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Book of Abstracts

2nd WORKSHOP ON MASS SPECTROMETRY IN LIFE SCIENCES

Zagreb, September 19th – 21st 2024

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Book of Abstracts
of the 2nd Workshop on Mass Spectrometry in Life Sciences

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Anita Horvatić, Josipa Kuleš

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Dear colleagues,

It is our immense pleasure to welcome you all to the Second Workshop on Mass Spectrometry in Life Sciences to be held from September 19th to 21st, 2024 at the Faculty of Science (PMF), Department of Chemistry in Zagreb!

Following the success of the first meeting in November 2022 and the positive reception of the event, we have decided to organize the workshop for the second time and gather experts, enthusiasts, and representatives from companies and the industry interested in mass spectrometry applications in life sciences. The concept is similar to the first meeting in 2022, with local and international lecturers sharing their knowledge and expertise in the fields of proteomics, translational research, medicine, veterinary medicine, anthropology, bioinformatics, metabolomics, lipidomics, glycobiology, and drug development. The topics covered vividly illustrate the breadth and versatility of mass spectrometry applications in contemporary science. We have expanded the practical part of the workshop, now spanning through three days to provide participants of the hands-on session with more time to absorb new knowledge and approaches they are probably unfamiliar with. Special mention goes to our sponsors which made the organization of the workshop possible. We are grateful for their support.

We hope you will enjoy our workshop and that the event will provide an opportunity to acquire knowledge, exchange experience and ideas, meet your peers, and establish new contacts and connections.

In the name of the Organizing Committee

Marko Močibob, PhD
Chair of the Organizing Committee
2nd Workshop on Mass Spectrometry in Life Sciences
Zagreb, September 19th - 21th, 2024

Organizers

Faculty of Science, Department of Chemistry

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Programme

Lectures

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12:30 - 13:30		**Registration**
13:30 - 13:40		**Welcome**
13:40 – 14:30	PL1	**Philipp Spät** (Tübingen, Germany) *Proteomics - from Niche to Norm*
14:30 - 14:55	L1	**Lovorka Grgurević** (Zagreb, Croatia) *Serum Proteomics in Compensated Advanced Chronic Liver Disease: Impact of Portal Hypertension*
14:55 - 15:20	L2	**Mangesh Bhide** (Košice, Slovakia) *MALDI-TOF-TOF Based de novo Sequencing of the Novel Factor H Binding Protein Bg19 of Borrelia garinii*
15:20 - 15:45	L3	**Josipa Kuleš** (Zagreb, Croatia) *A Walk on the Wild Side: Omics Adventures in Animal Science*
15:45 - 16:00	S1	**Luka Mihajlović** (MS and Applicative Solutions Specialist, ANALYSIS Adria Laboratory Equipment d.o.o.) *Advances in Single Cell Proteomics - Trends and Outlooks*
16:00 - 17:00		**Coffee Break & Poster Session**
17:00 - 17:25	L4	**Morana Jaganjac** (Zagreb, Croatia) *Metabolomics: A Tool for Understanding Disease Pathophysiology*
17:25 - 17:50	L5	**Christian Andrew Reynolds** (Rijeka, Croatia) *Quantitative Lipidomic Analysis Using Multiple Reaction Monitoring*
17:50 - 18:05	S2	**Marialuce Maldini** (Advanced Workflow Specialist, SCIEX) *Highlights of the ZenoTOF Capabilities for Proteomics*
18:05 - 18:30	L6	**Andrea Gelemanović** (Split, Croatia) *Making Sense of MS Data - Applying Statistics and Bioinformatics in Downstream Analyses*

Lectures

Friday	September 20th	
13:30 - 14:20	PL2	**Gabriel Mazzucchelli** (Liège, Belgium) *From Biomolecule Characterization to Spatial Proteomics to Decipher Biological Systems*
14:20 - 14:45	L7	**Željko Debeljak** (Osijek, Croatia) *Single-Cell Mass Spectrometry Imaging*
14:45 - 15:00	S3	**Ivan Grgičević** (Business Development Manager, Labtim Adria d.o.o.) *Advancements in High-Resolution Mass Spectrometry: Revolutionizing Life Science Applications*
15:00 - 15:25	L8	**Luka Bočkor** (Zagreb, Croatia) *MS Methods in Applied Bioanthropology*
15:25 - 15:45	S4	**Valeriia Kuzyk** (Field Application Scientist, Bruker Daltonics) *High Throughput Plasma Proteomics on timsTOF Platform*
15:45 - 16:45		**Coffee Break & Poster Session**
16:45 - 17:10	L9	**Sandro Makarić** (Zagreb, Croatia) *Role of Mass Spectrometry in Characterization of Peptide Drug Products*
17:10 - 17:35	L10	**Toni Todorovski** (Rijeka, Croatia) *Analysis and Characterization of Bioactive Peptides and Peptide-Drug Conjugates by Various Tandem Mass Spectrometry (MS/MS) Techniques*
17:35 - 18:00	L11	**David Klarić** (Zagreb, Croatia) *Mass Spectrometry in Analysis of Drug-Cyclodextrin Complexes*
18:00 - 18:25	L12	**Toma Keser** (Zagreb, Croatia) *Glyco-Fishing: Catching Glycopeptides with Glycoprotein Enrichment and LC-MS*
18:25 - 18:50	L13	**Jelena Šimunović** (Zagreb, Croatia) *LC-MS Analysis and Data Processing of Complex Glycosylation Profiles*

Practical Sessions: MS Data Processing & Analysis

Thursday	September 19th
8:30 - 9:00	**Registration**
9:00 - 9:45	**Marko Močibob** (Zagreb, Croatia) *Introduction to Shotgun Proteomics and MS Data Analysis*
9:45 - 12:00	***Marko Močibob*** *(Zagreb, Croatia)* *DDA Data Processing with Proteome Discoverer*

Friday	September 20th
9:00 - 12:00	**Philipp Spät** (Tübingen, Germany) *DDA Data Processing with MaxQuant*

Saturday	September 21st
9:00 - 11:00	**Anita Horvatić** (Zagreb, Croatia) *Introduction to DIA and DIA Data Processing with Spectronaut*
11:00 - 12:00	**Ruđer Novak** (Zagreb, Croatia) *Data Visualization and Analysis*
12:00 - 13:00	***Lunch Break***
13:00 - 15:00	**Josipa Kuleš** (Zagreb, Croatia) *Introduction to Metabolomics and Data Analysis with MetaboAnalyst*

Plenary lectures

PLENARY LECTURE 1

Proteomics - from Niche to Norm

Philipp Spät
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Proteomics enables the identification, quantification and functional characterization of the proteins present in a tissue, cell or cell compartment at a given time. Due to its unique information content, proteomics has revolutionized our understanding of complex biological systems. In contrast to the static genome and the transcriptome, the proteome is much more complex, as there are several protein isoforms per gene and a large variety of proteoforms as a result of post-translational modifications. The dynamic abundance range can span several orders of magnitude between the most and least abundant protein isoforms and proteoforms, making it particularly difficult to capture the full range of proteins present in complex proteomes. Therefore, proteomics can be a challenging task and requires both deep and broad detection methods.

High sensitivity and selectivity are the hallmarks of tandem mass spectrometry (MS), which today represents the most powerful technology for analyzing such complex proteomes. This has only been possible because not only high-precision MS technologies, but also nano-flow liquid chromatography, peptide ionization and bioinformatics have been significantly advanced over the last three decades.

As a result, MS-based proteomics has evolved from a niche discipline to the state-of-the-art approach in the life sciences for the routine analysis of thousands of proteins in less than one hour of measurement time. In combination with stable isotope or isobaric labeling, the proteomes of multiple samples can be compared in the same measurement with the highest precision at a great analytical depth. Such multiplexing is particularly important for the analysis of rare post-translational modifications (PTMs), such as phosphorylation of proteins, whose detection can easily be missed due to the stochastic nature of data-dependent acquisition (DDA) in bottom-up proteomics, known as the "missing value" problem. More recently developed data-independent acquisition (DIA) or nested DIA (nDIA) strategies have now widely replaced DDA and result in more comprehensive data sets.

Nevertheless, further improvements are needed to enable even deeper analysis, higher throughput and more comprehensive data in order to establish proteomics also in clinical diagnostics and routine biomarker analysis.

Dr. Philipp Spät

Dr. Philipp Spät recently joined Immatics Biotechnologies GmbH in Tübingen as a scientist for Immunopeptidomics & Proteomics with a focus on absolute peptide quantification methods. After his master studies at the Eberhard-Karls-University of Tübingen, he started his research career in the field of mass spectrometry based proteomics in 2012 as a research assistant in the group of Prof. Maček, Department of Quantitative Proteomics, University of Tübingen. After he obtained his PhD on the dynamic response of the cyanobacterial phosphoproteome in 2017 under the supervision of Prof. Forchhammer at the University of Tübingen, he returned to Prof. Maček's group as a post-doc. During this time, he also filled the position of scientific program coordinator of the DFG Research Unit FOR2816 “The Heterotrophy-Autotrophy Switch in Cyanobacterial Metabolism” (12/2018 - 11/2021) and was recipient of the Young Investigator Grant of the Cluster of Excellence “Controlling Microbes to Fight Infections” for the project “Characterization of the contact-mediated cellular response of co-cultured bacterial species” EXC 2124: 1-09.016_0.

Philipp Spät is an excellent researcher with extensive experience in hybrid mass spectrometry and state-of-the-art proteomics. His research focuses on the establishment and optimization of semi-quantitative proteomics and phosphoproteomics to study various aspects of intracellular signal transduction, metabolic adaptation and communication between bacteria. Other topics of interest include protein interactomics and proteogenomics in cyanobacteria.

PLENARY LECTURE 2

From Biomolecule Characterization to Spatial Proteomics to Decipher Biological Systems

Gabriel Mazzucchelli
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In this presentation, we will explore the uses of mass spectrometry, from protein characterization to advanced methods for studying various biological phenomena. First, we will present simple approaches for characterizing pure proteins, including whole protein mass measurement, which allows the detection of potential variants and modifications. For deeper analysis, sequencing methods will be required for a more detailed characterization of proteins.

We will highlight the benefits of the multi-enzymatic limited digestion (MELD) method, which enables detailed sequencing of primary structures, as well as the identification and localization of post-translational modifications, truncations, and mutations. By combining the "Terminal Amine Isotopic Labeling of Substrates (TAILS)" method with this advanced enzymatic digestion approach, studies on the identification of new substrates and protease cleavage sites are greatly facilitated. Another adaptation of this method involves the use of a continuous enzymatic reactor coupled to a mass spectrometer, allowing the collection of structural information on the studied biomolecules.

In the second part of the presentation, we will address the challenges of proteomics analysis when the amount of available biological material is very limited. We will highlight the influence of vial materials and additives on proteomics performance. An innovative approach developed in the lab, involving the use of a microfluidic chip for sample preparation, will also be presented.

Finally, we will discuss the combination of cutting-edge techniques, such as laser capture microdissection followed by shotgun proteomics, MALDI imaging, and single-cell proteomics, to combine functional and spatial analyses of tissues.

This presentation will emphasize the importance of these methods and advanced mass spectrometry technologies in protein studies and for the deeper understanding of complex biological systems.

Prof. Gabriel Mazzucchelli

Gabriel Mazzucchelli holds the position of Associate Professor and serves as the Chief Research Logistician within the management team at the Mass Spectrometry Laboratory of the University of Liège. In this capacity, he oversees scientific collaborations related to the advancement of mass spectrometry techniques for "Omics" applications. His involvement in this field dates back to 2001 when he initiated the development of mass spectrometry-based methods for biomolecule characterization and proteomic approaches aimed at analyzing complex biological samples.

In 2008, he assumed the role of Scientific and Quality Control Manager for the GIGA Proteomic Platform and the Omics Academic Research Unit of the Mass Spectrometry Laboratory (MSLab-OMICS). Subsequently, in 2015, he was appointed as an Associate Professor, and in 2016, he took on the responsibility of Internal QC Auditor at the University of Liège (RQlab). Currently, he holds the position of President of the Council of the Scientists Group at the University of Liège.

Furthermore, he is one of the co-founders of MSExpertise SRL, a consultancy firm specializing in Mass Spectrometry and Quality Assurance. His ongoing research projects are centered on the comprehensive characterization of proteins and proteomes within biological systems, utilizing advanced mass spectrometry techniques at the University of Liège's Mass Spectrometry Laboratory, under the direction of Professor Gauthier Eppe. More specifically, Gabriel's research projects focus on single-cell proteomics and downscaled analyses, MALDI imaging combined with laser capture microdissection of tissue sections followed by shotgun proteomics for spatial omics studies and human sweat proteomics in the context of personalized medicine.

He has authored 89 scientific publications in peer-reviewed international journals, with an H-index of 29 and a total of 2523 citations (as of May 2024, based on the Scopus database).

Lectures

L1 Serum Proteomics in Compensated Advanced Chronic Liver Disease: Impact of Portal Hypertension

Lovorka Grgurevic

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Chronic liver injury from various causes leads to scarring, impaired liver function, and portal hypertension (PH), often progressing to cirrhosis. Early-stage cirrhosis may present few or no symptoms, leading to undetected disease progression until liver decompensation occurs, marked by complications such as ascites, variceal bleeding, and hepatic encephalopathy. The gold standard for assessing PH severity is the hepatic venous pressure gradient (HVPG) measurement, an invasive and costly procedure. Non-invasive alternatives like elastography, combined with platelet count, are recommended but have limitations, particularly in patients with certain conditions. Serum biomarkers offer a potential solution to these limitations, providing insights into the disease's pathophysiology and response to treatment.

We prospectively included consecutive patients with histologically confirmed compensated advanced chronic liver disease (cACLD) and available HVPG measurements. Serum samples were pooled based on the presence or absence of clinically significant PH (CSPH) and analyzed using liquid chromatography-mass spectrometry. Gene set enrichment analysis was conducted, followed by an extensive literature review to identify proteins with the most significant differences between the groups.

In a study of 48 patients with confirmed chronic advanced liver disease, several proteins were identified as potential biomarkers for clinically significant portal hypertension (CSPH), including CD44, VEGF-C, LYVE-1, tenascin C, Plasminogen activator inhibitor 1, Nephronectin, Bactericidal permeability-increasing protein, Autotaxin, Myeloperoxidase, disintegrin and metalloproteinase with thrombospondin motifs-like protein 4.

These findings suggest that inflammation, vascular contractility, and lymphangiogenesis are key factors in PH development, and these proteins may serve as targets for future therapeutic interventions and diagnostics.

L2 MALDI-TOF-TOF Based *de novo* Sequencing of the Novel Factor H Binding Protein Bg19 of *Borrelia garinii*

Mangesh Bhide[1,2], Jana Jozefiaková[1], Lea Talpašová[1], Tomáš Maľarik[1], Katarína Kucková[1], Jakub Víglaský[1], Katarína Bhide[1]

[1]*Laboratory of Biomedical Microbiology and Immunology, University of Veterinary Medicine and Pharmacy in Košice, Košice, Slovakia*

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Factor H binding by *Borrelia* has been correlated with pathogenesis as well as with host diversity. Here we present a novel factor H binding protein, an additional member in FHBP (Factor H binding protein) family. With the help of affinity ligand binding assay (ALBA), 2-D electrophoresis, peptide mass fingerprinting and *de novo* sequencing we confirmed this novel, ~ 19 kDa factor H binding protein of *Borrelia garinii* serotype 4. The protein was unique in this serotype and none of the other serotypes possesses this protein. Seventeen *Borrelia* strains were grown in BSK-II medium, washed 5 times with ultra-pure water and then resuspended in water containing 1% TFA, 1% of nuclease mix and 1% of a protease inhibitor cocktail (GE Healthcare). Affinity ligand binding immunoblot (ALBI) was performed to detect factor H binding proteins of *Borrelia.* Unique 19 kDa protein was separated then with 2D gel electrophoresis and subjected for peptide mass fingerprinting. MALDI-MS data were obtained in an automated analysis loop using an Ultraflex time-of-flight (TOF) mass spectrometer (Bruker-Daltonics) equipped with a LIFT-MS/MS device. Subsequently, selected precursor ions were subject to fragment ion analysis in the tandem time-of-flight (TOF/TOF) mode to obtain the corresponding MALDI-MS/MS spectra. Automated analysis of mass data was performed using the flexAnalysis software (Bruker-Daltonics). MASCOT peptide search was failed to assign a statistically significant peptide match with 19 kDa protein. Further manual de novo sequencing was attempted based on MALDI-MS/MS spectra. Peptide sequences obtained by de novo sequencing were submitted to the BLAST search algorithm at the NCBI (http://www.ncbi.nih-gov/BLAST/). As coiled-coil elements were demonstrated to be involved in the presentation of the fH binding sites, putative coiled-coil formation analysis for novel sequences was performed by using PepCoil software (http://bioweb.pasteur.fr/seqanal/interfaces/pepcoil.html). Manual de novo sequencing using MS/MS fragmentation of m/z = 2603.2 revealed peptide sequence SNEKLEEDEENEAQQVNSLQNR. The short input BLAST search showed a complete sequence homology with a hypothetical protein of *B. garinii* PBi (Genbank AAU07257). Hitherto, no information regarding the function and topology of hypothetical protein PBi was available in the public protein databases. However, in silico analysis indicated that there was a high probability of two coiled-coil formations near the C-terminus (120 to 147 and 118 to 152 residues with a probability of 1.00), supporting the role of this novel protein in human fH binding. Research was funded from APVV-22-0084, VEGA1/0381/23, VEGA1/0348/22, EURONANOMED2021-105.

L3 A Walk on the Wild Side - Omics Adventures in Animal Science

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Our understanding of fundamental biological processes has largely depended on the study of model organisms. However, recent advancements in genome sequencing, assembly, and annotation have made it possible to investigate non-model organisms using the same sophisticated bioanalytical tools. As mass spectrometry is becoming more accessible than ever before, many advantages of applying omics to non-model organisms arise, driving progress in fields such as biomedical research, veterinary medicine, agriculture, behavioral ecology, and food safety.

For many years, wild animals have been reported as potential sources of infectious and parasitic diseases that can affect both domestic animals and humans. Historically, the primary focus has been on safeguarding human and domestic animal health, with the impact of pathogens on wildlife receiving less attention. However, the adoption of the integrated One Health approach acknowledges that the health of humans, animals (including wildlife), and ecosystems are interconnected and mutually dependent. There is also growing recognition of the significant role that emerging diseases play in wildlife populations. Numerous factors, such as invasive species, climate change, pollution, and resource overexploitation, are increasingly undermining the health of wildlife populations.

Parasites can influence the health and population dynamics of wildlife, and their presence in wild populations is often considered a key indicator of ecosystem health. When non-native parasites are introduced to previously unexposed hosts, they can lead to significant losses in livestock production and threaten the survival of local wildlife populations. Herein, one of such examples in Europe - the introduction of the giant liver fluke *Fascioloides magna*, imported from North America, is presented. Broad proteomic and metabolomic studies across different host types (roe deer, red deer, and wild boar) will improve our understanding of parasitic infection, zoonotic prediction, and highlight possible treatments. Furthermore, determining host-parasite omics signature is critical to understanding infection, mechanisms of innate and adaptive immunity as well as zoonotic spillover potential. Additionally, integrated omics approaches are becoming essential for gaining deeper insights into molecular mechanisms of disease development, offering a more comprehensive understanding than any single technique alone.

L4 Metabolomics: A Tool for Understanding Disease Pathophysiology

Morana Jaganjac
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Metabolomics is a rapidly evolving branch of science that deals with the characterization and quantification of metabolites in biological samples. Metabolites are small molecules (MW <1500 Da) that include endogenous compounds such as lipids and amino acids as well as xenobiotic compounds, such are those derived from environment or diet. The metabolome is highly dynamic, and the composition of the metabolites changes in response to various factors under both physiological and pathological conditions. Oxidative stress, inflammation or exposure to xenobiotics are among the factors that can significantly influence the metabolome. Thus phenotypic changes, caused by small changes at the cellular level or changes in the environment, may have huge impact on the metabolome. Metabolomics is now recognized as the omics discipline closest to the biological phenotype. This presentation will provide an overview of the various analytical chemistry techniques used for the high-throughput characterization of metabolites. Technical breakthroughs in mass spectrometry technologies have positioned mass spectrometry an essential tool for metabolomics research in combination with liquid chromatography and gas chromatography separation techniques. Different methodological approaches and research strategies used in metabolomics will be discused and the advantages of each given. Finally, it will highlight the importance of understanding metabolome remodeling in both physiology and pathology, as this could help in the biomarker discovery and the identification of therapeutic targets for a variety of diseases. Examples of various applications of metabolomics will also be given.

L5 Quantitative Lipidomic Analysis Using Multiple Reaction Monitoring

Christian A. Reynolds
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Lipidomics is a subset of metabolomics that is focused on analysis of hydrophobic or amphiphilic small molecules and targeted lipidomic analysis is the preferred tool for quantitation of lipids in biological systems. Multiple reaction monitoring (MRM) analysis is the most widely used approach in targeted lipidomics, in which liquid chromatography (LC) is combined with triple quadrupole mass spectrometers (MS). MRM-based lipidomic analysis involves the selection of ions corresponding to the lipids of interest with subsequent fragmentation to produce a range of daughter ions, which are then selected for quantitation purposes. This allows for isolation of parent ions and specific daughter ions corresponding to the lipid molecules of interest thereby improving sensitivity and accuracy. Here we review the principles behind MRM-based analysis using real-world targeted lipidomic datasets as examples.

L6 Making Sense of MS Data – Applying Statistics and Bioinformatics in Downstream Analyses

Andrea Gelemanović
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With the rapid technological advancements, today we are flooded in various big data, so called omics data, which we are using to tackle various biological questions and trying to understand different phenomenas at the molecular level. We have also shifted our focus from gene-centric view, as the main units of heredity, towards a protein-centric view, as the key functional unit. To investigate the proteins at a larger scale under specific condition, mass spectrometry (MS) has grown popularity as a method of choice for quantitative profiling of an entire proteome. MS-based data offers a wide range of applications, from understanding the disease etiology, identifying new biomarkers, and opening the revenues for potential therapeutic interventions and treatment strategies. However, the challenge lies in the interdisciplinary nature of the field as it requires the knowledge of biochemistry for sample preparation, analytical chemistry for instrumentation and computational biology for data analysis and interpretation. Since the output of MS-based data results in several thousands of proteins identified at once in a single experiment, one should be trained in applying the best standards for data preprocessing and analysis, in order to allow for proper downstream applications and interpretation. Thus, the aim of this lecture is to provide a theoretical overview of the best practices in statistical and bioinformatical evaluation of quantitative MS data with software-driven data analysis strategies. Focus will be on providing the overview of the key methodological concepts in the analysis of proteomic and metabolomic data after quantification step, and to discuss the importance of normalization methods, filtering, outliers and the problem of missing data which is very common in MS. Different downstream approaches for results interpretation will also be discussed, ranging from functional annotation, machine learning approaches, multi-omics integration methods to visualization options. Methodological concepts will be supported through various case studies.

L7 Single-Cell Mass Spectrometry Imaging

Željko Debeljak*, Ivana Marković
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First mass spectrometry imaging (MSI) experiments involving entire animal organism scans were conducted in 1990'ties. Since then, many improvements of the technique were devised. Among the most important are the improvements of lateral resolution: today, Matrix Assisted Laser Desorption/Ionization Time-of-Flight (MALDI TOF) Mass Spectrometry (MS) instruments with 5 µm resolution are commercially available. Depending on the ionization source used, lateral resolution of MSI may be even < 1 µm. These lateral resolutions enable enforcement of the MSI experiments on a single-cell or even on a sub-cellular level. Still, there are some obstacles to be resolved. First of all, quantity of the material desorbed per a single cell is very small raising a question of sensitivity. Another important obstacle is associated to the discrepancies between the selected Region-of-Interest (ROI) and actually scanned ROI: mismatch between the selected and scanned ROI of only 3-4 µm may assign the scanned MS to wrong cells. To avoid this, the use an integrated light microscope and MSI device is recommended. In case of MALDI TOF MSI, there is also the need for very small matrix crystals: large crystals produced by spraying may cover 2 or more cells which, in turn, leads to mixing of spectra from those cells. In case of cell suspension analysis, like the blood analysis, some additional obstacles arise. First of all, medium in which cells are suspended may cause interference in MS. Removal of the media may cause significant changes in cell's metabolism introducing the artificial MS changes. To be analyzed using the MSI, cells need to be fixed on the slide which also may change their metabolism. Different cell populations may be present in suspension. If differences in MS between cell populations are expected, each cell identity should be confirmed using dyeing and the light microscopy. This should be carefully done since dyes also produce their own signals in MS and, even worse, may cause chemical changes in analyzed cells. Finally, cells in suspension can not be cut by a microtome: without cutting only the cell surface can be analyzed. Given obstacles and actual single-cell MALDI TOF MSI experiments will be commented in the presentation.

L8 MS Methods in Applied Bioanthropology

Luka Bočkor
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Multiple factors (genetics, epigenetics, environment and lifestyle) impact on today's non-communicable diseases development. Our genetics is a result of acquired adaptations during millions of years of natural selection, not only of our species, but also of all our ancestral species. The social evolution generated by the rapid advancement of science and technology has dramatically altered human lifestyle in a in a relatively short time on a historical timescale. Unfortunately, adaptive changes of the genome are not able to compensate, and as life expectancy has increased, deleterious consequences of genetic traits that once provided survival advantage are now outweighing their once protective benefits. As a result, multiple non-communicable diseases (e. g. diabetes, cardiovascular diseases, tumours) are emerging within human populations globally, placing increasing burdens on both healthcare systems and affected individuals.

In order to reduce the incidence of these diseases, a comprehensive approach that considers social, behavioural, psychological, and biological factors is necessary. Modern bioanthropology, always interdisciplinary, is now breaking down barriers in science more efficiently then ever. By applying methods from analytical chemistry, molecular biology and biochemistry, along with various types of questioners and interviews and historical populations data, we can better understand the causes of diseases, both on the level of an organism and on molecular level.

Analytical chemistry methods provide essential data on the lifestyle habits and environmental exposures of ancient population, as well as additionaly toxicological, metabolomic and proteomic data in contemporary populations. To illustrate how bioanthropology can contribute to a better understanding of diseases and society with a help of MS methods a brief overview of our prespective will be presented, with an emphasis on studies of ancient populations and biomedical research at the Institute for Anthropological Research.

L9 Role of Mass Spectrometry in Characterization of Peptide Drug Products

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In the last decades, thanks to new technologies and manufacturing, peptide drug development has made great progress. Therapeutic peptides are unique class of pharmaceutical agents composed of a series of well-ordered amino acids, usually with molecular weights of 500 – 5000 Da, or up to 40 amino acids. Technologies used for peptide purification and synthesis, structure elucidation and sequencing made substantial progress, which lead to over 80 drug products on the market and even 100 in preclinical trials and clinical development. Given the current state of technologies for peptide characterization, various types of analytical procedures are used in the aspects of peptide related substances, aggregation, peptide sequence, physical attributes and so forth. In this oral presentation, High resolution Mass spectrometry instrument was used for Intact mass, Peptide mapping and Sequencing, Impurity profile evaluation including Identification of impurities.

L10 Analysis and Characterization of Bioactive Peptides and Peptide-Drug Conjugates by Various Tandem Mass Spectrometry (MS/MS) Techniques

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Peptides are making steady inroads into diverse medicinal categories, particularly as promising future therapeutics and diagnostic tools. Over the last decades, the field of peptide pharmaceuticals has grown considerably and currently in the PepTherDia, a database of approved peptide therapeutics and diagnostics, there are over 105 approved peptide pharmaceuticals. This number is going even higher if we include peptide-drug conjugates (PDCs), a class of molecules formed by chemical linkage, either directly or through a linker unit, between a drug cargo and a corresponding peptide. However, today's development of new bioactive peptides and various active PDCs is challenging due to the high demands and rigorous conditions set by state regulatory agencies (FDA, EMA) that newly synthesized pharmaceutics must fulfil in order to be considered as potential candidates for clinical trials. Currently, the main development approach consists of using solid-phase peptide synthesis (SPPS) to obtain a desired peptide pharmaceutical, high performance liquid chromatography (HPLC) to purify it, lyophilization to obtain it in the solid form and mass spectrometry (MS), tandem mass spectrometry (MS/MS) or liquid chromatography – mass spectrometry coupling (LC-MS) to confirm its identity and purity. Tandem mass spectrometry has been shown as very useful technique to analyze peptide sequences and to identify the structure of main impurities accompanying the final product. Through selective and guided ionization and dissociation of the corresponding peptide/impurity molecule, the formed backbone (a, b, c, x, y, z) and side-chain ions (d, v, w) help us not only to confirm the peptide identity but also to distinguish between possible isomeric/isobaric structures formed during the synthesis.

Here, we present the fragmentation behavior of synthetic peptides containing various oxidized tryptophan residues [1,2], related to the pathogenesis of Alzheimer's disease, Parkinson's disease, atherosclerosis and diabetes mellitus, by using post-source decay (PSD) and collision-induced dissociation (CID) in matrix-assisted laser desorption/ionization (MALDI) time-of-flight (TOF)/TOF-MS and CID in an electrospray ionization (ESI) double quadrupole TOF-MS. Additionally, the MS/MS analysis, using ESI triple quadrupole (QQQ), of the main impurities formed during the synthesis of antiviral peptide-porphyrin conjugates (PPCs), capable to pass blood-brain barrier (BBB) and to act against HIV and Zika virus [3,4], are discussed.

Finally, we can conclude that mass spectrometry remains one of the main analytical techniques used in the characterization and identification of the peptide-inspired endeavors in the pharmaceutical pipeline.

L11 Mass spectrometry in analysis of drug-cyclodextrin complexes

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Cyclodextrins (CDs) are a family of oligosaccharides consisting of a macrocyclic ring, with the most common natural CDs, α-, β-, and γ-CD containing 6, 7, and 8 glucopyranose subunits, respectively. Their interesting properties offer the potential for CDs to improve solubility and stability of active pharmaceutical ingredients through the formation of host−guest inclusion complexes via non-covalent interactions.

The knowledge of the non-covalent molecular interactions that occur in supramolecular systems is the basis of their applications. The assessment of the formation of a drug–CD inclusion complex and its full characterization is not a simple task and often requires the use of different analytical methods, whose results must be combined and examined together, since each method explores a particular feature of the inclusion complex. There has been growing interest in the advancement of efficient and reliable analytical methods that assist with elucidating CD host−guest drug complexation. One of techniques with an increasing application in this field is mass spectrometry (MS). The use of MS together with different soft ionization methods to investigate supramolecular structures such as drug–CD inclusion complexes in gas-phase is recently becoming popular because this technique can provide information about non-covalent gaseous ion, structural determinations, stoichiometry of the inclusion complexes, reactivity in gas-phase and gas-phase thermochemical data.

Here, we present our recent results on exploring host−guest inclusion complexes of various drugs such as praziquantel, nabumetone and cinnarizine with natural β-CD and its derivatives (namely randomly methylated β-CD, hydroxypropyl-β-CD and sulfobutylether sodium salt β-CD). Aparat from structural determination of host−guest inclusion complexes in gas-phase by mass spectrometry we are focused on investigating stability of various drugs in potential cyclodextrin pharmaceutical formulations with improved properties. In such studies liquid chromatography – high-resolution mass spectrometry is a technique of choice due to its sensitivity and potential to provide unequivocal structural characterization of degradation products.

L12 Glyco-Fishing: Catching Glycopeptides with Glycoprotein Enrichment and LC-MS

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Glycosylation is a prevalent and diverse co- and post-translational modification essential for proper protein function and stability, offering substantial biomarker potential. Over the past 15 years, high-throughput glycomics has surged, driven by methods optimized for the 96-well format. Analytical processes initially focused on human plasma, targeting glycans as potential biomarkers. This led to the development of various methods on platforms such as liquid chromatography with fluorescent detection, capillary electrophoresis with fluorescent detection, and lectin arrays. More recently, glycosylation analysis has increasingly employed MS-based methods. However, these often rely on costly affinity chromatography for glycoprotein isolation and tend to analyze glycosylation at the released glycan level, losing site-specific information and introducing confounding contaminant glycoproteins.

In the past few years, we have introduced several cost-effective, high-throughput methods for site-specific N-glycosylation LC-MS analysis of human plasma proteins at the glycopeptide level. These methods bypass the need for antibody-based and affinity chromatography isolation, instead enriching glycoproteins to a higher level before glycopeptide analysis. Post-trypsinization, glycopeptides are purified using hydrophilic interaction chromatography-based solid-phase extraction and analyzed by reversed-phase liquid chromatography-electrospray ionization-MS. Our methods for alpha-1-acid glycoprotein, complement C3, and fibrinogen site-specific N-glycosylation LC-MS analysis have been validated across large cohorts, demonstrating their capability to detect biologically meaningful data. These techniques can be applied to large cohorts, aiding in the search for novel disease biomarkers and enhancing our understanding of human plasma glycoprotein roles and functions in health and disease.

L13 LC-MS Analysis and Data Processing of Complex Glycosylation Profiles

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Glycosylation is one of the most common cotranslational and posttranslational modifications, representing a controlled enzymatic process that adds glycans to proteins and lipids. A characteristic of protein glycosylation is its microheterogeneity, meaning that a single protein synthesized in a specific type of cell can carry a wide range of different glycans, making the analysis and regulation of glycosylation even more complex.

Glycosylation analysis can be performed at various levels, including whole glycoproteins, smaller protein fragments, glycopeptides, or only glycans released from the protein. When analyzing glycopeptides and glycans using mass spectrometry, employing separation techniques before mass spectrometric detection enables the effective analysis of these complex samples.

Liquid chromatography-mass spectrometry (LC-MS) technique is applied to determine the structure of glycopeptides and glycans, as well as for their quantification. Typically, this involves relative quantification, which expresses the abundance of glycan structures at a specific glycosylation site on an individual protein or the proportion of glycan structures across all plasma proteins. In addition to analysis of individual and smaller sets of samples, high-throughput methods are now available for plasma protein glycosylation analysis, allowing studies with a large number of samples.

Processing of LC-MS data is always challenging due to the large amount of information that needs to be analyzed. Currently available tools for relative quantification can be partially automated. For instance, Lacy Tools can be used for data obtained from LC-MS systems, and serves as an example to present data processing workflow and the difficulties encountered when analyzing both small and large numbers of samples. Overcoming challenges and understanding how this process can be further improved is crucial for deepening our knowledge of glycosylation and its role in biological processes as well as in different pathological conditions.

Posters

P1 Mass Spectrometry Contribution to the Study Of Extracellular Lipase from Bacterium *Streptomyces rimosus*

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Lipases (EC 3.1.1.3) are enzymes that have important role in biological systems but also have wide industrial applications (organic synthesis, detergent additives, food processing), because they remain stable in organic solvents and possess broad substrate specificity and high enantioselectivity. They catalyze both hydrolysis and synthesis of esters depending on reaction conditions. Most lipases show dependence on the interface for full catalytic activity (interfacial activation phenomenon) and their kinetics cannot be described by Michaelis-Menten equations. Majority of the lipases with solved 3D-structure share common structural motif (α/β-hydrolase fold) and catalytic triad (Ser, Asp/Glu, His) similar to the one of serine proteases. We have shown an extracellular lipase from bacterium *Streptomyces rimosus* to be a true lipase, based on its kinetics and activity towards esters of long-chained fatty acids. The stability of this enzyme in wide pH and temperature ranges, and in various organic solvents was demonstrated, as well as broad substrate specificity, which makes this enzyme interesting for a possible industrial application.

While working on solving the 3D-structure of *S. rimosus* lipase, we turned to mass spectrometry (MS) for characterization of structural and kinetic properties of this enzyme. MS was used to confirm the protein sequence of over-expressed enzyme, and showed no post-translational modifications. The existence of 3 disulfide bridges and their pattern, a factor contributing to lipase's remarkable stability, was determined *via* comparison of MALDI-MS spectra of reduced and non-reduced alkylated tryptic peptides. The protein sequence of *S. rimosus* lipase comprises two putative consensus sequence motifs (i.e. GDS(L) and GXSXG, corresponding to SGNH-hydrolases and α/β-hydrolases, respectively). To distinguish which of these motifs contains catalytic Ser, MALDI-MS and MALDI-MS^2 spectra of enzyme incubated with covalently binding inhibitor (dichloroisocoumarin, DCI) were analyzed. The spectra clearly showed DCI bound to Ser10 in GDSY motif, thus proving *S. rimosus* lipase to belong to a SGNH-hydrolase family. The kinetics of release of the DCI from the active site, as well as the active enzyme concentration in the sample were determined by activity measurements, MALDI-MS and ESI-MS, with good agreement between the methods.

In conclusion, mass spectrometry added greatly to our understanding of *S. rimosus* lipase biophysical and kinetic properties.

P2 LC-MS/MS Method Development for Quantification of Omega-6 Polyunsaturated Fatty Acid (PUFA)-Derived Lipid Mediators

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Lipid mediators, derived from dietary omega-6 polyunsaturated fatty acids (PUFAs), play critical roles in inflammation, immune responses, and tissue homeostasis. Quantifying these bioactive lipids in biological matrices is essential for understanding their physiological and pathological roles. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) has emerged as a powerful technique for the accurate and sensitive quantification of lipid mediators. This study presents the development of an LC-MS/MS method for the quantification of diet-derived lipid mediators in plasma samples. Our method utilizes optimized sample extraction, chromatographic separation, and targeted multiple reaction monitoring (MRM) mass spectrometry method for detection of unique molecular ion-daughter ion combinations for lipid mediators derived from omega-6 PUFAs. This LC-MS/MS approach provides a robust analytical tool for studying the role of dietary omega-6 PUFAs in health and disease, offering insights into their contribution to inflammation, metabolic regulation, and chronic disease processes.

P3 Proteomic Profiling of *Trypanosoma rotatorium*

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Trypanosoma is a genus of parasitic flagellate protozoans, primarily known for causing diseases in both humans and animals. Trypanosomes are transmitted through insect vectors such as tsetse flies or kissing bugs. Two the most important diseases caused by trypanosomes are African trypanosomiasis (sleeping sickness) and Chagas disease. They have complex life cycles involving both the insect vector and the mammalian host. Except pathogenic species, there are facultative pathogenic or apathogenic species of trypanosomes. One of those is a frog trypanosome, *Trypanosoma rotatorium*. It has a complex life cycle that involves both an invertebrate vector, believed to be a leeches which ingest the parasite when feeding on the blood of an infected amphibian. To determine the reason of frog trypanosomes apathogenicity, we focused on proteomic profile of *in vitro* cultured *T. rotatorium* epimastigotes. Epimastigotes were washed in PBS buffer and lyzed in lysis buffer containing SDS. Total proteins were digested using FASP protocol and analyzed using high resolution LC-MS/MS by employing Ultimate 3000 RSLCnano system coupled with the Orbitrap Q Exactive Plus mass spectrometer. Acquired MS/MS spectra were further processed for protein identification using SEQUEST algorithm implemented into Proteome Discoverer as a database search engine against *Trypanosoma* FASTA files. For functional annotation, TriTrypDB was used. Identified proteins were classified as cellular components involved in biological processes related to flagellate's life cycle. As expected, we did not detect any of the important virulence factors present in *T. brucei* or *T. cruzi*.

P4 Mass Spectrometry in Newborn Screening in Croatia

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The main purpose of newborn screening (NBS) is to detect inherited metabolic diseases (IMD) in presymptomatic infants, thus enabling early start of treatment for curable disorders to prevent often irreversible health consequences, and in some cases, death. Dried blood samples (DBS) taken from newborns are tested by tandem mass spectrometry coupled with high performance liquid chromatography, LC-MS/MS. Due to its suitability for simultaneous determination of a large number of analytes from a single DBS, it provides early diagnoses for over 50 IMDs, including organic acidurias, aminoacidopathies and fatty acid oxidation disorders. In October 2017, Croatian NBS laboratory started a pilot project with six new IMDs added to the existing NBS program. NBS in Croatia is performed with commercial Recipe reagent kit for LC-MS/MS, using the analytical method for determination of aminoacids, acylcarnitines and its ratios on Shimadzu LC-MS/MS 8050. The DBS sample is spiked with internal standard and extracted from the dried blood matrix. To obtain better stability for certain metabolites, the samples are derivatized in butanolic HCl and transferred into their corresponding n-Butylester. After their preparation, the samples are injected directly into the ion source of the MS, via flow injection analysis (FIA) at a constant flow rate, thus eliminating the need for column separation. The analytes are ionised by electrospray ionisation (ESI) and transferred into the gas phase. The measurement of the analytes is carried out in multiple reaction monitoring (MRM) which ensures identification and quantification with high selectivity and sensitivity. Concentrations of individual acylcarnitine and amino acid species are calculated from isotope-labelled internal standards of known concentration for each analyte. Since the samples are determined semi-quantitatively due to the variable blood volume and haemocrit content, all positive screening results need to be verified by additional confirmatory tests. Some of those tests include molecular analysis and the analyses of amino acids and acylcarnitines in plasma, which are also perfomed on LC-MS/MS. To ensure the quality and accuracy of NBS results, aside from daily internal quality control, Croatian NBS laboratory participates in several external quality control programs. In the near future, the plan is to expand the national screening panel with more IMDs and introduce second-tier tests for re-evaluation after positive NBS results for specific IMDs.

P5 Targeted Analysis of Arachidonic Acid and Linoleic Acid Oxylipins

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Lipids are one of the prime targets for oxidative stress, leading to the formation of oxidatively modified lipids that result in the formation of oxidatively modified lipids that constitute a significant portion of the epilipidome. Among these modified lipids, oxylipins represent a class of bioactive molecules derived from polyunsaturated fatty acids (PUFAs), such as linoleic acid (LA) and arachidonic acid (AA), which are particularly susceptible to oxidation by reactive oxygen species. Oxylipins are important signaling mediators involved in the innate immunity responses. Oxylipins exhibit a spectrum of effects, ranging from pro-inflammatory to anti-inflammatory, with some displaying dual regulatory roles. Still, the involvement of oxylipins in the development of different diseases is under explored. Thus, the aim of this work was to develop a sensitive method for the detection and identification of AA and LA oxylipins by liquid chromatography mass spectrometry.

The method development focused on two areas: sample preparation and analytical detection. The selected analytes for this multi-analyte analysis included oxylipins derived from LA and AA, both of which serve as precursors for a diverse range of bioactive oxylipins and are among the most physiologically significant PUFAs. The targeted oxylipin analysis includes key metabolites derived from LA and AA. Targeted LA-derived oxylipins were 9(S)-HODE, 9-OxoODE, 13(S)-HODE, 13-OxoODE, (±)9(10)-EpOME, (±)9(10)-DiHOME, (±)12(13)-EpOME, and (±)12(13)-DiHOME, while targeted AA-derived oxylipins were 5(S)-HETE, 5-OxoETE, 8(S)-HETE, 9(R)-HETE, 11(S)-HETE, 12(S)-HETE, 12-OxoETE, 15(S)-HETE, and 15-OxoETE. Analytes were separated and analyzed by the Agilent Technologies Series 1200 HPLC binary solvent delivery system coupled with an Agilent 6520 Accurate-Mass Q-TOF was used.

Once fully validated, this method will provide a robust platform for the comprehensive analysis of LA- and AA-derived oxylipins, facilitating their quantification in complex biological matrices such as plasma. Given the diverse roles of oxylipins in various pathophysiological processes, this method holds significant potential for advancing our understanding of the involvement of these lipids in disease etiology. Given the diverse roles of oxylipins in various pathophysiological processes, this method holds significant potential for advancing our understanding of the involvement of these lipids in disease etiology.

P6 Metabolic Challenges to Fidelity of Isoleucine Decoding in *Escherichia coli*

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Fidelity of mRNA translation and protein biosynthesis depends on the accuracy of DNA transcription, stringency of the codon:anticodon pairing on the ribosome, and fidelity of tRNA and amino acid coupling catalyzed by aminoacyl-tRNA synthetases (aaRS). Some aaRS, like isoleucyl-tRNA synthetase (IleRS), have difficult task of differentiating cognate amino acid, e. g. isoleucine, from isosteric or structurally closely related or even non-proteinogenic amino acids, such as valine or norvaline (Nva), which therefore jeopardise fidelity of ribosomal translation.

IleRS evolved additional editing domain which cleaves off incorrect amino acids bound to cognate $tRNA^{Ile}$ and prevents erroneous incorporation of Val or Nva into growing polypeptides instead of Ile during ribosomal translation. If the IleRS editing domain is inactivated and the growth medium supplemented with excessive concentration of Val or Nva, high levels of mistranslation can be achieved in bacterium *Escherichia coli*, up to 20 %, as measured proteome-wide, by shotgun proteomics. However, such high levels of Ile mistranslation come with the price: proteomic analysis revealed upregulation of major chaperones, disaggregase ClpB and proteases, indicative of proteotoxic stress. Furthermore, excessive nonspecific protein aggregation, enriched in mistranslated polypeptides (close to 30 %) was observed, bacterial fitness and resistance to heat stress were reduced. *Escherichia coli* can sustain high levels of Ile substitution with Val or Nva, but it suffers dire pleiotropic consequences.

In our recent research we have discovered that under certain conditions, during growth in minimal medium, IleRS editing-deficient bacterial strain can accumulate about 15% mistranslation, even without exogenous Val or Nva supply. This indicates that metabolic changes and adaptation to differet growth conditions can lead to unanticipated challenges to fidelity of protein synthesis. Proteome analysis will be performed on bacteria grown in mistranslating and control conditions, in order to dechiper changes in metabolic pathways challenging the fidelity of protein biosynthesis. For now, it is unclear if observed mistranslation is the consequence of Val or Nva misincorporation, because isobaric Val and Nva can not be distinguished by MS analyses. Metabolite and/or protein amino acid composition analysis will be needed, to confirm hypotheses derived form proteomic analyses. The *in vivo* consequences of endogenous, metabolically induced mistranslation will be compared to previous results obtained in media supplemented with excessive concentrations of offending amino acids.

P7 Optimisation of a Novel LC-MS Method for the Determination of Anastrozole and Palbociclib in Human Plasma

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Palbociclib (PAL) and anastrozole (ANA) are antitumor drugs used for the treatment of the endocrine sensitive breast carcinoma. A number of clinical studies indicate that PAL shows synergistic antitumor activity when combined with ANA. For the purposes of therapeutic monitoring of these drugs and the personalization of therapy there is a requisite of an analytical method that can quantify them. The chosen technique was liquid chromatography coupled to a mass spectrometer (LC-MS) as it allows the separation, identification and quantification of analytes with high sensitivity and selectivity.

The aim of this work was to develop a novel method for the simultaneous quantification of ANA and PAL in human plasma samples using LC-MS. Reverse phase chromatography was performed on a biphenyl chromatographic column with binary gradient elution. The mobile phase consisted of 0.1 % formic acid in water and 0.1 % formic acid in acetonitril. MS detection was performed on an Agilent Ultivo Triple Quadrupole using electrospray ionization in positive recording mode. Nitrogen was used as a gas for drying and dispersing the mobile phase and for collision, and was set at a temperature of 350 °C and a flow rate of 10 L/min. The pressure of the nebulizer was 40 psi, and the voltage on the capillary was 5000 V. In order to achieve optimal detection conditions, the parameters of the fragmentor and the voltage on the capillary were optimized with the Source optimizer Ultivo ESI program. The best precursor and product ions were selected, with the corresponding fragmentor voltages and collision energies, and the fragmentation patterns of the analytes were assessed from the obtained MS spectra. Optimal conditions were selected by analyzing the computer-optimized parameters and assessing the fragmentation of ANA and PAL at different collision energies. By observing their mass spectra the ion transitions m/z 448.2→380.0 for PAL and m/z 294.1→210.0 for ANA were selected as optimal ion transitions for monitoring. Recording and processing of the obtained results was done using the Mass Hunter Acquisition and Qualitative Analysis computer programs. Analytes were identified based on mass spectra and retention times. The developed method was deemed selective and sensitive, and thus shows good potential for future clinical application after validation.

P8 Proteomic Analysis of the Role of Isoleucine Mistranslation in the Adaptation of *Escherichia coli* to Oxidative Stress

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Translation is a cellular process in which the genetic information contained in mRNA is decoded for the synthesis of polypeptide chains. Errors in translation are mainly associated with harmful effects on the organism (e.g. protein aggregation, loss of protein function, change in cell morphology, impaired growth and organelle function). However, moderate levels of mistranslation may have a beneficial effect enabling adaptation of organisms to various stress conditions. Esterification of tRNA molecules with corresponding amino acids is catalyzed by aminoacyl-tRNA synthetases (aaRS), thus these enzymes have a decisive influence on the accuracy of the translation of the genetic code. At the aaRS level, mistranslation occurs due to the synthesis of incorrectly aminoacylated tRNA, which is why error correction mechanisms evolved. For example, some aaRSs contain an editing domain (ED) that hydrolyzes the ester bond of incorrectly aminoacylated tRNA. Isoleucyl-tRNA synthetase (IleRS) can misrecognize valine (Val) and norvaline (Nva), amino acids structurally similar to isoleucine, but possesses an ED domain to correct such errors. Interestingly, incubation of an *Escherichia coli* strain that expresses IleRS with a non-functional ED domain in a medium with Val or Nva causes an increased level of mistranslation but also enables bacteria to better cope with subsequent oxidative stress conditions. To identify proteomic changes enabling adaptation to oxidative stress, total proteins were isolated from bacteria exposed to mistranslating conditions and the proteome was analyzed by LC-MS/MS. It was shown that levels of isoleucine mistranslation go up to 30%, depending on the Val or Nva concentrations in the medium. Using quantitative mass spectrometry, differential protein expression in samples with or without Val or Nva has been analyzed in order to identify cellular processes that enable improved adaptation of bacteria to oxidative stress. Both Val and Nva mistranslation caused upregulation of peptide transport across plasma membrane and downregulation of glutamate metabolism, branched-chain amino acid transport and enterobactin biosynthesis, involved in iron transport. Additionally, several proteins involved in protein folding and stress response were also increased. Specifically, Val mistranslation resulted in upregulation of arginine biosynthetic process, while Nva mistranslation had a major impact on translation process.

P9 Natural Derived Mono- and bisQACs from Quinuclidine Backbone as an Alternative to Commercial Disinfectants

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Quaternary ammonium compounds (QACs) are amphiphilic molecules widely recognized for their broad-spectrum antibacterial properties. However, excessive use of QAC-based products has contributed to increasing bacterial resistance, prompting research efforts aimed at understanding and optimizing the structure-activity relationship of QACs for enhanced efficacy.

Recent studies have identified bisQACs as a promising alternative to monoQACs, owing to their superior antimicrobial activity. Structurally, bisQACs consist of two hydrophobic tails and two hydrophilic heads connected by a linker, or structural spacer. The double positive charge in bisQACs is believed to promote stronger interactions with bacterial membranes compared to monoQACs, leading to greater antimicrobial potency. This has made bisQACs an exciting point for further research.

In our previous work, we demonstrated that quinuclidine, a natural scaffold, serves as a valuable starting material for the synthesis of monoQACs with potent antimicrobial properties. Building on these findings, we now aim to synthesize both mono- and bisQAC variants based on quinuclidine and investigate their antibacterial activity and mechanisms of action. We believe this research will provide key insights into the design and optimization of future QACs for enhanced antimicrobial performance.

P10 Characterization of Antiviral Peptide-Porphyrin Conjugates (PPCs) by Tandem Mass Spectrometry (MS/MS)

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As viral diseases remain one of the biggest concerns of humankind, significance of finding new effective antiviral drugs has become undoubtedly prominent. A milestone in the antiviral drug development was achieved by the introduction of peptide-drug conjugates (PDCs), an association of two molecular entities (an antiviral drug molecule and a cell-penetrating peptide (CPP) carrier) conjugated directly or indirectly through a linker. Among the various antiviral PDCs, peptide-porphyrin conjugates (PPCs) have been drawing attention due to their remarkable pharmacological potential[1]. Moreover, several antiviral PPCs were recently shown to be effective against brain-residing viruses (Zika virus and Human Immunodeficiency Virus - HIV) in their *in vitro* studies[2,3].

Currently, synthesis of various antiviral PPCs is performed using solid-phase peptide synthesis (SPPS) approach, whereas high performance liquid chromatography (HPLC) is used for their purification. In addition, analytical techniques of mass spectrometry (MS), liquid chromatography – mass spectrometry (LC-MS) and tandem mass spectrometry (MS/MS) are usually applied for PPCs' identification and purity characterization. Nevertheless, despite these well-established SPPS, HPLC and MS procedures, obtaining antiviral PPCs in high yield and purity, remains one of the main bottlenecks. Thus, successful identification and characterization of the main accompanying PPCs' impurities by various MS, LC-MS and MS/MS techniques could help to overcome this obstacle through subsequent synthetic and purification method optimization.

Herein, we have performed LC-MS and MS/MS analysis of two antiviral PPCs (PPIX-PepH1 and PPIX-PepH3), using 6400 Triple Quad LC/MS Agilent Technologies. The main identified impurities during the LC-MS analysis (represented by positively singly, doubly, triply and quadruply charged ions of PPIX-PepH1 and PPIX-PepH3 sample, respectively) were additionally fragmented using collision-induced dissociation (CID) and characterized by the obtained a-, b-, c- or x-, y-, z-series of ions. In the case of PPIX-PepH1 (PPIX-VQQLTKRFSL) we were able to establish the *m/z* 521.7 impurity ion's origin to be the conjugate's C-terminal, while in the case of PPIX-PepH3 we identified the *m/z* 401 impurity ion to be a result of a loss of W at the C-terminal of the PPIX-PepH3 (PPIX-AGILKRW) sequence. Thereby, the presented tandem mass spectrometry analysis and characterization of the corresponding impurities should contribute to the optimization of antiviral PPC synthesis's methodology and further emphasize the significance of peptide analysis by MS/MS in drug development

P11 Domain Mapping Using Limited Proteolysis and MALDI-TOF MS

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Protein-protein interactions provide irreplaceable information about pathogenesis of human viruses. Conventional ways of studying protein-protein interactions include immunoassays like co-immunoprecipitation, pull-down and ELISA. Despite providing information about host-pathogen interactome, these methods cannot provide structural details (e.g. binding sites) of such interactions. Several immunochemical, structural and computational approaches can be applied to map binding sites of interacting proteins. Structure-based methods like X-ray crystallography and NMR are most accurate in determining atomic resolution of binding sites. However, these methods are time consuming and require high amount of material. Here, we show simple and rapid protocol for mapping of plausible binding sites between spike (S) and nucleocapsid (N) proteins of SARS-CoV-2. First, bait protein (N) was immobilized on PVDF-FL membrane and incubated with prey protein (S). Then, on-membrane limited trypsin digestion was performed to cleave prey protein bound to bait protein. Resulting non-interacting peptides were washed and interacting peptides recovered from membrane for MALDI-TOF MS analysis. As control, in-solution and *in silico* trypsin digestion of prey protein was done and generated peptides were compared to interacting peptides recovered from membrane. As negative control assay was performed omitting protein S. Total of 12 peptides were identified as potential binding sites, of which 7 with potential biological relevance. All the relevant binding sites are located on the N-terminal domain of S protein. This protocol proved to be useful in preliminary prediction of potential binding sites in protein-protein interactions. However, more detailed identification of recovered peptides would provide information about peptides, that were not matched to *in silico* trypsin digestion control. This method can be supplemented with different approaches like site-directed mutagenesis, to precisely determine binding sites. Research was funded from APVV-22-0084, VEGA1/0381/23, VEGA1/0348/22, EURONANOMED2021-105.

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