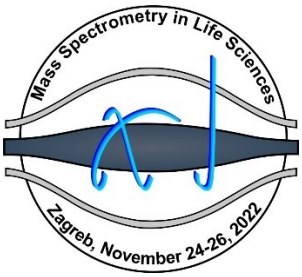
1st Workshop on Mass Spectrometry in Life Sciences : Book of abstracts

Book of Abstracts

1ST WORKSHOP ON MASS SPECTROMETRY IN LIFE SCIENCES

Zagreb, November 24th – 26th 2022



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Book of Abstracts of the 1st Workshop on Mass Spectrometry in Life Sciences

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Marko Močibob, Marija Pranjić

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Dear colleagues,

It is our immense pleasure to welcome you all to the First Workshop on Mass Spectrometry in Life Sciences to be held from November 24th to 26th 2022 at the Faculty of Science (PMF), Department of Chemistry in Zagreb!

Mass spectrometry (MS) is a state-of-the-art analytical tool with many applications in biological, biomedical, and clinical research. Over the past twenty years, it has gained increasing popularity through high-throughput "omics" experiments. MS allows researchers to dive deeply into biological or clinical specimens, generating qualitative and quantitative insights into their molecular contents. The range of applications of mass spectrometry only promises to grow as the technology continues to improve.

The variety of sample types that can be analyzed, and the plethora of information that can be obtained, have helped MS to be represented within an extensive range of research areas. This workshop will lead you through the recent advances in MS-based proteomics, metabolomics, and lipidomics and their application in biotechnology, biomedical research, nutrition, and translational medicine, exploring the structure, composition, dynamics, function, and interactions of (bio)molecules in diverse biological contexts. Various MS-based approaches and strategies in the pharmaceutical industry will be also covered. Finally, you will enrich your proteomic data analysis and visualization skills through various specialized software and bioinformatic tools.

Once again, we invite you to enjoy the unique opportunity offered by our workshop which brings together experts from research and industry to acquire knowledge, gain new skills and ideas, as well as future collaborations!

In the name of the Organizing committee

Marko Močibob, PhD
Chair of the Organizing Committee
1st Workshop on Mass Spectrometry in Life Sciences
Zagreb, November 24th - 26th, 2022

Organizers

Faculty of Science, Department of Chemistry

Croatian Chemical Society

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Richard Burchmore, Glasgow Polyomics, University of Glasgow

Morana Dulić, Faculty of Science, University of Zagreb

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Ruđer Novak, School of Medicine, University of Zagreb

Marija Pranjić, Faculty of Science, University of Zagreb

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Venue

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Horvatovac 102A, Zagreb

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Programme

Thursday	November 24th	
13:00 - 14:00		**Registration**
14:00 - 14:10		**Welcome & Introduction**
14:10 - 15:10	PL1	**Boris Maček** (Tuebingen, Germany) *Shotgun Proteomics in Biomedical Research*
15:10 - 15:35	L1	**Lovorka Grgurević** (Zagreb, Croatia) *Proteomics in Biomedical Research and Translational Medicine*
15:35 - 16:00	L2	**Marko Močibob** (Zagreb, Croatia) *Proteome Response to Mistranslation*
16:00 - 16:15	S1	**Darko Delgalo** (Kemolab Ltd) *Kemolab and Thermo Fisher Scientific for Life Science Mass Spectrometry in Croatia*
16:15 - 17:00		**Coffee Break**
17:00 - 17:25	L3	**Mario Cindrić** (Zagreb, Croatia) *Data Dependent and Data Independent* De novo *Sequencing*
17:25 - 17:50	L4	**Saša Kazazić** (Zagreb, Croatia) *Protein Conformation Dynamics Probed by Mass Spectrometry. Deuterium Labelling Approach*
17:50 - 18:15	L5	**Ana Butorac** (Zagreb, Croatia) *Mass Spectrometry Application in Pharmaceutical Research of Biomolecules*
18:15 - 18:40	L6	**Mislav Runje** (Zagreb, Croatia) *Mass Spectrometry in Development of Active Pharmaceutical Ingredients*

Friday	November 25th	
14:00 - 15:00	PL2	**Richard Burchmore** (Glasgow, UK) *Adventures in Metabolomics*
15:00 - 15:25	L7	**Anita Horvatić** (Zagreb, Croatia) *Deciphering the Complex Molecular Interplay in Health and Disease by Integrative Multi-omics*
15:25 - 15:50	L8	**Christian Andrew Reynolds** (Rijeka, Croatia) *Tandem Mass Spectrometry in Combination with Ion Mobility for Characterization of Cardiolipin Molecular Diversity*
15:50 - 16:00	S2	**Borna Ferčec, Tomislav Gregorić** (AlphaChrom Ltd) *Mass Spectrometry Appliance in the Life Science*
16:00 - 16:45		**Coffee Break**
16:45 - 17:10	L9	**Mislav Novokmet** (Zagreb, Croatia) *High-throughput LC-MS Based Analysis of Plasma Protein Glycosylation*
17:10 - 17:25	S3	**Ivan Grgičević** (Labtim Adria Ltd) *Accelerating Research and Comprehensively Identify Analytes in Your Complex Samples*
17:25 - 17:50	L10	**Stjepan Orhanović** (Split, Croatia) *Phytoplankton Toxins in the Adriatic Sea*
17:50 - 18:15	L11	**Maja Šemanjski Čurković** (Ljubljana, Slovenia) *Molecular Mechanisms of HipA-mediated Bacterial Persistence in* E. coli *Investigated by Mass Spectrometry-based Phosphoproteomics*

Saturday	November 26th	
	P1	
9:30 - 10:15	**Marko Močibob** *Introduction to MS Data Analysis*	
10:15 - 11:15	**Donna Potts** (Agilent Technologies, Inc.) *Bioconfirm Software Workshop*	
	P2	**225**
11:15 - 12:00	**Marko Močibob** *Data Processing with Proteome Discoverer*	**Anita Horvatić** *Data Processing with Proteome Discoverer*
12:00 - 13:00	**Lunch Break**	
13:00 - 14:00	**Marija Pranjić** *Data Processing with MaxQuant*	**Andrea Gelemanović** *R for Data Analysis*
14:00 - 14:45		**Ruđer Novak** *Data Visualisation*
14:45 - 15:00	**Short Break**	
15:00 - 16:00	**Andrea Gelemanović** *R for Data Analysis*	**Marija Pranjić** *Data Processing with MaxQuant*
16:00 - 16:45	**Ruđer Novak** *Data Visualisation*	
	P1	
16:45 - 17:00	**Concluding Remarks**	

Plenary lectures

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PLENARY LECTURE 1

Shotgun Proteomics in Biomedical Research

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Rapid progress in sample preparation, high resolution MS instrumentation and related bioinformatics have enabled the application of shotgun of proteomics at the forefront of biomedical research. I will give a brief overview of the field and use several examples from our research to explain how shotgun proteomics is used to identify new genes and gene variants, detect kinase substrates and monitor protein synthesis in dormant and resuscitating cells. I will conclude with my personal assessment of the field, its current challenges, such as single-cell proteomics, as well as future directions and potential disruptive technologies.

Prof. Dr. Boris Maček

Boris Maček received M.Sc. in Molecular Biology at the University of Zagreb (Croatia) and Ph.D. in Biology at the University of Muenster (Germany). He pursued his postdoctoral research in quantitative proteomics with Matthias Mann at the University of Southern Denmark and Max-Planck-Institute of Biochemistry in Martinsried (Germany), where he was involved in early applications of Orbitrap mass spectrometry in analysis of eukaryotic and prokaryotic signal transduction. Since 2008 he is Professor of Quantitative Proteomics at the University of Tuebingen (Germany) and Director of the Proteome Center Tuebingen. His current research interests involve proteogenomics, mitochondrial proteomics and analysis of regulatory networks involved in regulation of bacterial persistence. He co-authored about 180 peer-reviewed articles in the field of shotgun proteomics and biological signal transduction.

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PLENARY LECTURE 2

Adventures in Metabolomics

Richard Burchmore

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Proteins, and the metabolites with which they interact, comprise the molecular basis for biological phenotypes. These are highly complex, diverse and dynamic constellations of molecules, and are thus incompletely characterised or understood. Proteomics and metabolomics are complementary research areas, which aim to understand how proteins and metabolites contribute to health and disease. Understanding the complexity and regulation of proteomes or metabolomes remains an aspiration.

Untargeted characterisation of proteins or metabolites, which is essential to approach global coverage, is achieved through mass spectrometry, preceded with biochemical separations to select analytes as required. These technologies evolve continuously, advancing research frontiers but demanding continuous investment to maintain state-of-the-art laboratories. Proteomics and metabolomics projects thus tend to be collaborative research ventures, between basic or clinical researchers and centres of excellence in mass spectrometry.

Glasgow Polyomics (www.polyomics.gla.ac.uk) is an academic core facility which supports and develops proteomics and metabolomics workflows. We have contributed to diverse projects, ranging from animal nutrition to archaeology. My presentation will introduce the opportunities and challenges that are presented by metabolomic approaches. I will outline the application of biological mass spectrometry and supporting technologies, to achieve metabolomic analysis. I will provide examples from projects developed within my own research and collaborations.

Prof. Dr. Richard J. S. Burchmore

Richard J. S. Burchmore received B. Sc. in Parasitology at the University of London in 1989, and Ph. D. at School of Life Sciences, King's College London in 1993. He was a postdoctoral fellow at Oregon Health Sciences University (1994-1999), St George's Hospital Medical School (1999-2000) and University of Glasgow (2000-2003). In 2003 he moved to Sir Henry Wellcome Functional Genomics Facility (University of Glasgow) as Senior RA (2003-2006). Currently he is Senior Lecturer and Head of Proteomics at Glasgow Polyomics.

His research is focused on understanding the mechanisms by which pathogens thrive in the challenging environments which they encounter. He was an early adopter of mass spectrometry for proteomics and metabolomics and, over 20 years, he has been instrumental in establishing both these research areas at the University of Glasgow. His group was among the first to apply proteomic approaches to *Leishmania* and to other parasites.

With colleagues in Glasgow, he pioneered the use of Orbitrap mass spectrometry for untargeted metabolomic analyses. In collaboration with many colleagues across the world, he has applied mass spectrometry to address diverse questions, from animal nutrition to archaeology. He has published over 150 papers and he has supervised more than 30 PhD students.

Lectures

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L1 Proteomics in Biomedical Research and Translational Medicine

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A significant proportion of basic proteomic research is directed towards the clinic, largely aimed at the identification of proteins as potential biomarkers of pathological conditions. Reliable protein profiling is of particular importance in the field of plasma biomarker research where selected proteins must be consistently identified and quantified in large patient cohorts. As a first step, databases of existing plasma proteomes can serve as a valuable basis for the characterization of previously identified plasma biomarker candidates. As they are a non-invasive biological samples, blood and urine are commonly used for analysis. However, the research of human plasma/serum is challenging because a large number of parameters must be satisfied in order to validate a certain molecule as a biomarker. Other samples used in clinical studies include solid tissues, a range of other body fluids and samples from genetic animals models and cell lines. A common example is the use of mass spectrometry-based proteomics as a tool to study the biological changes occurring in cancer/metastasis. Such basic scientific research aimed to discover potential biomarker candidates, is often collaborative in nature, since clinicians collaborate with the academia. Biopsies from sites affected by primary disease such as tumours, cysts or transformed organs can be obtained directly from patients. Standardized protocols and working algorithms defining sample collection, processing and storage are essential for obtaining reliable and reproducible results. They are further processed to obtain the first round of candidate molecules, specific for a pathological condition. It is of the utmost importance that the study leader facilitates mutual communication and understanding between different disciplines and provides regular feedback on study progress.

The use of high throughput protein assays in the clinical setting and the advancement of proteomics-based technologies have raised expectations in the search for new protein biomarkers. However, to date, progress has been limited due to lack of an effective technological platform, poorly defined guidelines for determining groups of clinical samples, no standardized procedures for biomarker development, and quality assessment of performed studies. Successful production of diagnostic tests should be based on a clear technical and conceptual plan. The goal of the study should be to solve a clearly defined clinical question with an appropriate study design, which is a prerequisite for biomarker discovery that connects the joint work of clinical and basic research.

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L2 Proteome Response to Mistranslation

Marko Močibob[1], Marija Pranjić[1], Maja Šemanjski Čurković[2*], Philipp Spät[2], Boris Maček[2], Ita Gruić-Sovulj[1]

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[2] *Interfaculty Institute of Cell Biology, University of Tuebingen, Germany*

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Mistranslation is an error in protein biosynthesis caused by erroneous incorporation of the amino acids. Aminoacyl-tRNA synthetases (aaRS) play the crucial role in this process, as they supply the ribosome with its substrates, aminoacylated tRNAs (aa-tRNA). If there is a mistake in aa-tRNA synthesis, the erroneous amino acid will be misincorporated into the nascent polypeptide chains, since there are no additional surveillance mechanisms of synthesized aa-tRNAs.

Here, we explore the effects of isoleucine mistranslation on *Escherichia coli* proteome induced by editing-defective isoleucyl-tRNA synthetase (IleRS). Two types of mistranslation were investigated: substitution of isoleucine with valine, the standard proteinogenic amino acid, or with norvaline (Nva), a non-proteinogenic amino acid which accumulates in the cell under anaerobic conditions. We have found that *E. coli* can tolerate unexpectedly high levels of mistranslation, up to 22 % Ile-to-Val or 18 % Ile-to-Nva substitutions. Analysis of differentially expressed proteins revealed upregulation of chaperones GroES/EL, DnaK/DnaJ/GrpE, HtpG and disaggregase ClpB. Overall, response to Val and Nva mistranslation was similar, with Nva being more toxic. We performed a detailed analysis od DnaK clients. Curiously, we found very few changes in DnaK interactome, suggesting that DnaK upregulation counteracts increased amount of proteins inherently prone to misfolding, rather than the increased number of different misfolded proteins. The number of DnaK interactors did increase when the bacteria were subjected to mistranslation prior to heat-shock, indicating that mistranslation under heat-shock conditions has caused extensive proteome destabilisation. Consequently, prolonged exposure of mistranslated cells to heat-shock was lethal, pointing to reduced ability of bacteria with increased mistranslation level to adapt to unfavourable environmental conditions. Upregulation of ClpB, a disaggregase working in tandem with DnaK chaperone prompted us to analyse the insoluble proteome fraction, i. e. protein aggregation caused by mistranslation. In IleRS editing-deficient strain mistranslation caused nonspecific and proteome-wide accumulation of protein aggregates, with increased level of mistranslation compared to the soluble proteome fraction (up to 26 % Ile to Val and 20 % Ile to Nva). Disruption of *clpB* gene promoted aggregation, with mistranslation in aggregates reaching up to 29 % and 28 % for Val and Nva misincorporation, respectively. Thus, the main consequence of protein mistranslation is deposition of mistranslated proteins to insoluble aggregates, normally counteracted by ClpB disaggregase.

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L3 Data Dependent and Data Independent *De novo* Sequencing

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One of the most challenging tasks of proteomics is peptide or protein *de novo* sequencing. The method can acquire peptide or protein sequences without a protein database assistance. This approach overcomes the limitations of database-dependent methods like peptide or Protein Mass Fingerprinting (PMF). However, the drawbacks of this method are first, *de novo* sequencing algorithm will sometimes not be able to deliver a complete sequence, or will have uncertainty in a portion of the derived sequence with a consequence that derived sequence amino acid string will not be the only algorithm solution and second, it will never achieve better peptide or protein identification score results relative to database-dependent search methods. So, why do we need *de novo* sequencing? Genome and consequently protein databases are a small fragment, almost negligible of the tree of (sequenced) life, *i.e.*, 3,278 unique animals have had their nuclear genome sequenced (0.2% of all animal species), mutations in proteins are almost invisible to the database search, discrepancies between DNA, mRNA and protein code are frequent, exact antibody amino acid sequence can be deduced only through protein *de novo* sequencing etc. In addition, *de novo* sequencing algorithm could search for posttranslational modifications or for identifications of mutations with the assistance of homology-based software. Peptide or protein mass spectra suitable for *de novo* sequencing can be produced in two ways, data dependent or data independent acquisition (DDA or DIA). In DDA only selected peptides are further fragmented during the second stage of tandem MS, and in DIA all peptides are fragmented and analysed during the second stage of tandem MS. In opposite to a limited number of selected precursors in DDA, DIA is faster, easy-to-use and –optimize, and it will dissociate and analyse all ions. However, DDA is selective, more informative and sensitive in terms of a single ion. Differences and similarities between DDA and DIA *de novo* sequencing will be explained at peptide, protein and species level.

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L4 Protein Conformation Dynamics Probed by Mass Spectrometry. Deuterium Labelling Approach

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Proteins are not static structures. They can adopt more than just one conformation. These changes in conformation, collectively called protein dynamics, can include bond vibrations, ring flips, translocations of whole subunits, and changes in tertiary structure. Because so many functional aspects of proteins are tied to structural changes, it is just as important to investigate these structural changes as it is to understand the general features of protein structure. Simple mass analyses provide no detailed information about the protein tertiary structure. To use mass spectrometry to investigate protein structure and dynamics, a labelling method that "captures" the structural information must be implemented before mass analysis. These experiments use mass modification to determine the local structural environment of parts of the protein. When backbone amide hydrogen is exchanged into deuterium, every residue except proline can be labelled. Protection of residues from deuterium labelling reaction is related to protein structure features and can be revealed as the alteration in labelling reaction rate. This technique allows for analysing proteins that are difficult to purify or otherwise hard to obtain in suitable quantities for biophysical characterization by other methods. Protein-ligand interactions, a critical step toward understanding biological function, can be analysed in detail with the H/D eXchange method. The theoretical basis with practical steps in a typical continuous HDX experiment will be explained. In the remaining part of the lecture, I will illustrate how HDX was utilized to characterize the conformation changes found in several proteins studied in our laboratory.

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L5 Mass Spectrometry Application in Pharmaceutical Research of Biomolecules

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Mass spectrometry (MS) is widely used in pharmaceutical research of biomolecules such as peptide and protein therapeutics. MS-based techniques are utilized in peptide and protein identification, quantification and characterization but are also used to provide data on the structural integrity and post-translational modifications. Therefore, MS is often used as analytic tool throughout all stages of biopharmaceuticals development: from discovery and design of new biological entities up to quality control of final products and biosimilar. Additionally, due to high selectivity, low sample volume requirements, sensitivity and speed, MS-based methods have begun to rival traditional immunoassays in pharmacokinetic studies of biopharmaceutical products.

The objective of this presentation will be to provide a brief overview of MS-based methods developed in our laboratory to assess the structure and purity of chosen biopharmaceuticals. The focus will be laid on protein N- and C- terminal sequencing, N-glycosylation site occupancy and impurity characterization such as truncated forms and quantification of site-specific oxidation of methionine and tryptophan residues. Moreover, in this presentation pharmacokinetics study of biopharmaceutical product performed by multiple reaction monitoring (MRM) MS-based targeted protein quantification will be presented.

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L6 Mass Spectrometry in Development of Active Pharmaceutical Ingredients

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Development of active pharmaceutical ingredients has become challenging due to a new regulatory demands and a concern for a patient's health. USA and European regulatory guidelines are requesting that impurity profiles of a drug substance should be investigated and the control of all potential mutagenic impurities should be controlled according to ICH M7 (Assessment and control of DNA reactive (mutagenic) impurities in pharmaceuticals to limit potential carcinogenic risk). Knowledge on impurities must be presented in a drug master files submitted to regulatory agencies with the special emphasis of a fate of impurities in starting materials and intermediates as well as identification of degradation impurities and degradation pathways. Appropriate control strategies are critical to ensure process performance and product quality. In order to understand and fulfil all those requests and to ensure the patients' health and safety mass spectrometry became a powerful tool in development of active pharmaceutical ingredients. Improvements in technology of mass spectrometry over the last decade enabled usage of MS detector in everyday work both qualitative and quantitative analysis during the development of pharmaceutical products. Now day's mass spectrometry (MS) plays a key role in advancing the production of active pharmaceutical ingredients, which is enabling quality product for patients. Over last few years, the focus of pharma industry is on the nitrosamine impurities. Guidelines from FDA and EMA have identified potential nitrosamine impurities that could theoretically be present in drug products. As part of the guidelines, it is crucial to have established methods to screen active pharmaceutical ingredients on potential nitrosamine impurities. Usage of hyphenated techniques such as GC-MS/MS and LC/MS/MS showed their full potential in determination of nitrosamine impurities in active pharmaceutical ingredients.

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L7 Deciphering the Complex Molecular Interplay in Health and Disease by Integrative Multi-omics

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The development of high-throughput omics methods and introduction of the systems biology approach revolutionized biomedical and nutritional research. The objective of any omics study is to characterize biomolecules and identify their networks and pathways which may play important roles in biological processes occurring in cell, tissue and/or organism. However, the biological interpretation of a single-layer omics data provides limited insights due to the complex biochemical regulation at multiple levels. The advances in technological innovations, sequencing technologies, increased computational power and bioinformatic tools, as well as introduction of publicly available databases enabled the emergence of integrated omics studies. Integrated multi-omics refers to comprehensive collection of large-scale datasets at multiple levels of information, combining genomics, transcriptomics, proteomics, metabolomics, lipidomics, glycomics, etc. These integrative approaches can provide detailed insights into the complex molecular mechanisms in health and disease.

Mass spectrometry (MS) is one of the key analytical techniques on which the omics approaches are based, providing the detection and quantification of thousands of proteins and metabolites in a global or targeted manner. Herein, the application of integrative MS-based proteomics and metabolomics in pathological conditions and animal nutrition will be presented.

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L8 Tandem Mass Spectrometry in Combination With Ion Mobility for Characterization of Cardiolipin Molecular Diversity

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Cardiolipin (CL) is a unique class of membrane phospholipid distributed almost exclusively in the mitochondrial inner membrane of eukaryotic cells. Extensive remodeling of CL occurs via a highly-conserved deacylation – transacylation process, and deficiency of the CL transacylase leads to Barth syndrome. Due to their unique structure, comprehensive characterization of CL molecular diversity is analytically challenging. Coupling traveling wave ion mobility and high-resolution tandem mass spectrometry (IM-MS/MS) enables a simple and rapid multidimensional characterization CL molecular diversity. Using IM-MS/MS we confirm that *Saccharomyces cerevisiae* mutants completely devoid of CL remodeling exhibit a shift towards CL species with more saturated and shorter acyl chains. We further demonstrate that CL acyl chain composition is strongly influenced by the presence of exogenously supplemented fatty acids. IM-MS/MS provides a powerful tool for the structural identification of CLs in complex biological samples.

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L9 High-throughput LC-MS Based Analysis of Plasma Protein Glycosylation

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High-throughput glycomics gained a momentum in the last 15 years with the development of the methods adapted and optimized for the 96-well format. Analytical processes were optimized on human plasma as major sample for the analysis primarily having glycans as new potential biomarkers in the focus of the research. Since then, number of different methods were developed and optimized on different analytical platforms covering liquid chromatography with fluorescent detection, capillary electrophoresis with fluorescent detection or hyphenated to mass spectrometry, mass spectrometry based analytical approaches both with and without LC separation and lectin arrays. In the last couple of years in our lab we introduced number of analytical techniques covering several plasma proteins, both human and rodent. Immunoglobulin G as the major analyte isolated from human, mouse, and rat plasma, analysed on released glycan level and on the site-specific N-glycosylation level using UHPLC-FLR-MS and nanoLC-MS, respectively. Just recently, analytical approaches for two additional human plasma proteins, enriched to a higher level but not completely purified, were introduced for the analysis of glycopeptides. Methods for the Alpha-1-acid glycoprotein (AGP) and Complement component 3 (C3) site-specific N-glycosylation LC-MS analysis were proven on several large cohorts as quantitative analytical methods capable of detecting biologically meaningful data. Glycosylation is one of the essential factors for the functioning of every organism and the development of effective analytical methods for qualitative and quantitative detection of glycosylation and their changes under various physiological and pathological conditions is a prerequisite for the successful research and introduction of glycans as clinically relevant biomarkers.

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L10 Phytoplankton Toxins in the Adriatic Sea

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Poisoning by phytoplankton toxins is widespread; it is estimated that about 60,000 people are poisoned each year. Phytoplankton toxins have different mechanisms of action and cause different symptoms, ranging from mild ones such as skin irritation to severe gastrointestinal and neurological symptoms and paralysis, which can lead to death in some cases. Poisonings occur during blooms of toxic phytoplankton species, usually dinoflagellates. The toxins accumulate in filter-feeding marine organisms such as bivalves, and their wild or cultured populations are used as food. Toxic blooms and thus human intoxication are becoming more frequent due to eutrophication of the oceans, the rise in global temperature and the spreading of toxic species through the ships ballast water. The most widespread and economically important mollusk species is the blue mussel, with a monitoring program established worldwide to follow the breeding populations of this mollusk species. The occurrence of the most important classes of toxins is monitored, most commonly using liquid chromatography coupled with mass spectrometry, usually with QQQ mass spectrometers. This technique is suitable for routine analysis and quantification of known toxin species, however, for detection of analogues or for detecting of the occurrence of new toxins in an area, it is better to use high-resolution mass spectrometry, i.e., QTOF or Orbitrap instruments. Several groups of structurally different toxins are known, with a large number of analogues in each group, which makes detection much more difficult, especially with low-resolution instruments. In addition, analytical standards are available for only about 10% of the known toxins. In the Adriatic Sea, several types of toxins have been detected so far in shellfish and other organisms. Cases of paralytic shellfish poisoning (PSP) have been reported after consumption of ascids contaminated with PSP toxins, which contained a complex profile of toxins, including the extremely potent carbamates saxitoxin and gonyautoxins (GTX 2 and 3) and their analogues. Toxins of the gymnodimines and spirolides groups, okadaic acid and its derivative DTX1, and hydrophilic PSP and ASP (Amnesic shellfish poisoning) toxins were also found in the mussel samples from the Adriatic Sea. In collaboration with the Institute of Oceanography and Fisheries, a project is being launched at Faculty of Science Split to detect analogues of phytoplankton toxins not included in routine analyses. The high-resolution QTOF mass spectrometer Sciex 6600+ will be used to detect the occurrence of new toxin analogues, and the SWATH technique will be used to create a digital record of the compounds present in extracts from biological material.

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L11 Molecular Mechanisms of HipA-mediated Bacterial Persistence in *E. coli* Investigated by Mass Spectrometry-based Phosphoproteomics

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Bacterial persistence, the ability of bacteria to survive antibiotic treatment by entering a physiologically dormant state, is an acute biomedical problem. Protein Ser/Thr kinase HipA, the first toxin connected to bacterial persistence, phosphorylates glutamate--tRNA ligase GltX and triggers a chain of events that lead to a halt in translation, accumulation of ppGpp and induction of persistence. Intriguingly, its variant HipA7 is able to induce significantly higher levels of persistence despite being less efficient in inhibiting cell growth. We postulated that this phenotypic difference may be driven by diverse substrate pools of the two kinase variants. To address this, we ectopically expressed *hipA* and *hipA7* in *E. coli* and monitored their in vivo substrates during persistence and resuscitation using a stable isotope labelling by amino acids in cell culture (SILAC)-based quantitative phosphoproteomic workflow in combination with high-resolution mass spectrometry. Our assays confirmed that GltX was indeed the main substrate of both kinase variants and likely the primary determinant of persistence. Unlike HipA7, HipA phosphorylated several additional substrates involved in translation, transcription, and replication, such as ribosomal protein L11 (RplK) and the negative modulator of replication initiation SeqA. Conversely, HipA7 had no additional substrates under tested conditions and showed a similar substrate pool only when expressed at significantly higher levels. The kinase variants also differed in autophosphorylation level, which was significantly lower in HipA7. When expressed from the chromosome, HipA showed no activity due to inhibition by antitoxin HipB, whereas HipA7 phosphorylated GltX and phage shock protein PspA, providing a direct evidence that HipA7 targets GltX *in vivo*. Initial testing did not reveal a connection between HipA-induced phosphorylation of RplK and persistence or growth inhibition, suggesting that other substrates unique to HipA were likely responsible for growth inhibition. Taken together, this study shows that HipA and HipA7 differ substantially in their kinase activities and substrate pools, which may contribute to their distinct phenotypes. Moreover, these results contribute to understanding molecular mechanisms of HipA and HipA7 and the phosphoproteome data obtained here yielded a comprehensive collection of phosphorylation events in *E. coli* that can serve as a valuable resource for further studies of phosphoregulation in bacteria.

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