid biomarkers of PDAC specifically contain omega-6 linoleic acid (18:2(n-6,9)).

CONCLUSION(S)

The use of OAD aims to enhance pancreatic cancer research by providing more comprehensive identification needed to understand the biological roles of the lipids identified as potential markers of PDAC.

P023

Quantitating Over 200 Pesticides in Black Tea Using GC/MS/MS with Steady Performance and Maximized Uptime

Remko van Loon, Anastasia Andrianova and Carola Damen

Agilent Technologies, The Netherlands

Analysis of GC-amenable pesticides at low concentrations can present analytical challenges given the diverse and labile nature of many pesticides and the complex matrices in which they are analyzed. A critical goal in GC/MS/MS analysis of pesticides is to augment instrument uptime to optimize sample throughput. A combination of the best practices in GC/MS/MS analysis with the state-of-the-art triple quadrupole GC/MS system enhances detection limits and increases maintenance-free instrument uptime.

This poster demonstrates the performance and robustness of the developed GC/MS/MS method for the analysis of over 200 pesticides in a black tea extract. Enhanced sample detection provided sensitivity sufficient for meeting pesticide maximum residue limits (MRLs) in black tea when accounting for sample dilution factor. State-of-the art high efficiency ion source technology in combination with the optimized GC method resulted in sensitivity at low-ppb and sub-ppb levels for the evaluated pesticides. The calibration performance was excellent, covering a wide dynamic range of up to four orders of magnitude. Method ruggedness was shown with over 500 consecutive injections of a black tea extract spiked with pesticides.

P024

De novo sequencing of human milk oligosaccharides using IMS-MS

Julia Vreugdenhil[a], Kevin Hooijschuur[a], dr. Gaël Vos[a], dr. Bernd Stahl[a,b,] dr. Javier Sastre Toraño[a], Prof. dr. Geert-Jan Boons[a]

[a] *Chemical Biology and Drug Discovery, Utrecht University, Universiteitsweg 99, Utrecht, The Netherlands*
[b] *Nutricia, Uppsalalaan 2, Utrecht, The Netherlands*

Human milk is the gold standard for an infant's nutrition. It consists of bioactive substances such as lipids, proteins, and human milk oligosaccharides (HMOs). These HMOs are crucial for protection against infections and for the development of the intestinal microbiome. To establish structure-function relationships and exploit glycoscience for the development of future nutraceuticals and therapeutics, it is essential to determine exact HMO structures in human milk. Currently, accurate mass measurements are used for compositional assignment and additional MS/MS fragmentation experiments can provide structural information. Due to their isomeric nature, however, the assignment of exact HMO structures from MS/MS spectra remains very challenging. Furthermore, the lack of well-defined standards is another major hurdle for exact structure identification. In this work we present an ion mobility-MS de novo sequencing methodology, that can unambiguously determine isomeric HMO structures in the absence of synthetic standards.

A limited set of chemoenzymatically synthesized isomeric HMOs was used to develop a drift tube ion mobility spectrometry (DTIMS)-MS methodology and a reference library with common terminal glycan epitopes, including Lewis and human blood group epitopes, for de novo sequencing of isomeric HMO structures. The DTIMS-MS methodology uses high-resolution demultiplexing to obtain high resolution arrival time distributions (ATDs; up to 240 Ω/ΔΩ) and accurate mass and CCS values of intact isomeric HMOs and their fragments.

The DTIMS-MS methodology was used for the elucidation of HMO structures with undefined carbohydrate sequence and linkages in neutral and acidic fractions of milk donors. Fragments of HMOs were identified by their accurate mass and CCS values, using fragment ion entries from the reference library, and used for de novo sequence assembly to elucidate the HMO structures. The CCS values of elucidated intact structures were then added to the reference library and used to identify larger and more complex isomeric structures, creating a self-expanding reference library in the absence of further standards. This resulted in the elucidation of exact HMO structures, ranging from DP 2-15 in the samples and >200 new (fragment) ion entries for the reference library. The structure assignment by DTIMS-MS was validated with well-defined standards and revealed 100% correct assignment by the de novo sequencing method.

This new methodology can also be applied to other glycan classes and will allow the glyco-workfield to rapidly and unambiguously identify exact structures in biological samples, independently of reducing end labels, without the need for synthetic standards.

The odd one out – Mass spectrometric detection of the ever-changing IgG4 landscape

Linus Wollenweber, Albert Bondt, Albert J.R. Heck

Immunoglobulin G4 (IgG4) is the least abundant subclass of the four human IgG antibodies in plasma, but the second most used format for therapeutic antibodies. IgG4s exhibit unique structural properties that diminish a) binding to most Fcy receptors, b) binding to C1q and enable c) facile Fab-arm exchange (FAE). These properties result in low antibody dependent cellular cytotoxicity (ADCC) activation, reduced complement activation, and inability to cross-link antigens. The ability of IgG4 to bind inhibitory FcyIIB in combination with the reduced binding to activating Fcy receptors leads to a shift towards cellular inhibition. While this anti-inflammatory character has benefits in allergy and parasitic infections, studies in recent years have shed light on IgG4-mediated pathologies, such as IgG4-autoimmune diseases (IgG4-AID) and IgG4-related diseases (IgG4-RD).

Studying immunoglobulin repertoires in plasma with clonal resolution can provide novel insights into basic questions in immunology. Ig repertoire profiling allows to follow on antibody plasticity, to get novel insights into antibody maturation, and to detect mature Ig sequences directly from donors.

In recent years, our group has presented new mass spectrometric approaches to profile antibody repertoires. Therefore, we capture all IgG1 and/or IgA1 and employ specific heavy chain (HC) hinge-directed proteases to generate their Fab fragments. Subsequently, liquid chromatography coupled online to mass spectrometry (LC-MS) is applied to measure plasma Ig clones in a quantitative manner while preserving information on heavy and light chain pairing.

Here we build further on this approach by introducing plasma IgG4 clonal profiling at the level of the intact Fabs. Our initial attempts on IgG4 profiling were focused on the intact Fab2 fragments, which would allow extensive studies on FAE. In middle-up MS experiments, most of the measured signal was however dispersing in unusually high noise. We deduced, that the inflated complexity originated from FAE facilitated stochastic Fab:Fab pairings. This assumption was supported by the observation that reduction of the same Fab2 samples resulted in far less complex data. By that, the complexity originating from stochastic Fab:Fab pairings was reduced to the mere light chain and Fd diversity. Notably, this is in line with earlier claims that almost all serum IgG4 undergo FAE.

One way to reduce the spectral complexity is to measure IgG4 Fabs instead of Fab2s. For that we take advantage of the IgG4 property that a fraction of all HCs is held together exclusively by non-covalent interactions in the CH3 domain. This unique structural property gives rise to two distinct digestion products after hinge digestion; Fab2 for the molecules with covalent HC:HC interaction, and Fabs for those with only non-covalent interaction. These products can then be separated by SEC to enrich for the lower abundant Fabs prior to MS analysis.

P026

Optimization of Faecal Headspace Sampling with High-Capacity Sorptive Extraction Probes (HiSorb) and GC-MS for Volatolomics Applications

Daniëlle Pachen[1] , Robert van Vorstenbosch[1] , Alex Mommers[1], Frederik-Jan van Schooten[1], **Agnieszka Smolinska[1]**

[1] Department of Pharmacology and Toxicology, NUTRIM School of Nutrition and Translational Research, Maastricht University, Maastricht, The Netherlands

Disease detection and monitoring using volatile organic compounds (VOCs) is gaining momentum, particularly in the context of gastrointestinal diseases where the microbiome plays a significant role. Since the microbiome produces a wide variety of volatile compounds, faecal volatile holds substantial potential for disease detection. One promising approach for faecal headspace sampling is High-capacity Sorptive extraction (HiSorb) probes, which offer a solution to the technical limitations of traditional headspace analysis that often require specialized and costly equipment. In this study, we optimized and evaluated HiSorb probes for their ability to harmonize sampling of faecal VOCs, ensuring compatibility with analytical techniques commonly used in exhaled breath studies. The optimization aimed to maximize reproducibility, the number of detected VOCs, and inter-subject variation by applying a statistical design of experiment approach. HiSorb probes were found to result in a faster sampling procedure, a higher number of detected VOCs, and increased stability of the metabolic profiles compared to other techniques. Furthermore, faecal headspace samples collected using HiSorb probes were analyzed using state-of-the-art Orbitrap mass spectrometry, enabling sensitive, accurate, and reproducible detection of VOCs across a broad dynamic range. The results demonstrate that HiSorb not only allows for efficient sampling but also for a higher sensitivity in the detection of VOCs, making it the preferred method for faecal volatolomics studies. Recommendations for pre-processing and study design have been provided to ensure optimal use of HiSorb in future research. Overall, HiSorb probes combined with Orbitrap mass spectrometry represent an effective and practical platform for faecal headspace collection and analysis in volatolomics.

P027

Insight in the mechanism of action of Semaglutide in Alzheimer's disease as assessed by shotgun proteomics

D. Katsavelis[1,2] , M.G.C. van der hart[1] , H.A. Kooijker[3] , J. Roeser[3] ,H.P. Permentier[2] , T.I.F.H. Cremers [1,2]

*[1] Quantall BV, Groningen, The Netherlands, **2** IMSC University of Groningen, The Netherlands, **3** Quantall LLC, Mountain View, CA, US*

Semaglutide is a peptide similar to glucagon-like-protein 1 which is effective in the treatment of obesity and diabetes type 2. Recently, semaglutide has also been studied for its efficacy in Alzheimer's disease. Although preclinical and clinical data seems to be positive, little is known about its central mechanism of action in Alzheimer's disease.

Shotgun proteomics uses high resolution mass spectrometry in combination with liquid chromatography to measure thousands of proteins within a single sample. This method allows for an unbiased study of biomarkers and can shed light on mechanistic cascades induced by drug administration or the development of certain pathologies. In this study we investigated the effect of semaglutide administration on protein expression in the mouse brain and plasma. For this purpose, we used shotgun proteomics to study changes in protein expression as a result of semaglutide administration.

Semaglutide (0.1 mg/kg) or vehicle was subcutaneously administered in mice (C57Bl/6). Three hours after administration, dissected brain tissue and plasma were harvested and snap frozen until further analysis. Upon sample preparation and protein in-gel digestion, samples were analyzed using a nanoflow LC-system coupled to an Orbitrap Exploris 480 high resolution mass spectrometer. Acquisition was done in DIA mode and data was processed with Spectronaut 19 (Biognosys AG, Zurich, Switzerland).

From the 4210 proteins detected in the Prefrontal Cortex, 357 proteins were significantly altered. In plasma, 270 proteins were detected and 43 were found to be differentially expressed. Differentially expressed proteins were further assessed using an in- house developed quality quotient score (QQQ-score). Besides pathways relevant to energy metabolism, multiple differentially expressed proteins were found of relevance for Alzheimer's Disease, of which most have been described to be reciprocally altered in Alzheimer's Disease. These findings indicate that semaglutide might counteract the disturbance of several protein pathways in Alzheimer's Disease.