# Joint Conference of Polish Mass Spectrometry Society and German Mass Spectrometry Society

# Abstracts

## 4-7 March 2012, Poznań, Poland

### Contents



**Scientific Publishers OWN • Poznań 2012**

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**Dear Colleagues,**

On behalf of German Mass Spectrometry Society, Polish Mass Spectrometry Society and Institute of Bioorganic Chemistry Polish Academy of Sciences host and main organizer of Joint Conference of the Societies we would like to welcome all participants of the Conference in our city. Scientists and practitioners from various countries, interested in applications of mass spectrometry in different fields of research and other kinds of activities will meet at the Congress Center of Poznań International Fair.

We are especially satisfied that high number of young researchers (graduate students and postdoctoral fellows) is interested in applications of mass spectrometry. We do hope that the exchange of experiences between German and Polish mass spectrometrist will be fruitful and interesting for both sides.

We wish all participants and sponsors interesting scientific discussions.

Enjoy your stay in Poznań.

Conference Organizing Committee

**Conference is under Honorable Auspices of:**

MARSHAL
OF THE WIELKOPOLSKA REGION
MAREK WOŹNIAK

**and Mayor of Poznań Mr Ryszard Grobelny**

POZnań*
*Eastern energy, Western style

**The Conference is organized with financial support from Ministry of Science and Higher Education, Republic of Poland and European Molecular Biology Organization**

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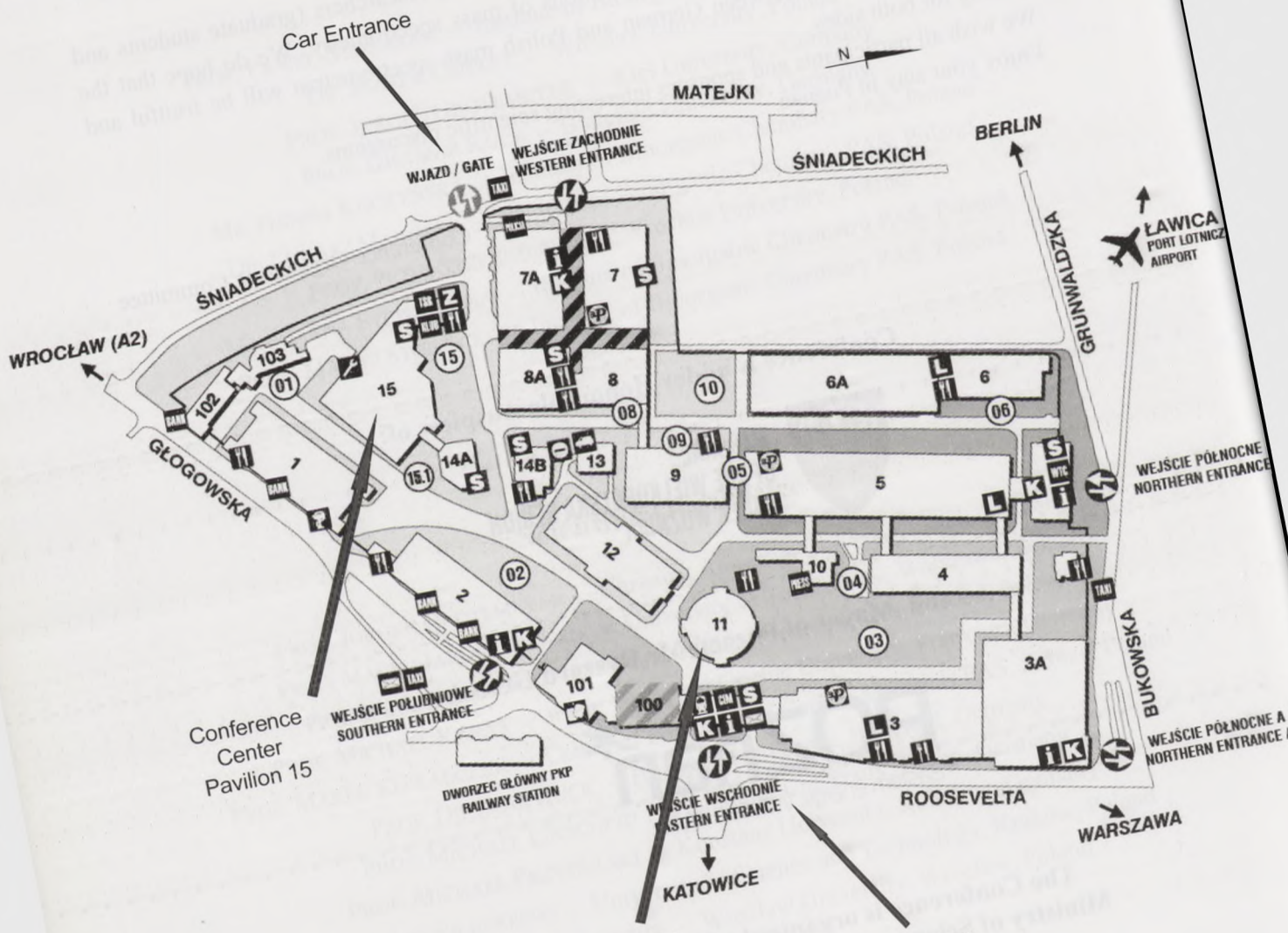
# Map of Poznań International Fair area

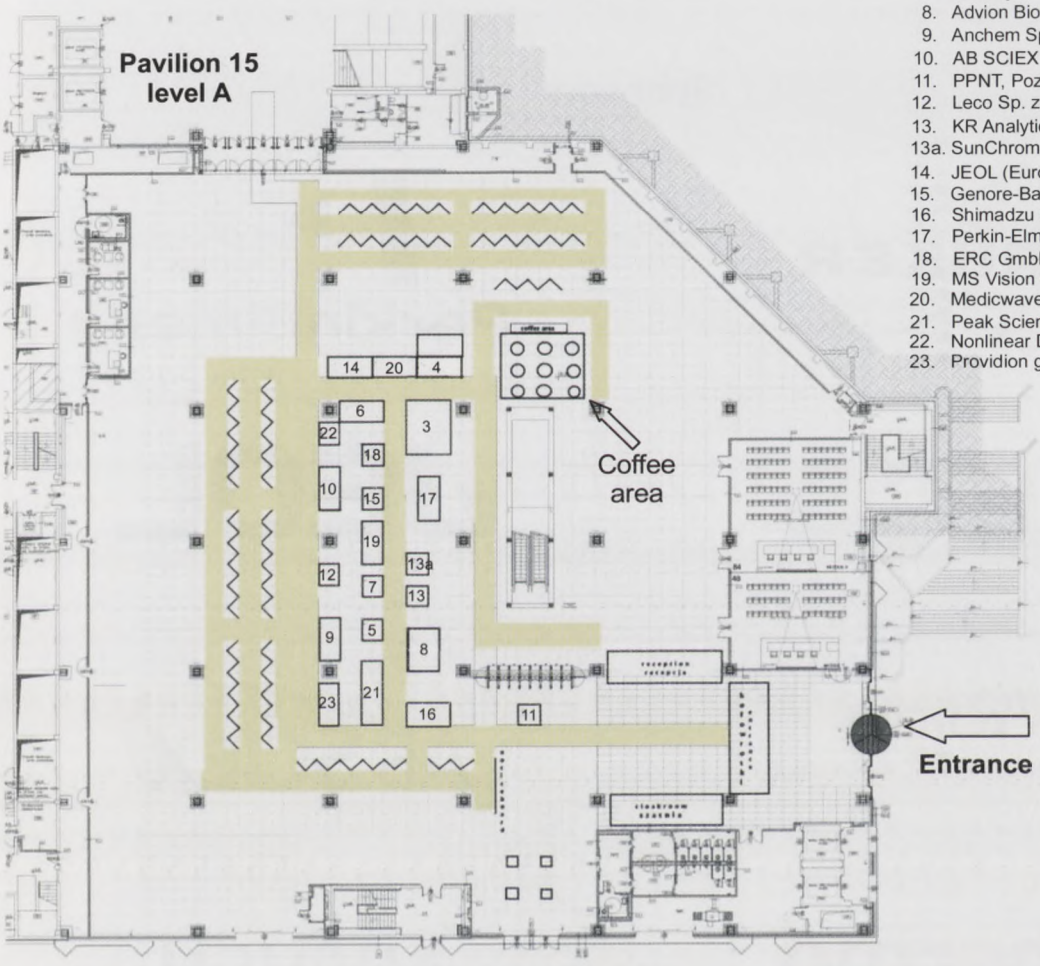


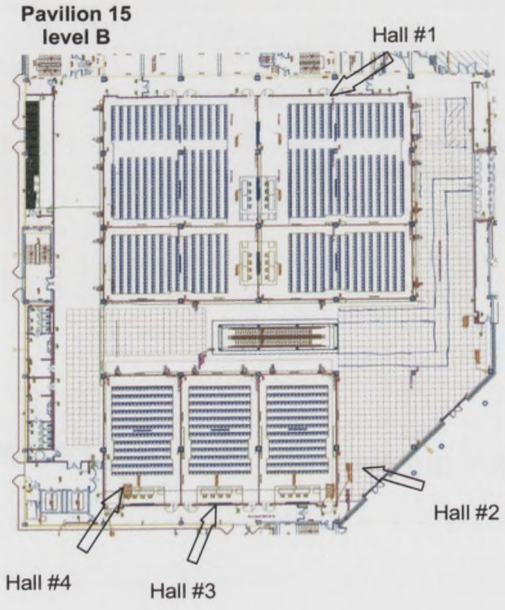
# Plan of Pavilion 15: level A and B

3. Agilent GmbH, Perlan Technologies Niemcy
4. Waters Gmbh, Austria
5. SMS Service für Massenspektrometrie GmbH, Niemcy
6. Bruker Daltonik GmbH, Niemcy
7. MasCom Technologies GmbH, Niemcy
8. Advion BioSciences Ltd., UK
9. Anchem Sp. J., Thermo
10. AB SCIEX UK Ltd
11. PPNT, Poznań
12. Leco Sp. z o.o., Polska
13. KR Analytical Ltd, UK

13a. SunChrom GmbH, Niemcy

14. JEOL (Europe) S.A., Polska
15. Genore-Baltica, Polska
16. Shimadzu GmbH, Shimpol
17. Perkin-Elmer, Polska
18. ERC GmbH, Niemcy
19. MS Vision GmbH, Niemcy
20. Medicwave 6 m, Szwecja
21. Peak Scientific Instruments Ltd, UK
22. Nonlinear Dynamics Ltd, UK
23. Providion group



**Joint Conference**
**of Polish Mass Spectrometry Society**
**and German Mass Spectrometry Society**

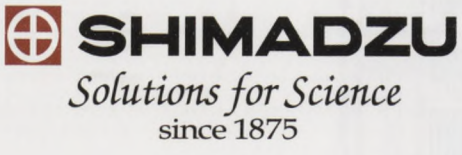
## Sponsors














# PROGRAM

## Joint Conference of Polish Mass Spectrometry Society and German Mass Spectrometry Society

**4-7 March 2012, Poznań, Poland**

## SUNDAY, 4 MARCH 2012

| | |
|---|---|
| 13:00-16:30<br>Lecture Hall 3 | **Workshop 3**<br>*Desorption Ionization – An Introduction to FD and MALDI* (Organizers J.H. Gross; Heidelberg; H.B. Linden Heidelberg, M. Karas Frankfurt) |
| 13:30-16:30<br>Lecture Hall 1 | **Workshop 1**<br>*Fundamentals of Mass Spectrometry* (Organizers: D. Kuck, Bielefeld; W. Danikiewicz, Warszawa) |
| 13:30-16:30<br>Lecture Hall 2 | **Workshop 2**<br>*Fourier-Transform Mass Spectrometry and Accurate Mass* (Organizers: W. Schrader, Mülheim; P. Stefanowicz, Wrocław) |
| 13:30-16:30<br>Lecture Hall 4 | **Workshop 4**<br>*Mass Spectrometry Imaging* (Organizers: B. Spengler, A. Roempp, Giessen) |
| 17:00-17:15<br>Lecture Hall 1 | **Opening Ceremony**<br>Session Chair: M. STOBIECKI<br>Session Chair: J. GROTEMEYER |
| 17:15-18:15<br>Lecture Hall 1 | **Wolfgang Paul Lecture**<br>Session Chair: J. GROTEMEYER and M. STOBIECKI<br>MICHAEL L. GROSS, Professor, Department of Chemistry, Professor of Immunology and Internal Medicine, Washington University School of Medicine<br>*Can Mass Spectrometry Play a Role in Structural Biology?* |
| 18:15-18:45<br>Lecture Hall 1 | **Bestowal of the Honoary Membership**<br>Session Chair: J. GROTEMEYER<br>*Bestowal of the Honorary Membership to Prof. Dr. Franz Hillenkamp, Münster* |
| 20:00-23:30<br>Pavillion 15 | **Conference Mixer** |

# MONDAY, 5 MARCH 2012

8:30-9:20
Lecture Hall 1

**Plenary Lecture 1**

Session Chair: W. DANIKIEWICZ

DETLEF SCHRÖDER, Institute of Organic Chemistry and Biochemistry Prague, Czech Republic
*Mass spectrometry going towards the condensed phase*

9:20-10:10
Lecture Hall 1

**Wolfgang Paul Award**

Session Chair: J.H. GROSS

**Introduction:** J.H. GROSS, President of the Jury

**Lectures from the Awardees:** H. WINKLER, A.K. SCHALLEY, T. JASKOLLA, A.K. KARAS

10:10-10:30

**Coffee Break**

10:30-12:30
Lecture Hall 2

**Session 1: Fundamentals of Mass Spectrometry and Instrumentation**

Session Chair: D. KUCK

*HPLC/FT-ICR MS as an approach for detailed analysis of a crude oil sample.* S. Lababidi, W. Schrader

*Fragmentation of Xanthene Dyes by Laser Activation and Collision-Induced Dissociation on a High-Resolution Fourier Transform Ion Cyclotron Resonance Mass Spectrometer.* J. Peters, J. Grotemeyer

*Ion Mobility Analysis of Glycans: A Calibration Protocol for Travelling Wave Instruments.* Kevin Pagel, David J. Harvey

*Improving Bioanalytical Selectivity by Differential Mobility Spectrometry – Mass Spectrometry.* C. Lenz, S. Kreppenhofer, M. Glueckmann, T. Bienkowski, Y. LeBlanc

*Rapid switching (80 Hz) between hard electron- and soft photo-ionization for gas chromatography/ time-off-flight mass spectrometry: Technical realization and applications.* R. Zimmermann, M. Eschner, T. Gröger, M. Gonin

*ESI-mass spectrometry for structural characterization of selected aliphatic homo- and copolyester.* G. Adamus, M. Kowalczuk

10:30-12:30
Lecture Hall 1

**Session 2: Proteomics 1**

Session Chair: A. SINZ

*Quantitative Label-free Mass Spectrometry for Unbiased Profiling of the Human Monocyte Secretome.* M. Groess, H. Luksch, A. Rösen-Wolff, A. Shevchenko, M. Gentzel

*A Simple Way to Perform Quantitative Proteomics and Lipidomics Measurements from Small Tissues Samples.* M. Nakade, S. Hebbar, D. Schwudke

*Online-Bioaffinity-mass spectrometry: Analytical development and application for structural and affinity characterisation of immune- and peptide-protein complexes.* S. Slamnoiu, C. Vlad, A. Moise, M. Stumbaum, M. Przybylski

*Improved Precision of Quantitative 'Bottom-up' Proteomics with Stable Isotope Labeled Proteins as Internal Standards.* A. Konopka, M.E. Boehm, M. Rohmer, M. Karas, W.D. Lehmann

*Benchmarking Label-free Quantification using the Meta-Proteome Quantification Standards (MPQS).* J. Kuharev, P. Brownridge, R. Engelke, W. Thompson, M. Kipping, A. Moseley, J. Dengjel, R. Beynon, H. Schild, O. Jahn, S. Tenzer

*The importance of total system peak capacity for complex proteome analysis.* R. Tonge

10:30-12:30
Lecture Hall 3

**Session 3: Environmental and Pharmaceutical Applications**

Session Chair: P. STEFANOWICZ

*SPME-MS as a tool for food quality and authenticity assessment.* H.H. Jeleń, A. Ziółkowska, M. Majcher

*Mass Spectrometry in Sports Drug Testing: Investigating Diagnostic Product Ions of Testosterone by ESI-CID HRMS and IRMPD spectroscopy.* M. Thevis, S. Beuck, S. Höppner, M. Schäfer, J. Held, J. Oomens, A. Thomas, W. Schänzer

10:30-12:30
Lecture Hall 3

*Forensic Screening of Isobaric Compounds in Crude Samples by Ultrahigh-Resolution UHPLC-QTOF Technology.* A. Ingendoh, M. Macht, P. Decker, C. Baessmann, A. Pelander

*Hyphenated techniques for determination of volatile organic compounds in breath.* T. Ligor, E. Trawińska, K. Cieslinski, B. Buszewski

*Solid phase microextraction coatings and LC-MSn for determination of drugs and their metabolites in biological samples.* M. Szultka, B. Buszewski

*Negative ion tandem mass spectrometry of prenylated fungal metabolites and their derivatives.* R. Heinke, A. Norbert, W. Ludger, S. Jürgen

12:30-13:30
Lecture Hall 1

**Lunch Seminar Bruker**

12:30-13:30
Lecture Hall 2

**Lunch Seminar Leco**

13:30-15:00

**Poster Session 1 a, b**

Instrumentation, Fundamentals of MS, Inorganic and Elemental MS, Imaging MS, Proteomics

**Authors of odd numbered posters should be present**

15:00-15:30

**Coffee Break**

15:30-16:20
Lecture Hall 1

**Plenary Lecture 2**

Session Chair: M. PRZYBYLSKI

THOMAS J.D. JØRGENSEN, University of Southern Denmark, Odense, Denmark
*Insights into Protein Structural Dynamics with Hydrogen/Deuterium Exchange Monitored by Mass Spectrometry.*

16:20-18:00
Lecture Hall 2

**Session 4: General Mass Spectrometry**

Session Chair: W. DANIKIEWICZ

*Circular Dichroism Laser Mass Spectrometry: Report of Recent Progress and Outlook to Future Projects.* U. Boesl, C. Logé, K. Titze

*Identification of psychoactive substances by LC-MS/MS-TOF after closing down 1378 smart shops in Poland.* A. Błażewicz, P. Baran, Z. Fijałek

*9-aminoacridine: a powerful MALDI matrix – but with pecularities.* K. Mueller, U. Jakop, K. Bresler, A. Nimptsch, B. Fuchs, J. Schiller

*Detailed mechanistic studies of a multifunctional catalyst in a one-pot reaction by Electrospray mass spectrometry.* M. Wasim Alachraf, C.E Müller, P.R Schreiner, W. Schrader

16:20-18:00
Lecture Hall 2

**Session 5: Proteomics 2**

Session Chair: M. KARAS

*Characterization of surgical aerosols – Towards mass spectrometry-guided surgery.* A. Fendt, K.P. Hinz, K.C. Schäfer, Z. Takats, B. Spengler

*Chemical Phosphorylation of Histidine Residues in Proteins and Peptides Assists in the Analysis of Acid-labile Phosphorylation Sites.* S. König, U.M. Hohenester, K. Ludwig

*Systematic study of copper and platinum complexes of peptide-heterocycle conjugates.* A. Kluczyk, K. Paszek, J. Hipner, P. Szczepaniak, D. Bąk, M. Ratajska, P. Stefanowicz, Z. Szewczuk

*Affinity – Mass Spectrometry-based Cord Blood Serum Profiling Leads to an IUGR Proteome Signature.* C. Koy, M. Wölter, C. Röwer, T. Reimer, W. Rath, U. Pecks, M.O. Glocker

*High-resolution LC-MS Analysis Reveals Novel Cellular Substrates of Clostridium difficile Toxins.* J. Zeiser, R. Gerhard, I. Just, A. Pich

18:00-19:30

**PS 2: Poster Session 1 a, b**

Instrumentation, Fundamentals of MS, Inorganic and Elemental MS, Imaging MS, Proteomics

For abstracts see poster session PS 1: Posters from 1:1 to 1:120

**Authors of even numbered posters should be present**

# TUESDAY, 6 MARCH 2012

8:30-9:20
Lecture Hall 1

**Plenary Lecture 3**

Session Chair: MICHAEL W. LINSCHEID

ROBERT B. CODY, JEOL USA, Inc.
*DART Mass Spectrometry: Recent Developments and Applications*

9:20-10:10
Lecture Hall 1

**Mattauch-Herzog Award**

Session Chair: M.W. LINSCHEID

**Introduction:** M.W. LINSCHEID, President of the Jury, Lectures from the awardee: A. Römpp, *Comprehensive and Specific Biological Information at Cellular Resolution by MS Imaging*

10:10-10:30

**Coffee Break 1**

10:30-12:30
Lecture Hall 1

**Session 6: Imaging Mass Spectrometry**

Session Chair: BERNHARD SPENGLER

10:30-12:30
Lecture Hall 1

*Analysis of colorectal adenocarcinoma samples with Desorption Electrospray Ionization and multivariate statistical methods*. S. Gerbig, O. Golf, J. Balog, J. Denes, Z. Baranyai, A. Zarand, E. Raso, J. Timar, Z. Takats

*Software for 3D MALDI imaging data: visualization and 3D spatial segmentation.* D. Trede, S. Schiffler, M. Becker, S. Wirtz, J. Strehlow, J.H. Kobarg, K. Steinhorst, M. Aichler, S. Heldmann, A. Walch, H. Thiele, P. Maass, T. Alexandrov

*Spatial Proteomics: A new LC-MS/MS Tissue Imaging Workflow providing Protein Distributions and their Identities in Tissue.* M. Becker, M. Schürenberg, C. Lübbert, R. Paape, D. Suckau

*Ion yields in UV-MALDI mass spectrometry as a function of laser wavelength and physico-chemical properties of classical and halogen-substituted matrices*. J. Soltwisch, T.W. Jaskolla, F. Hillenkamp, M. Karas, K. Dreisewerd

*Water Ice as a Matrix for the Analysis of Glycosaminoglycans in Infrared Matrix-assisted Laser Desorption/Ionization Mass Spectrometry*. L. Witt, A. Pirkl, K. Dreisewerd, M. Mormann

*DART-TOF-MS: A time-saver in analytical chemistry.* C.W. Klampfl

10:30-12:30
Lecture Hall 2

**Session 7: Inorganic and Elemental Mass Spectrometry**

Session Chair: M. JAROSZ

*ToF-SIMS studies of the surface properties of Co/SiO2 catalyst for Fischer-Tropsch synthesis*. J. Grams, A. Ura

*Sources of inaccuracy of isotopic ratio measurements by MC ICPMS: Comparison of wet and dry plasma conditions.* R. Anczkiewicz

*Determination of Total Mercury Content in Wood Materials Using ICP-MS in Comparison to ICP-OES with Mercury Cold Vapor Technique.* H. Fleischer, K. Thurow

*In-vitro investigation of possible intracellular transformations of ruthenium-based anticancer drug–protein adducts by CE-ICP-MS and ESI-MS.* K. Pawlak, M. Matczuk, A. Siwek, L. Xifeng, S. Aleksenko, A. Timerbaev, M. Jarosz

*Binding abilities of copper to phospholipides*. J. Jaklová Dytrtová, M. Jakl, D. Schröder

*A multi-reflection time-of-flight mass separator for high-resolution beam purification at ISOLTRAP.* R.N. Wolf, for the ISOLTRAP collaboration L. Schweikhard

10:30-12:30
Lecture Hall 3

**Session 8: Metabolomics and Lipidomics**

Session Chair: W.D. LEHMANN

*Mass Spectrometric Analysis of Spatial Metabolite Distribution Pattern during Barley Grain Development.* M. Peukert, A. Matros, H.P. Mock

*Expanding role of in vitro simulation of cytochrome P450 metabolism for drugs of abuse.* P. Jakub Mielczarek, H. Raoof, J. Silberring

*Software for Pathway Informed Metabolomics.* J. Thiemann

*Development of a spectral interconversion algorithm for desorption ionization techniques.* O. Golf, M. Walther-Schmidt, Z. Takáts

*Quantification of Phospholipids in Biological Samples Using μLC-FTICR-MS.* N. Zehethofer, B. Lindner

*Development and validation of a LC-MS/MS method for routine determination of aldosterone and its application in pediatric endocrinology.* A.E. Kulle, C. Wewetzer, P.M. Holterhus, F.G. Riepe

12:30-13:30
Lecture Hall 1

**Lunch Seminar Agilent**

12:30-13:30
Lecture Hall 2

**Lunch Seminar Waters**

13:30-15:00

**Poster Session 2 a, b**

Proteomics, Lipidomics, Metabolomics, Environmental and Pharmaceutical Applications

**Authors of odd numbered posters should be present**

15:00-15:30

**Coffee Break**

15:30-16:15
Lecture Hall 1

**Plenary Lecture 4**

Session Chair: M. STOBIECKI

A. BYRT, former ambassador of Poland in Germany

*Biochemistry of Polish – German relations*

16:15-16:45
Lecture Hall 1

**Agilent Award Lecture**

Session Chair: W. SCHRADER

**Introduction:** W. SCHRADER, President of the Jury

**Lecture of the awardee:** J. SPROß, Martin-Luther-University Halle-Wittenberg

16:45-17:15
Lecture Hall 1

**Special Main Lectures**

Session Chair: ZBIGNIEW SZEWCZUK

M. DADLEZ, Institute of Biochemistry and Biophysics, Warszawa

*RAGE receptor structure and oligomerisation studied by hydrogen-deuterium exchange monitored by mass spectrometry*

17:15-18:00
Lecture Hall 1

**DGMS Business Meeting**

Session Chair: J. GROTEMEYER

17:15-18:00
Lecture Hall 2

**PTSM Business Meeting**

Session Chair: P.R. STEFANOWICZ

18:00-19:30

**Poster Session 2 a, b**

Proteomics, Lipidomics, Metabolomics, Environmental and Pharmaceutical Applications

**Authors of even numbered posters should be present**

20:00-23:30
Pavillion 15

**Conference Dinner**

# WEDNESDAY, 7 MARCH 2012

8:30-9:20
Lecture Hall 1

**Plenary Lecture 5**

Session Chair: **J.H. GROSS**

JEAN CLAUDE TABET, Université Pierre and Marie Curie, Paris, France
*Conformational influence on dissociations of multicharged biological complex systems according to ion activation mode.*

9:20-10:10
Lecture Hall 1

**Waters Award**

Session Chair: WOLF D. LEHMANN

**Introduction:** WOLF D. LEHMANN, Lectures from the awardees: Prof. Dr. F. Lottspeich, Prof. Dr. H.E. Meyer, Dr. R. Kellner

10:30-12:30
Lecture Hall 1

**Session 9: Proteomics 3**

Session Chair: M. DADLEZ

*Strategies of protease substrate identification by mass spectrometry.* V. Frochaux, M. Trusch, D. Hildebrand, M.W. Linscheid, H. Schlüter

*Mass spectrometry in studies of protein denaturation.* E. Jankowska, M. Sosnowska, P. Karpowicz, K. Radziszewska, P. Stefanowicz, A. Szymańska

*Acid-catalyzed 18O-labeling – a new tool for peptide and protein quantitation.* A. Schlosser, E. Haaf

*Specific detection of arginine-derived advanced glycation end-products and their localization in proteins.* R. Schmidt, D. Böhme, D. Singer, R. Hoffmann, A. Frolov

*Determination of Catalase Expression by a Targeted UHPLC-MS/MS Based Proteome Analysis.* N. Zänglein, M. Pischetsrieder

*How to handle huge amount of mass spectrometric data? A new strategy with the Achroma software.* R.K. Scheerle, C. Kaufmann, M. Krappmann, J. Grassmann, T. Letzel

10:10-10:30

**Coffee Break**

10:30-12:30
Lecture Hall 2

**Session 10: Environmental and Pharmaceutical Applications**

Session Chair: P. KACHLICKI

*A milestone in pharmaceutical trace level analysis in water – LC-MS/MS and LC-QTOF.* A. Kot-Wasik, A. Jakimska, A. Wasik, J. Namieśnik

*Nontarget screening with LC-Q-TOF-MS to characterize input and fate of contaminants in surface waters.* C. Zwiener, M. Zedda

*Analysis of pharmaceuticals and personal care products in river water samples by UHPLC-TOF.* S. Daugherty, S. Redd

*Protein profiling using LC/MS method for identification of genetically modified foods.* A.U. Koc-Latoszek, J.F. Garcia-Reyes, A. Molina Diaz, M. Trojanowicz

*Heck coupling in the gas phase: Examination of the reaction mechanism by ion/molecule reactions and mass spectrometry.* L. Fiebig, H-G. Schmalz, M. Schäfer

*Investigation of Figopitant and its Metabolites in Rat Tissue by combining Whole Body Autoradiography with Liquid-Extraction-Surface-Analysis Mass Spectrometry (LESA-MS).* S. Schadt, F. Porbeck, R. Almeida, S. Kallbach, J. Sandel

10:30-12:30
Lecture Hall 3

**Session 11: Fundamentals of Mass Spectrometry and Instrumentation**

Session Chair: U. BOESL

*Formation and fragmentation of sodiated amino acid cluster ions in the gas phase.* M. von Gernler, R. Kirschbaum, T. Drewello

*Increasing Importance of Electrochemistry/Mass Spectrometry in Life Science.* A. Kraj, H.J. Brouwer, N. Reinhoud, P. Mielczarek, J. Silberring, J-P. Chervet

*High-resolution time-of-flight mass spectrometry using folded flight path technology for profiling of steroids and steroid metabolites in urine.* J. Wendt, K. Siek, J.S. Patrick, E. Guice

*Maldi-ToF Analysis in the study of luminescent polyethers and poluthioeters with the crabazolyl unit in the substituent for oled applications.* A.S. Swinarew, J. Gabor, S. Golba, B. Swinarew

*Mechanistic and Analytical Features of Water Ice as a Matrix for IR-MALDI mass spectrometry.* A. Pirkl, J. Soltwisch, F. Draude, K. Dreisewerd

*Charge-Tagged Peptides for Sensitive Detection by ESI-MS.* Z. Szewczuk, M. Rudowska, R. Bąchor, M. Cydzik, D. Wojewska, A. Kluczyk, P. Stefanowicz

12:30-13:30
Lecture Hall 1

**Lunch Seminar Thermo**

12:30-13:30
Lecture Hall 2

**Lunch Seminar AB Sciex**

13:30-14:00
Lecture Hall 1

**Closing Conference.**

Session Chair: M. STOBIECKI

Session Chair: J. GROTEMEYER

# Wolfgang Paul Lecture

## Can Mass Spectrometry Play a Role in Structural Biology?

MICHAEL L. GROSS

*Washington University in St Louis, United States of America*
*mgross@wustl.edu*

Our goal is a rapid, sensitive, and specific means of determining protein interactions, folding, and unfolding by using chemical footprinting coupled with MS. Driving this approach is the wide availability of mass spectrometers for analytical proteomics; these should also be applicable to protein footprinting. To this end, we are developing fast photochemical oxidation of proteins (FPOP) and hydrogen/deuterium exchange (e.g., PLIMSTEX) to interrogate protein interactions, interfaces, and dynamics of folding/unfolding. A significant advantage of H/DX is its simplicity and generality. FPOP is fast owing to the use of free radicals; radical generation occurs in low ns, and the radical reactions are complete in 1 microsec. Only a single conformation of the protein exists during the footprinting. Besides OH radicals, other radicals can be used including the sulfate and carbonate radical anions. FPOP can also be the probe in a classic, two-laser "pump-probe" experiment whereby a temperature-jump perturbation is produced as a pump by one laser and a second laser initiates FPOP as the probe. Subsequent analysis is by MS. This experiment is capable of probing protein structural dynamics at the sub millisecond level with improved sensitivity and more detailed structural resolution than any physical chemical method. Better understanding of folding will emerge from this approach, which is more informative thanthe usual physical probe methods (e.g., fluorescence).

**Method:** *H/DX* Proteins were diluted 1:10 in $D_2O$ buffer. At each time, aliquot was removed, quenched by lowering pH to 2.5 and T to 0°C. For peptide-level, deuterated proteins were digested on line by using column-immobilized pepsin; resulting mixture analyzed by LC/MS (Bruker MaXis Q-TOF). For single-residue, the digested deuterated peptides were analyzed by LC/MS/MS (Thermo LTQ Orbitrap) using ETD. *FPOP:* »OH radicals were produced by homolysis of $H_2O_2$ at 248 nm pulsed KrF excimer; excess »OH scavenged with 20 mM glutamine. Samples infused through fused silica; the laser irradiated a window at a pulse frequency giving single-shot exposure. Modifications found following trypsin digestion, using nanospray LC-MS/MS on Thermo LTQ-Orbitrap spectrometer, using Mascot and custom data processing.

**Preliminary Data:** We will illustrate the potential of H/DX, FPOP, and other footprinting strategies with applications to the ApoE family of proteins, a family with important implications in Alzheimer's and other diseases. Advances in H/DX will be specifically illustrated with the application of PLIMSTEX (protein-ligand interactions by MS, titration, and H/DX) to troponin binding $Ca^{\wedge\wedge}$ to determine in a single experiment the binding constants and then, using a digestion protocol, the order of binding of four calcium ions to the protein. We also are developing a top-down approach to understand the kinetics of H/DX at the amino-acid level FPOP will be specifically illustrated by an application to determining unknown protein/ligand structures and by a two-laser, pump-probe experiment designed to use footprinting to follow protein folding on the microsec time scale. Other labeling strategies will be illustrated to understand the dimerization of Her4 protein in a membrane and its subsequent phosphorylation. The results of a combination of these approaches will then be applied to understand the oligomerization of apoE.

**Novel Aspects:** Protein footprinting occupies a middle ground between high resolution methods (X-Ray, NMR) and low resolution approaches (fluorescence, CD).

# Mass spectrometry going towards the condensed phase

**DETLEF SCHRODER**

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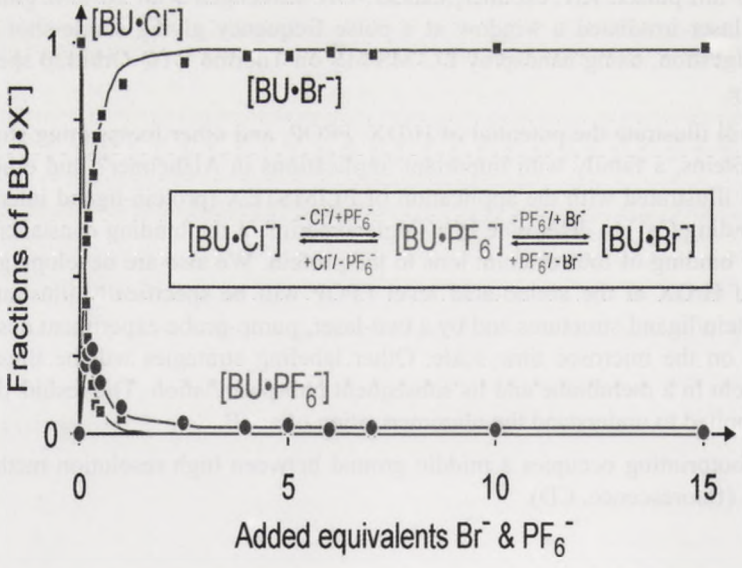
Mass spectrometry is often considered as a mere analytical method which is used to obtain structural information, the molecular weight in particular. In recent years it has been demonstrated, however, that electrospray ionization can indeed provide a close link between observations in the idealized conditions of a mass spectrometer and "real" chemistry occurring in solution.

**Method:** The experiments described use electrospray ionization (ESI) with a particular emphasis on concentration series and the effects of the concentration of the feed solution on the mass spectrometric data obtained. In addition, hyphenating ESI with infrared multiphoton dissociation (IRMPD) is used to show parallels between the gaseous and condensed phase.

**Preliminary Data:** The gas-phase chemistry of metal ions is briefly reviewed and a few examples for correlations between ESI-MS measurements and condensed phase properties. These include several solvated metal ions [1, 2] and metal-mediated coupling reactions [3, 4]. In addition to qualitative aspects concerning the type and intensities of certain species in solution, valuable mechanistic information can be achieved which bear relevance for preparative chemistry. In fortunate cases, it is even possible to link the chemistry in solution directly with that occurring in the gas phase and also in the transfer between the condensed and gaseous state [5]. In this context, we also propose the first quantitative correlations between gas-phase measurements and condensed-phase properties [2, 5].

**Novel Aspects:** Quantitative correlations between gas-phase and condensed-phase properties. Long-term vision: Implementation of ESI-MS in synthesis optimization.

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# Insights into Protein Structural Dynamics with Hydrogen/Deuterium Exchange Monitored by Mass Spectrometry

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Amide hydrogen/deuterium exchange monitored by mass spectrometry (HDX-MS) has become an important method for the characterization of protein structural dynamics. The basis of the HDX-MS method is that backbone amide hydrogens that are engaged in stable hydrogen bonds in the folded conformation are protected against exchange with the solvent. Structural fluctuations and local unfolding events that transiently break these hydrogen bonds cause exposure of the amide hydrogens to the solvent and this leads to exchange. The exchange kinetics of amide backbone hydrogens is therefore a direct probe for the structural dynamics of the protein backbone.

**Method:** When a protein is incubated in $D_2O$, its global deuterium uptake is readily monitored by mass spectrometry as each incorporated deuteron causes a mass increase of 1 Da. To obtain local information on the deuterium incorporation, the labeled protein is typically digested by pepsin at cold acidic conditions where the amide hydrogen exchange reaction is quenched (i.e., pH ~2.5 and 0 °C) and the labeled peptides are analyzed by a refrigerated LC-MS set-up.

**Preliminary Data:** With this bottom-up approach, we have investigated the structural dynamics of proteases and protease-inhibitors implicated in extracellular proteolysis, kinases and allosteric activation mechanisms. The spatial resolution of the bottom-up approach was until recently on the peptide level. We have recently demonstrated the utility of gas-phase electron-based fragmentation to obtain site-specific deuterium levels in peptides. We have now developed novel top-down MALDI and ESI fragmentation approaches (i.e., without pepsin digestion) for investigating protein hydrogen exchange at a spatial resolution approaching single-residue resolution and proof-of-principle results will be presented.

**Novel Aspects:** We have now developed novel top-down MALDI and ESI fragmentation approaches.

# DART Mass Spectrometry: Recent Developments and Applications

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Following its development in 2003 and its commercial introduction in 2005, the DART ion source has been applied to an extraordinarily broad range of applications. This may be attributed to the accessibility of the atmospheric-pressure ionization region and the relative simplicity of the mass spectra that are the end result of a set of complex reaction mechanisms.

**Method:** Recently, we have operated the DART with a relatively high gas temperature in a "pyrolysis mode" for the identification of wood samples. The first such application was to distinguish red oak (Quercus rubra) and white oak (Quercus alba). Chemometric tools were applied to the negative-ion DART mass spectra for classification. This approach was subsequently used by the US Fish and Wildlife Service to identify Brazilian Rosewood, which is an endangered species that is banned for importation. In a separate application, chemometric analysis of DART data has shown essentially 100% accuracy for ovarian cancer screening by Fernandez and coworkers at Georgia Institute of Technology. The unexpectedly high accuracy may result from the use of a relatively large set of biomarker ions.

**Preliminary Data:** Other recent applications of DART have included the identification of so-called "spice" designer drugs, detection of condom lubricants in fingerprints, and screening for banned pesticides in imported fruit. The latter application makes use of DART in transmission mode, providing more reproducible sampling and improved quantitation. A novel variation of transmission-mode DART involves spotting samples on wire mesh on a disposable card. The mesh has a resistive area that can be heated rapidly in the DART gas stream by passing an electrical current through the mesh. This simplifies and speeds analysis by eliminating such variables as sample placement and DART gas temperature. Small-molecule H/D exchange can be carried out in the DART source by simply adding deuterated solvents into the open-air ionization region. This has previously been demonstrated for the identification of melamine contamination in pet food and powdered milk. We have found that H/D exchange can provide useful structural clues that can assist in the interpretation of fragmentation resulting from collisional activation. An example will be presented for methylated xanthines: caffeine and the isomers theobromine and theophylline.

## Mattauch-Herzog Award

# Comprehensive and specific biological information at cellular resolution by MS imaging

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Mass spectrometry imaging (MS imaging) has become a method of outstanding importance in life sciences. The information content of the generated MS images greatly depends on the quality of the underlying mass spectral data. We have recently introduced a method that combines the high performance features of Fourier transform mass spectrometers with a spatial resolution in the low micrometer range [1]. We have now optimized our workflow for the 'Exactive Orbitrap' and applied it to the detailed analysis of phospholipids in a number of biological samples. In addition data processing and analysis strategies have been expanded and optimized.

**Method:** Sections of mammalian, insect and plant tissue were prepared with a cryomicrotome. Matrix was applied by a home-built spraying device [2]. MS imaging experiments were performed with a home-built atmospheric-pressure imaging source [1] attached to an 'Exactive Orbitrap' mass spectrometer (Thermo Scientific GmbH, Bremen). Pixel size was between 5 and 10 µm. Mass accuracy was better than 2 ppm (root mean square) under imaging conditions, i.e. compounds were identified with high confidence. MS images were generated with a bin size of dm/z = 0.01, which largely eliminates interferences from neighboring peaks. In all experiments the high resolution and mass accuracy proved to be essential for specific image generation and reliable identification of analytes.

**Preliminary Data:** A dedicated sample preparation protocol was established for the analysis of single cells. We were able for the first time to identify larger metabolites (phospholipids) and investigate their spatial distribution within native single cells in one measurement. A full profile of phospholipids and smaller metabolites such as nucleic acids and cholesterol was obtained from a single 7 µm pixel. Intact phospholipids and their acyl chain fragments were detected simultaneously in all ion fragmentation experiments in a human brain tumor sample. This allowed the (tentative) differentiation of isomeric lipid structures throughout the whole section within one experiment. A complete set of positive and negative ion images was obtained simultaneously by periodically switching the polarity of the ion optics throughout the imaging experiment. This significantly increased the number of lipids that could be identified in a single experiment in mammalian tissue and thus improves the differentiation of tissue types. The effect of microbial infection on plants was studied based on changes in the phospholipid profiles. Pathogen specific signals were detected at a spatial resolution of 10 µm. Detailed distributions of phospholipids were detected within a root section of less than 1 mm in diameter. Whole body-sections of insects (e.g. paederus) were also imaged at 10 µm spatial resolution. Phospholipid patterns were used to obtain morphological information and to assign signals of interest (e.g. insect defense agents) to specific organs. Statistical analysis tools (including PCA and LDA) were adopted and applied for semi-automic assignment of tissue types in mouse and human tissue sections. Additional data analysis tools were made accessible by conversion to the common data format for MS imaging – imzML (www.imzml.org). The presented workflow with its improvements in sample preparation, measurement parameters and data processing significantly improves the biologically relevant information that can be obtained by mass spectrometry imaging.

**Novel Aspects:** Highly reliable biological information from MS imaging for mammalian, insect and plant samples.

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# RAGE receptor structure and oligomerisation studied by hydrogen-deuterium exchange monitored by mass spectrometry

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Receptor for advanced glycation end products (RAGE) is a multiligand cell surface receptor, known to bind glycoxidised form of proteins as well as many other ligands such as Ab peptide, the S100 family of proinflammatory cytokine-like mediators, Mac1 integrin and many others. RAGE is an important mediator of the proinflammatory response involved in diverse pathophysiological states such as neurological disorders, stroke,amyloidosis, immune response, diabetes and inflammatory disorders, infectious disease and tumors. It is a potential therapeutic target, but in spite of significant effort its structure, ligand binding mode and signal transduction pathway has not been characterised yet on the molecular level.

**Method:** In the presented work we used hydrogen-deuterium exchange and mass spectrometry to gain insight into the structural properties of exRAGE – the full extracellular part of the protein containing V, C1 and C2 domains and the structural consequences of its oligomerisation.

**Preliminary Data:** The highlight of the present study is the intertwining of protected and fully exposed regions of the protein underscoring a highly dynamic character of large portions of this protein. However, we show that upon oligomerisation the C1-C2 domain linker region, fully flexible in the monomer, becomes stabilised.

**Novel Aspects:** Our result allow to propose a new model of the molecular mechanism of signal transduction by RAGE receptor.

# Conformational influence on dissociations of multicharged biological complex systems according to ion activation mode

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The *in vitro* function of biological molecules is dependent on the particular structural arrangement, included conformation. Mass spectrometry based on electrospray ionization can be a useful tool for establishing the non-covalent complex stoichiometry as well as origin of conformational stabilization. However, desolvation is a crucial step for maintaining at least partially the system conformation preformed in solution. Thus, under such careful conditions, ESI mass spectra can reflect partially the properties of survivor charged complexes in gas phase. On the other hand, from naked multiply charged systems (solvent-free), the ion activation can provide some insights on the interaction sites on the folded molecule or in complex ions. For reaching such information, a veritable arsenal is helpful for ion activation. Indeed, ergodic(e.g. CID, EID and IRMPD) or considered as non-ergodic (e.g., ECD, EDD or ETD) modes are available for such purposes. The presented studies will different illustrations of application with its limitation and the risk of conclusive mistake.

**Method:** Peptides, proteins, and single strand DNA were purchased from Sigma-Aldrich Chemicals and Genepep and are used without purification. Sample solutions in water/methanol 80/20 (or 50/50) have been prepared using deionized water (18.2 Mμ) and HPLC methanol. The final concentration of the peptide, protein and nucleotides solutions were 20 μM and diluted par addition a solution of ammonium acetate buffer 40mM when it was required. Mass spectra and CID-MS/MS spectra forDNA, dsDNA, peptide/DNA and protein/inhibitor complexes were performed on ion-trap instrument (Esquire 3000, Bruker) and on FT/MS instruments (LTQ-Orbitrap-XL in ESI, from ThermoFischer, and hybrid quadrupole ApexQeFT-ICR instrument with an actively shielded 7 T superconducting magnet, fromBruker-Daltonics). From the latter, a heated hollow cathode operating at a current of 1.7 A was used for electron bombardment activation (ECD, EDD and EID) during variable duration and energy. Double resonance experiments were used for ejection of selected fragment ions which could be intermediate to formation of a second generation product ion.

**Preliminary results:** From large monomeric systems as peptides, it is shown how their conformation, as well as their rigidity may influence dramatically the decomposition pathways occurring from the excited multicharged species. The ECD process for positive species (as well as EDD for negative ones) leads to some differentiation of conformers and topoisomercompared to the behaviour observed by CID. On the other hand, from various covalent and non-covalent ssDNA and dsDNA systems (associated with drugs and peptides) were investigated by using different activation modes according to the charge state and size, as well as the ion polarity. It emerges from many results, a similar trend which characterizesion orientation dissociations. This appears to be specific to the used activation. From several examples, it seems, that independently of the charge polarity, the formation of salt bridge plays an important role in the conformational stabilization. This stabilisation can be evidenced by cleavage of covalent bond rather than the salt bridge. However, this effect is dependent upon the relative gas phase basicity/acidity terms which are modulated by the charge state carried out by the studied systems. Indeed, the previous thermochemical terms decrease strongly with the increasing of charge state.This behavioractsas independent to the polarity and the chemical nature of the investigated systems. During all the gas phase operations: (i) aggregate formation and (ii) desolvation steps, it seems that conformation like to solution can survive at least partially, by the increasing of electrostatic interactions during the disappearance of hydrophobic interactions. Indeed, the neighboring groups which interact in solution can be maintained closer by the electrostatic effects if already they pre-exist in solution, even relatively distant due to solvent. Finally, some prudence must be used forconclusion as it will be shown from a study of protein/inhibitor interaction.

**New aspects:** (i) evidence on role of salt bridge on the folded system and (ii) H transfer conformational dependant, (iii) new evidence of complex conformational change through cation transfer into protein/ inhibitor non-covalent system and finally, (iv) the role of ion-dipole complexes on nucleotide ion cleavages.

**Acknowledgments:** Viet Nguyen, BessemBrahim, BaiyiXue, Ying Xu, Sandra Alves, Marie Perot-Taillandier, Carlos Afonso, for their various contributions and Dr Amina Woods for her helpful discussions.

# HPLC/FT-ICR MS as an approach for detailed analysis of a crude oil sample

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Crude oil is a very complex mixture which overall characterization represents a challenge for researchers. Non-hydrocarbon compounds present in a crude oil are among the most deleterious to refining catalysts and confer adverse stability properties. The complexity of the individual petroleum fractions is quite high, even after initial fractionations (i.e. SARA) [1], therefore the coupling of chromatography with an ultra-high resolution mass spectrometer is a successful choice. Sulfur polycycles were separated using a bonded β-cyclodextrin stationary phase [2]. On the other hand, nitrogen aromatic heterocyclic fractions were separated as well on a polar aminocyano-bonded silica HPLC column (PAC) [3]. This separation within the same class gives more detailed information about nitrogen compounds in crude oil samples.

**Method:** A polar aminocyano bonded column was used for the separation of crude oil samples with high nitrogen content by employing an elution gradient of n-hexane and iso-propanol, with IPA concentration up to 5%. The column was kept at room temperature, and was connected to a UV diode array detector set at 254 nm. High resolution mass spectra were obtained on a 12 T LTQ-FT-Ion Cyclotron Resonance Mass Spectrometer (Thermo Scientific). Different ionization sources were applied for the investigation including ESI, APPI, APCI and APLI. Offline samples were collected via Nanomate autosampler, whereas a micro-splitter was used to control the flow rate for online measurements.

**Preliminary Data:** Normal-phase chromatography was used as the separation method, because the majority of the compounds of interest in this case are of lower polarity. Using a mobile phase system consisting of n-hexane and isopropanol leads to better resolved chromatograms. First results were obtained by using an off-line LC/MS approach. A fraction of both peaks were collected offline every 20 seconds and by using a nano-ESI (Advion Nanomate). The N-selective separation was optimized into two major peaks: one broad peak appears after about one minute, and another peak elutes at around 20 minutes. Here, an attempt to couple the HPLC system to the FT-ICR spectrometer succeeds to generate comparable UV/ion chromatograms. This approach represents the first online LC/FT-ICR MS coupling in the field of crude oil analysis, which enables scan-to-scan characterization of the detected peaks. The first peak comprises mainly of hydrocarbons, whereas the second peak contains exclusively nitrogen species. ESI as an ion source was not adequate for this kind of investigation, when using n-hexane as an eluent. On the contrary, APCI measurements deliver good results, because it is an ideal method of ionization for low- to medium- polar compounds. Using APPI and APLI as ionization sources, it was possible to ionize crude oil samples investigated via LC/FT-ICR MS coupling on both modes, online and offline. A relative wide spectrum of polar species was observed using APPI as an ion source, However the lack of using a dopant was obvious on the outcome of the results. On the other hand APLI can deliver efficient ionization of poly aromatic species of the non-polar peak detected at earlier retention times. Increase of aromaticity of hydrocarbons and sulfur species in course of time was observed in the APLI measurement, in addition to the characterization of two distinct sulfur peaks in the non-polar fraction.

**Novel Aspects:** Online coupling of HPLC/FT-ICR MS system in a scan-to-scan structural elucidation feature for the analysis of a crude oil sample.

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# Fragmentation of Xanthene Dyes by Laser Activation and Collision-Induced Dissociation on a High-Resolution Fourier Transform Ion Cyclotron Resonance Mass Spectrometer

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The Fragmentation of rhodamine B as well as deuterated and non-deuterated analoga by laser and collisional activation was under investigation previously [1], [unpublished results]. The experiments were centered around the interesting double loss of $C_3H_8$ from the rhodamine B structure, seen in an FT-ICR mass spectrometer. The investigations have shown that this double loss is a general feature of the rhodamine B structure and that the fragmentation reaction takes place in the in the sidechain subsitution of the nitrogen atoms. This work shows the extension of these first experiments to molecules containing methyl and ethyl groups in different combinations, including deuteration. The properties of the fragment spectra are interpreted towards their meaning for the underlying reaction mechanism.

**Method:** The substances under investigation all had the basic structure of decarboxy-rhodamine B. The substitution patterns at the nitrogen atoms were as follows: $N(CH_3)_2$---$N(CH_3)_2$, $N(H,C_2H_5)$---$N(H,C_2H_5)$, $N(CH_3)_2$---$N(CD_3)_2$, $N(C_2H_5)_2$---$N(CD_3)_2$ and $N(C_2D_5)_2$---$N(CH_3)_2$. They were synthesized using literature procedures [2, 3]. The experiments were conducted on a 9.4 T FT-ICR mass spectrometer (Bruker Daltonics, Bremen, Germany). The analytes were transferred into the gas phase via the electrospray technique. Fragmentation was induced inside the ICR cell by two methods. First, collisional activation was performed using the SORI method and argon gas as collision partner. Secondly, laser activation was applied by an INNOVA argon ion laser (Coherent, Inc. Santa Clara, CA, USA) that was guided into the cell.

**Preliminary Data:** The tetramethyl variant of rhodamine B showed two losses of CH4. Additionally, a cleavage of $\cdot CH_3$ could be detected. This shows that, in this case, radicals are involved in the main losses and that these radicals can be seen on the timescale of the experiments. Calculations of the reaction regarding a concerted mechanism on the one hand and a stepwise radical one on the other hand gave further insight to these findings. While the concerted mechanism leads to products thermodynamically more stable by 430 kJ/mol, its transition state lies close in energy to the first radical intermediate, corresponding to the methyl loss. This explains why this first intermediate is accessible during the reaction. Further measurements were performed with a substance carrying only one ethyl group at each nitrogen atom. Among the occurring peaks, the most interesting ones are due to the loss of $\cdot CH_3$ and $\cdot C_2H_5$. They are higher in intensity than the respective even electron losses. Furthermore, the spectra show a peak that results from a combined cleavage of $\cdot CH_3$ and $\cdot C_2H_5$. This shows that radicals in this system are far more likely to occur than in the ones investigated before. Here the proposed mechanism for a concerted reaction cannot be realized, which makes the radical losses that intense. The methylated-deutero-methylated substance $N(CH_3)_2$---$N(CD_3)_2$ displayed main losses of $CH_4$, $CD_4$ and $CHD_3$. The latter shows, that a non-concerted component is present here. The only radical detected among these main fragments corresponds to the loss of $CD_4$+$\cdot CH_3$. The mixed methyl-ethyl variants $N(C_2H_5)_2$---$N(CD_3)_2$ and $N(C_2D_5)_2$---$N(CH_3)_2$ displayed no radical losses in the main dissociation steps. Only in combination with a $C_3$ unit loss from the ethylated side of the molecule, a non-concerted fragmentation reaction in the methyl part of $N(C_2H_5)_2$---$N(CD_3)_2$ is detectable, corresponding to a loss of $C_3H_8$+$CHD_3$.

**Novel Aspects:** Laser and collision induced fragmentation of deuterated and non-deuterated xanthene dyes containing methyl and ethyl substituents.

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# Ion Mobility Analysis of Glycans: A Calibration Protocol for Travelling Wave Instruments

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Glycosylation is one of the most abundant types of post-translational modifications found in proteins. The involved oligosaccharides are termed glycans and usually exhibit a branched and stereochemically complex structure. Currently, glycans are typically characterized using mass spectrometry-based techniques (MS), which are often directly descending form those established in proteomics. Measuring the molecular weight of a sugar, however, immediately poses a fundamental problem: entire classes of the constituting monosaccharide building blocks exhibit an identical atomic composition and, consequently, also an identical mass. Therefore, carbohydrate MS data can be highly ambiguous and often it is simply not possible to clearly assign a particular molecular structure.

**Method:** A promising approach to overcome the above-mentioned limitation is to implement an additional gas phase separation dimension using ion mobility spectrometry (IMS) – a method in which molecules of identical mass can be separated according to their shape and collision cross section (CCS). With the emergence of commercially available hybrid IM-MS instruments a couple of years ago IMS technology became readily available. Due to the non-homogeneous, travelling wave field (TW) utilized in these instruments, however, CCSs can currently not be determined directly. Instead, an external calibration using compounds of known CCS and similar molecular identity is required. For peptides, proteins, and protein assemblies, such calibration frameworks are readily available, but to date relatively few data are available for large carbohydrates.

**Preliminary Data:** Here we report a calibration protocol for TW-IMS instruments using a series of naturally occurring glycan-type carbohydrates. The underlying CCSs where determined using a modified Synapt G1 instrument, which was described in detail previously [1]. The utilized carbohydrate calibrants where obtained by enzymatic release from abundant and commercially available glycoproteins such as ribonuclease B and chicken ovalbumin using an easy-to-follow protocol. Our data indicate that only a few of the so-obtained carbohydrate mixtures are required to calibrate TWIMS instruments over a broad range of CCS values. Furthermore, the measured CCSs clearly demonstrate that carbohydrate isomers with identical mass but different conformation can be separated and distinguished using ion mobility spectrometry.

**Novel Aspects:** Calibration of TWIMS instruments with glycans, separation of oligosaccharide isomers with identical mass and different structure

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# Improving Bioanalytical Selectivity by Differential Mobility Spectrometry – Mass Spectrometry

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Differential mobility separation (DMS) can separate species based on the difference between their high field and low field mobility. At a specific separation voltage (SV) it is possible to ensure transmission of particular species through the device for detection (MS detection in this work) when a compensation voltage (CoV) is applied, while other species will simply be lost to the electrode walls. Using compound-specific combinations of SV, CoV and chemical modifiers added to the gas phase, it is possible to discriminate e.g. isomeric compounds or compounds of different charge states prior to their entry into the mass spectrometer. This work explores the use of DMS without and with chemical modifier for the separation of different compounds from chemical background.

**Method:** An AB Sciex 5500 QTRAP® system with Turbo V source and the electrospray ionization (ESI) probe was used, controlled by Analyst 1.5.2 Software. For DMS-MS, a SelexIon Ion Mobility Device (all AB SCIEX) was mounted in the source/interface region enabling the use of Differential Mobility Spectrometry (DMS). Both the DMS (SV, CoV, modifier) and Ion Source (GS1, GS2, CUR, IHT) parameters were optimized for the respective applications. Compounds were separated by chromatography using either an Agilent 1290 HPLC system at analytical flow rates (300-700 ul/min) or an Eksigent Express ht system at reduced flow rates (175 ul/min). Data Evalulation was performed using MultiQuant 2.1 Software.

**Preliminary Data:** Differential Mobility Spectrometry – Mass Spectrometry (DMS-MS) was successfully applied to a variety of analytical problems, e.g. a) different pairs of isomeric compounds that are difficult to differentiate by established LC-MS methods were successfully separated using DMS-MS, e.g prostaglandins. Using optimized SV/CoV/Modifier settings, PGF2α and 8-iso-PGF2α could be completely separated by DMS-MS, enabling dramatic reduction of chromatographic runtimes as full chromatographic separation is no longer required. b) A range of peptide biomarkers and peptide drugs (angiotensin I, angiotensin II, angiotensin IV, neurotensin, dynorphin A, melittin and exenatide) were spiked into both perchloric acid-precipitated plasma and tryptically digested plasma. DMS-MS was compared to traditional modes of analysis by MRM and SIM, and its dependence on instrumental parameters and peptide charge evaluated. For selected peptide charge states and sets of experimental parameters used, a significant reduction of analytical background could be achieved. Using these and similar examples, the mode of operation, experimental parameters and figures of merit of the SelexIon Ion Mobility Device will be discussed.

**Novel Aspects:** Differential Mobility Spectrometry – Mass Spectrometry (DMS-MS) for increase bioanalytical sensitivity.

# Rapid switching (80 Hz) between hard electron- and soft photo-ionization for gas chromatography/time-off-flight mass spectrometry: Technical realization and applications

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Mass spectrometry (MS) is the most versatile detector for gas chromatography (GC) and comprehensive two-dimensional gas chromatography (GC×GC). In addition to the standard electron ionisation (EI) technique, which fragments the analytes in a reproducible manner (compound identification), also soft ionisation techniques, such as single photo ionisation (SPI), have advantages. This includes the detection of the molecular mass of fragile compounds as well as the use of mass spectrometry with soft ionisation as a further, comprehensive (molecular mass) separation technique for GC and GC×GC [1]. However, the most analytical benefit is achievable if both information, i.e. SPI mass spectra as well as the standard, hard EI mass spectra could be gathered during the very same GC or GC×GC run.

**Method:** This work describes the realization of rapid switching between hard electron ionization (EI) and soft single photon ionization (SPI) in a compact orthogonal acceleration time-of-flight MS detector for gas chromatography (GC-oaTOFMS) and comprehensive two-dimensional gas chromatography (GC×GC-oaTOFMS). The SPI source uses vacuum ultraviolet (VUV) photons of 9.8 eV (126 nm), emitted from an electron beam pumped rare gas excimer light source (EBEL) filled with argon. The VUV light is focused into the ion chamber by an ellipsoidal mirror optic for accomplishing of SPI. A classical filament setup is used for EI (70 eV electron kinetic energy). Within this demonstration study, a maximum switching frequency of 80 Hz between SPI and EI was applied.

**Preliminary Data:** For the first time a MS with the novel switching capability was hyphenated to one-dimensional gas chromatography (GC) and comprehensive two-dimensional gas chromatography (GC×GC). By application of a polar GC column for GC-MS separation the SPI data can be displayed in a two-dimensional (2D) contour plot leading to a comprehensive 2D characterization (GC×MS), whereas the typical group type assignment (such as in GC×GC) for diesel is met [1]. In addition, standard GC-MS data (EI) are available too. In conjunction with GC×GC actually a 3D separation is achieved with SPI (GC×GC×MS) [1] while the standard EI-MS data for GC×GC is available too. The new technology is demonstrated exemplarily by analyses of a mineral oil type sample (diesel).

**Novel Aspects:** first realization of rapid switching between hard EI and SPI ionisation in GC-TOFMS instrument, two- and three-dimensional separation approach demonstrated.

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# ESI-mass spectrometry for structural characterizationof selected aliphatic homo- and copolyester

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Recently, there has been significant expansion in the number of studies using poy(3-hydroxyalcanoates) and their synthetic analogues for biomedical applications. The knowledge of structure-property relationships is essential for the successful application of polymeric materials, especially in biomedical applications such as tissue engineering and controlled drug delivery.In recent years, matrix assisted laser desorption/ionization time-of-flight (MALDI-TOF) and electrospray ionization (ESI) mass spectrometry have become a routine analytical tool for characterization of polymers, complementing NMR and other traditional techniquesby providing detailed structural information about the individual macromolecules in a (co)polyester samples studied and polymerization mechanism involved [1].

**Method:** Due to the separation of the signals representing individual polymer chains in the mass spectra, the structure of the polymers end groups and repeating units can be determined on the basis of the masses of the chains. However, the differentiation between random-, alternating-, and block copolymers cannot be determined by the studies of the simple mass spectra of copolymer. Deeper insight into the structure of copolymers can be achieved by means of a mass spectrometric fragmentation techniques such as tandem (ESI-MS/MS) and multistage (ESI-$MS^n$) mass spectrometry. Several successful applications ESI-$MS^n$ techniques to determine the architecture of the individual (co)polyester macromolecules including their topology, composition, chemical structure of end groups, as well as the sequence distribution of comonomers was recently reported [1-4].

**Preliminary Data:** The purpose of research, presented in this communication was to determine and compare the molecular structure and macromolecular heterogeneity found in different types of aliphatic copolyesters [3, 4]. The new model copolyesters with different architecture (diblock and random) were synthesized via anionic ring opening copolymerization of two racemic lactones e.g. β-butyrolactone and β-ethoxymethyl-β-propiolactone initiated by tetrabutylammonium cetate. The $^1$H NMR analysis supported by simple ESI-MS mass spectrometry provided information about the composition and general structure of copolyester macromolcules including the chemical structure of their end groups. Regardless of copolyester type or composition, resulting copolyesters contained predominantly linear macromolecular chains terminated by acetate end groups derived from the initiator used. Moreover some amount of copolyester chains terminated by unsaturated (crotonate and 4-etoxy-2-butenoate) and carboxyl end groups were identified predominantly in the low mass range. The significant differences in molecular level structures of diblock and random copolyesters studied were discerned by application of ion trap ESI-MS/MS method. The ESI-MS/MS experiments performed revealed that sequence distribution presented in the fragmented individual copolyester macromolecular ions has influenced on their fragmentation product patterns. Thus, arrangements of comonomer structural units along the diblock and random copolyester chains studied was determined based on the investigation of their fragmentation product patterns.

**Novel Aspects:** Determination of the comonomers sequence distribution based on fragmentation patterns of selected copolyester molecular ions.

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# Quantitative Label-free Mass Spectrometry for Unbiased Profiling of the Human Monocyte Secretome

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Secreted proteins act as messengers and regulators in intercellular communication and are involved in a variety of biological processes and diseases. Cytokine release from monocytes is essential for the correct response of the innate immune system to injury or pathogens [1]. Consequently, the secretome of monocytes provides a promising resource for discovery of molecular markers specific to immunological reactions. Quantitative label-free mass spectrometry is an attractive method for secretome analysis as it allows the unbiased comparison of a large number of analytes across many experiments while requiring only minimal processing and modification of the sample [2].

**Method:** Secretomes were obtained from human monocytes (THP1) under serum-free conditions in response to different inflammatory stimuli. Prior to MS, secretomes were analysed for their total protein content (BCA assay) and presence of known cytokines (CBA assay). For MS analysis, secreted proteins were concentrated by ultrafiltration and tryptically digested. After clean-up (C18 spin columns), samples were separated on a Dionex 3000 nano-LC system equipped with C18 Acclaim Pepmap columns (Thermo Scientific). Analytes were ionised using a Nanomate Triversa ion source (Advion) and MS analysis was performed on an Orbitrap Velos (Thermo Scientific). Data analysis was carried out using Mascot search engine, Transproteomic Pipeline (validation of protein identification), PVIEW (quantitation) and DanteR (statistical evaluation) [3, 4].

**Preliminary Data:** We present a robust label-free quantitative nano-LC-MS/MS approach that enables global and unbiased profiling of the human monocyte secretome. The technique allows accurate determination of relative changes in cytokine- and pro-inflammatory protein secretion in response to characteristic for tissue injury and microbial infection, thereby providing insight into the underlying biological processes. More than 700 of the identified proteins passed stringent quality criteria (minimum of two peptides per protein, presence in at least 3 biological replicates and statistical validation of protein identifications), making them suitable for protein quantification. The quantitative information allowed extraction of relevant marker proteins from unspecific background in the pool of proteins released from monocytes. This results in the bona-fide identification of 239 proteins which are over-secreted upon simulated tissue injury (LPS/ATP stimulation) or microbial infection (LPS/MDP stimulation). The majority of these proteins show significantly altered secretion in dependence of the type of monocyte stimulation. For example, of 26 over-secreted proteins validated as cytokines or inflammation related proteins by literature and database search, 19 show higher abundance in the LPS/ATP group, whereas 7 are over-secreted under LPS/MDP conditions; relative differences for specific proteins can be correlated to characteristic immune response mechanisms. In order to verify that the quantitative results are accurate, the technique was validated with an independent, antibody-based method (CBA assay), demonstrating that label-free mass spectrometry is able to quantify selected cytokines such as interleukins and tumor necrosis factor with high sensitivity (high attomole range). In conclusion, the unbiased approach allowed identification and quantification of a series of stimulus-dependent, previously unreported cytokines and immune-response related proteins which as could serve as lead candidates for further functional and pre-clinical evaluation.

**Novel Aspects:** First-time application of label-free quantitative mass spectrometry to profile the human monocyte secretome.

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# A Simple Way to Perform Quantitative Proteomics and Lipidomics Measurements from Small Tissues Samples

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In the course to investigate the influence of lipid metabolism in disease progression Omics approaches are important discovery tools. Perturbations of protein and lipid biosynthetic pathways due to stress and pathological processes are of complex and quantitative nature. The aim of this study is to develop efficient sample preparation strategies integrating proteomics and lipidomics approaches. As model we have chosen dissected Drosophila melanogaster brains which were analyzed in the context of sphingolipid metabolism perturbations and neurodegeneration.

**Method:** Several Drosophila melanogaster strains were genetically manipulated to perturb sphingolipid metabolism and/or induce neurodegeneration. Dissected adult brains were processed with the MTBE lipid extraction [1]. The upper organic layer was utilized for shotgun lipidomics [2, 3]. The lower aqueous layer was processed for total protein determination and proteomics starting with 1D SDS-PAGE analysis. Total protein amounts were determined by image analysis of coomassie stained gels using ImageJ. Gel lanes were sliced into 11 pieces which were processed for In-gel digestion [4]. The extracted peptides were spiked with BSA peptides and analyzed by NanoLC-MS/MS using Agilent 1200 system and a LTQ-Orbitrap Discovery coupled with an Advion Nanomate Triversa. MASCOT database searches and labefree quantitation were perfomed using Proteome Discoverer 1.3.

**Preliminary Data:** Optimization for dissection, lipid extraction, 1D SDS-PAGE and GEL-LC approaches were performed which enabled us to routinely perform lipidomics and proteomics measurements from only 5 dissected adult brains. In the course of such experiment we can access quantitative information of 783 proteins and 183 lipids. Currently we apply this methodology to analyze the influence of lipid metabolism perturbation on the proteome of neuronal tissue.

**Novel Aspects:** We present an analytical strategy enabling us to perform quantitative lipidomics and proteomics experiments from minute sample amounts.

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# Online-Bioaffinity- mass spectrometry: Analytical development and application for structural and affinity characterisation of immune- and peptide- protein complexes

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In the last decades biosensors have become important analytical tools with multiple applications, from basic research to broad bioanalytical applications. The recently developed K5-S-sens® biosensor [1] employs surface acoustic waves (SAW), generated by a reverse piezoelectric effect, for the detection of bioaffinity interactions by measuring mass loadings lower than 1 pg/mm2. Nonetheless, biosensors do not provide details about the chemical structure of ligands, which can be determined by mass spectrometry [2], the most important tool in the analysis of biopolymers.

**Method:** Here, we report affinity interaction and structural studies of antigen-antibody and peptide-protein complexes using the K5 S-sens® directly coupled to (i) ESI ion trap and (ii) ESI FT-ICR-MS.

**Preliminary Data:** The complexes included: Aß (1-16) peptide / anti- Aß (1-16) antibody; Aß (12-40) / anti- Aß (17-28) antibody; Tau protein (isoform 2N/4R) / anti-Tau specific antibody (clone TAU5); melitin / calmodulin, and α-Synuclein / anti- α-Synuclein antibodies. Antibodies or proteins were covalently immobilised (via a thiol linker) on the surface of a gold chip inside the biosensor, and their interactions with the specific peptide or protein were monitored with the SAW biosensor. Using an online interface, the bound peptide or protein was eluted under acidic conditions from the gold chip and analysed by ESI mass spectrometry, to identify their primary structures [2]. The combination of SAW biosensor and ESI-MS was found to be an excellent tool for the affinity determination of Tau, Aß and α-Synuclein, and analysis of structure modifications from biological material.

**Novel Aspects:** SAW biosensor was directly coupled to (i) ESI ion trap and (ii) ESI FT-ICR-MS.

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# Improved Precision of Quantitative 'Bottom-up' Proteomics with Stable Isotope Labeled Proteins as Internal Standards

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Quantitative bottom-up proteomics based on stable isotope dilution using intact labeled proteins as internal standards is a well-established method [1]. There is broad consensus that peptide/protein labeling by $^{15}N$, $^{13}C$, or $^{18}O$ leads to analogs exhibiting negligible isotope effects in quantitative proteomics. Technical precision of MS for isotope ratio measurements of peptides is in the order of 1%, whereas often heavy/light ratio changes larger than factor of 1.5 to 2 are given as confidence limits to differentiate between significant and non-significant changes in protein quantification studies [2]. Normally, the responsibility for this is given to biological variability of samples. In this study we attempt to explore other factors which may contribute to the reported relaxed precision in bottom-up proteomic studies.

**Method:** Cell-free expression protein system (Expressway, Invitrogen, Germany) was used to produce non-labeled and labeled form of the studied protein. Protein purification was achieved using nickel metal-chelate column chromatography (Propur IMAC spin mini columns, Nunc, Fisher Scientific). In-gel protein tryptic digestion was performed according to a standard protocol. LC-MS/MS analyses were performed using a UPLC-system (Waters, Milford, USA) connected to an LTQ-Orbitrap XL instrument (Thermo, Bremen, Germany). Statistical data evaluation was performed in SigmaPlot applying one way ANOVA-Holm-Sidak test.

**Preliminary Data:** In this study we try to address the question why the average light/heavy ratio quantification variance between the peptides belonging to the same protein is much larger than the measurement variance for single peptides in repeated ratio measurements. A non-labeled (light) and SILAC-type labeled (heavy, Arg+10, Lys+6) form of human calmodulin like-3 protein (Calml3) were generated by cell-free protein synthesis to preclude the possibility of differential protein modifications as source of light/heavy ratio variance. The proteins were combined, subjected to in-gel digestion with trypsin and analyzed in triplicate by UPLC-ESI-MS. As a result, 16 light/heavy peptide pair ratios were obtained. Among them 10 light/heavy ratios exhibited relative standard deviation (RSD) lower than 3%. Pairwise statistical comparison of these 10 peptide pairs revealed that many of them exhibited ratios, which were different at a high level of statistical significance ($p < 0.001$). The subset of peptides with statistically identical ratios resulted in an average ratio with a RSD of 1.2%, which is within the technical error of the MS measurement. This subset of peptides was characterized by high signal intensity and absence of missed cleavage sites. Variation of the digestion time from 0.25 to 4 h showed that the light/heavy ratios of most peptides decreased with increasing time, indicating a kinetic isotope effect explained by preferred cleavage of the light Calml3 version. UPLC-MALDI-MS measurements confirmed this observation, thus precluding the ionization method as a source of the observed ratio differences. In addition, Calml3 heavy version was prepared with $^{15}N$ labels incorporated in all amino acids, mixed with light version, subjected to tryptic digestion and analyzed by UPLC-ESI-MS. The $^{14}N/^{15}N$ peptide ratios determined for a set digestion times confirmed the occurrence isotope effects during tryptic digestion.

**Novel Aspects:** Improved technical confidence limits of quantitative proteomics by bottom up proteomics with intact labeled proteins as standards are defined.

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# Benchmarking Label-free Quantification using the Meta-Proteome Quantification Standards (MPQS)

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Label-free quantification has emerged as the method of choice for many laboratories for quantitative proteomics analyses. Recent developments allow for identification and quantification of >2000 proteins in a single LC-MS experiment. However, only few test data sets or sample sets exist that allow performance bechmarking of label-free quantification workflows. We present a custom sample set, named the meta-proteome quantification standards (MPQS) which are built from three different proteomes and Sigma UPS standards. Each sample contains equal amounts of mouse proteins and varying amounts of yeast, E.coli and UPS proteins, generating a system with thousands of proteins with exactly defined ratios and a highly complex, non-regulated background that can be used for both testing system and software performance for label-free quantification.

**Method:** Complete proteomes of mouse (EL4 cells), yeast and E.coli were digested using the FASP [1] method. UPS standards were purchased from Sigma and tryptically digested in solution in the presence of RapiGest. As initial quality control, all separately digested proteomes were intially verified on a Q-TOF Premier mass spectrometer, identifying approx 400 proteins for mouse, yeast, E.coli proteins and 43/48 UPS proteins. After initial analysis, digested proteomes and UPS standards were mixed in different ratios, using the mouse proteome as constant background. Samples were aliquotted and shipped to all participating laboratories for LC-MS analysis. Data were analyzed in five different labs on a total of seven instrument platforms. Label-free quantification was performed using different software, including MaxQuant, ProgenesisLCMS, ProteinLynxGlobalServer and IsoQuant.

**Preliminary Data:** LC-MS analysis of the complete MPQS sample sets identified between 400 and 1800 proteins on the different instrument platforms tested. Inspection of the raw data revealed a very high complexity with many overlapping isotopic patterns, making data analysis challenging. While sample complexity exceeds the situation in a standard label-free proteomics experiment for e.g. cell lysates, it is very similar to e.g. triple-SILAC labeling and by far not as complex as samples containing meta-proteomes of multiple species, e.g biofilms or gut bacterial samples. By defining the area under the quantification curve (AUQC) as a figure of merit, we generated metrics that conclusively describe performance of label-free quantification and allows to benchmark instrument or software performance. To avoid any systematic bias introduced into the data by normailzation of the different evaluation packages used, data were re-normalized before AUQC-curve generation, so that the median log2ratio of all mouse proteins was zero. Analysis of the data from the different laboratories revealed slightly better performance of QTOF-based platforms compared to Orbitrap platforms. For Orbitrap data, evaluation by ProgenesisLCMS provided higher AUQC values than MaxQuant label-free, while for Q-TOF instruments, data analysis using IsoQuant was more reliable than ProteinLynxGlobal server. Additionally, analysis of detection efficiency of proteins contained in the UPS standards allowed assessment of the dynamic range of the individual platforms/software combinations. On Q-TOF instruments, use of ion mobility separation improved both number of identified proteins and calculated AUQC values. The MPQS sample set described here can be easily reproduced using standardized protocols by other proteomics laboratory. The datasets created in this study will provide a rich resource for developing and validation of bioinformatics solutions for label-free quantitative proteomics. All datasets produced in this study will be made publicly available.

**Novel Aspects:** MPQS standards and evaluation procedures allowing conclusive benchmarking of label-free quantification using hundreds of proteins at exactly known ratios.

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# The importance of total system peak capacity for complex proteome analysis

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Shotgun proteome profiling is an analytically challenging discipline in which tens of thousands of peptides are analysed in a given analytical run. Usually, these peptides are not evenly distributed through the mass and chromatographic dimensions generally available, and result in areas of extreme analyte density. Analytical systems with high peak capacity are required to minimise chimeric interferences and to extract accurate and reproducible data from these samples.

**Method:** Total system peak capacity (PcSYSTEM) is determined by the respective peak capacities of LC (PcLC) and MS (PcMS) dimensions, and each of these can be significantly extended by the use of ultra high pressure multidimensional UPLC and ion mobility-MS methodologies. Orthogonality of dimensions is also an important factor to consider in the design of optimal analytical systems.

**Preliminary Data:** Given the extreme complexity typical in proteome samples, sometimes even analytical systems with high PcSYSTEM are unable to resolve all the peptides in the sample. In these cases, data-independent acquisition strategies such as HDMSE are required to deconvolute the resultant composite spectra, and can also provide quantitative data via label-free methodologies.

**Novel Aspects:** Shotgun proteome profiling.

# SPME-MS as a tool for food quality and authenticity assessment

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Analysis of food volatile compounds is used for monitoring food product quality, usually based on the determination of off-odorants or other deterioration markers. For the authenticity testing profiling volatile compounds is used most frequently. Methods of volatiles analysis are based on GC/MS. SPME-MS (Solid Phase Microextraction-Mass spectrometry) offers a rapid method of extraction and subsequent analysis (without chromatographical separation) of volatile compounds in a headspace of the product. Obtained "average" (low resolution) mass spectrum is subjected to multivariate analysis. The paper is focused on the application of SPME-MS in monitoring quality of edible oils, beer and wine and authenticity testing of spirits, wine and cheese.

**Method:** SPME-MS and SPME-GC/MS analyses were performed on a HP/Agilent Technologies single quadrupole instruments (5971/5975MSD). For each application type SPME extraction parameters were optimized. Analyzed food products were subjected to SPME-MS as well as to SPME-GC/MS analyses. Data obtained using these methods were processed using MVA (PCA and LDA) analyses. In GC/MS approach profiles of volatile compounds were determined as well as characteristic markers for quality (2, 4, 6-trichloroanizole in wine, and E-2-nonenal in ageing beer).

**Preliminary Data:** SPME-MS proved to be a rapid method for the assesment of selected quality features of investigated food products and authenticity testing. It was a good measure of lipid oxidation in edible oils, where TIC area was highly correlated with PV (peroxide value; 0.997). Intensity of selected ions in monitoring oxidation (m/z 56, 57, 110) were higly correlated with formed hexanal, 1-pentene-3-ol and 2, 4-heptadienal (rancidity indicators) [1]. Monitoring m/z 195, 197, 210 in wines allows detection of wines tainted with 2,4,6-trichloroanisole in concentrations as low as 10 ng/L. SPME-MS followed by LDA allowed also a differentiation of samples in authenticity/origin testing. It allowed a fast and reliable differentiation of raw spirits produced from rye, corn and potatoes (recognition and prediction >96%) [2]. It allowed also a differentiation of white wines produced from different grapes (Muscat, Sauvignon Blanc, Chardonnay) and produced in different countries, as well as red wines (Cabernet Sauvignon, Merlot and blends). It allowed differentiation of beers (Pils type) of different manufacturers and of different shelflives. It was also possible to use SPME-MS for the determination of authenticity of PDO smoked cheese Oscypek produced in sh eperds huts in Podhale region.

**Novel Aspects:** Rapid assesment of quality/authenticity based on MS-based "electronic nose". Processing MS spectrum as a "sensor" response.

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# Mass Spectrometry in Sports Drug Testing: Investigating Diagnostic Product Ions of Testosterone by ESI-CID HRMS and IRMPD spectroscopy

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Anabolic agents represent the most frequently detected class of prohibited substances in sports drug testing. Particularly challenging is the detection and identification of unknown analogs of testosterone. Steroids such as testosterone bearing a 3-oxo-4-ene structure commonly generate abundant product ions at *m/z* 97 and 109, which have been subject of extensive studies employing stable isotope labeling, $MS^n$ experiments, and accurate mass measurements in the past. Due to their considerable intensity and the diagnostic value, in-depth investigation concerning the gas-phase ion structure and potential formation of *m/z* 97 and 109 were conducted. The obtained information was subsequently successfully applied to the identification of a urea conjugate of testosterone detected in a product of Traditional Asian Medicine and urine samples of athletes.

**Method:** The model substance testosterone was studied concerning its dissociation into diagnostic product ions using ESI-CID followed by high-resolution/high-accuracy mass spectrometry corroborating the elemental composition of the ions at *m/z* 97 and 109. QTrap $MS^3$ experiments were performed to compare the dissociation patterns of *m/z* 97 derived from testosterone with those obtained from reference substances such as 3-methyl-2-cyclopenten-1-one and related isomers. Moreover, IRMPD spectroscopy conducted with a 4.7 T FTICR equipped with a free electron laser was used to provide further information on the nature of the gas-phase ion structure of *m/z* 97. Finally, two carbon atoms (C-10 and C-19 of testosterone) were selectively labeled with $^{13}C$ by synthesizing the model substance 4a-methyl-4,4a,5,6,7,8-hexahydronaphthalen-2(3H)-one, which resembles the A/B-ring structure of testosterone.

**Preliminary Data:** The elemental compositions of *m/z* 97 and 109 as obtained from the protonated molecule of testosterone $[M + H]+ = m/z$ 289 were confirmed with $C_6H_9O$ and $C_7H_9O$ as reported in earlier studies (Fig. 1) [1]. Based on $^{13}C$ and $^{2}H$ labeling, the origin of these ions was determined as the steroidal A- and B-ring with *m/z* 97 comprising the carbons 1-4, 10 and 19, and *m/z* 109 the carbons 1-6. It was, however, only assumed that a) carbon 10 is included in *m/z* 97 while carbon 5 is excluded, and b) that the gas-phase ion structure of *m/z* 97 is represented by the 3-hydroxy-1-methylcyclopent-2-en-1-ylium ion. Isotope labeling of a model substance 4a-methyl-4,4a,5,6,7,8-hexahydronaphthalen-2(*3H*)-one revealed two dissociation pathways in nearly equal intensities yielding two species of *m/z* 97 with identical elemental compositions (Fig. 2). The MS/MS analysis of protonated 3-methyl-2-cyclopenten-1-one, suggested to be present in the gas-phase as the O-protonated molecule as supported by DFT calculations, convincingly matched the $MS^3$ product ion mass spectrum of *m/z* 97 derived from testosterone, thus supporting the hypothesis that the monitored ion is in fact O-protonated 3-methyl-2-cyclopenten-1-one. Moreover, the proposed cyclopentenone structure was further corroborated by IRMPD spectroscopy using a free electron laser (Fig. 3). The importance of the ion at *m/z* 97 for the identification of steroidal substances related to testosterone was subsequently successfully applied to an unusual case of doping rule violation with androgens derived from musk pod. Here, a urea conjugate of androstenedione was characterized as 3a-ureido-androst-4-en-17-one, which gives rise to an intense ion at *m/z* 81 corresponding to *m/z* 97 after elimination of urea from C-3 of the steroidal A-ring.

**Novel Aspects:** The gas-phase structure of the steroid-typical ion at m/z 97 was elucidated and applied to characterize a typical musk-pod steroid.

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# Forensic Screening of Isobaric Compounds in Crude Samples by Ultrahigh-Resolution UHPLC-QTOF Technology

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Comprehensive forensic screening by LC/TOFMS based on a large target database of ~700 standard compounds has been established as a routine method. Compound identification is done on accurate mass, isotope pattern and retention time information. Although the method was proven to be very reliable (~7000 samples p.a.), the target database contains a few isobaric compound pairs with similar retention time, which require a mass resolution much higher than R = 10.000 achievable with standard ESI-TOF systems. We investigated on real-life samples which resolution is needed in those cases to reduce the number or even completely avoid any false negatives in screening of complex samples.

**Method:** 17 target compounds in 8 groups are found in the target database which require a high mass resolution to be distinguished. Their molecular weights ranged from 211 to 414 Da. A mass resolution up to ~50.000 was required to separate all compound groups clearly. Since the MS was coupled to a fast UHPLC separation, this mass resolution must be achieved at simultaneously high data acquisition rate of 10-20 Hz. Automated data processing was performed using dedicated screening software.

**Preliminary Data:** The mass accuracy of the systems allows the use of highly selective EIC trace widths down to 0.5 mDa. Observed average mass accuracies were mostly better than 0.5 ppm and independent of the acquisition rate. 12 of the 17 target compounds were detected even at 0.1 pg/µl, whilst 14 compounds gave linear calibration curves for the full concentration range (~ 4 orders of magnitude), demonstrating the suitability of the system for quantitative analysis. Additionally, the QTOF was successfully applied to an example of interference between a database compound and a supposed metabolite: according to experience for urine samples with positive findings of doxepin the identification of the internal standard dibenzepin is affected by an obviously coeluting compound of similar mass on standard TOF systems. The QTOF method resolves the two mass peaks and allows for confirmation of the assumed metabolite sum formula.

**Novel Aspects:** The mass accuracy of the systems allows the use of highly selective EIC trace with resolution of isobaric peaks.

# Hyphenated techniques for determination of volatile organic compounds in breath

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Exhaled breath analysis is a non-invasive diagnostic method without risk to the patients. Important part of procedure is a choice of sample preconcentration methods including solid phase microextraction (SPME) and solid sorbents combined with thermal desorption (TD), which usually requires cryofocusing for trapping of the sample. SPME is a simple and reliable preconcentration method. Gas chromatography and mass spectrometry (GC/MS) is well-suited technique for the investigation of volatiles. Typical instruments are equipped with quadrupole (Q) or ion trap (ITD), and more recently time of flight (TOF) analyzers. The main aim of this work was the comparison of variuos methodologies applied for the deteremination of volatile organic compounds in exhaled breath.

**Method:** The GC/MS analyses were performed on Agilent 5975 Inert XL MSD (quadrupole MS) and TrueTOF MS (Leco, USA) both coupled with 6890 N gas chromatographs (Agilent) with split-splitless injector Oven stemperature programme were: initial 40°C for 2 min., ramped 10°C $min.^{-1}$ to 140°C, then ramped 5°C $min.^{-1}$ to 270°C for 3 min. The MS analyses were carried out at electron impact ionisation 70 eV. Nist 2005 libraries were used for mass spectra identification. A SPME holder and Carboxen-PDMS (Supelco) coated fibers were used for extraction. The Tedlar air bags were applied for sample transportation (SKC, USA).

**Preliminary Data:** Exhaled breath contains volatile organic compounds (VOCs) such as methane, ethane, propane, butane, hexane, isoprene, ethanol, methanol, acetone, metanal, etanal, propanal, hexanal, etc. The up-to-date methods of separation and detection of VOCs allow to achieve low detection limits and high precision of measurements. The method showed satisfactory precision (RSD below 11%, linearity in the range of 2.8-136 ppb and limit of detection ranging from 0.6 to 2.1 ppb. Among determined compounds the heterocycles, aromatic hydrocarbons, alkanes, alkenes, alcohols, aldehydes, ketones and sulphur compounds have been found in human breath samples, especially for smoking cancer patients. A comparison of the results of cancer patients with healthy volunteers revealed differences in the concentration patterns. Sensitivity for detection of lung cancer patients based on different compounds not arising in exhaled breath of healthy volunteers was 51%, the specificity always being 100%. Isoprene and acetone, which are compounds appearing in everybody's exhaled breath, did not show a different concentration pattern for lung cancer patients as compared with healthy controls.

**Novel Aspects:** Presented reaserch work might lead to future diagnostic test for respiratory system diseases.

# Solid phase microextraction coatings and LC-MSn for determination of drugs and their metabolites in biological samples

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Determination of biologically active compounds from various matrices, including environmental and biological samples is a serious problem in a modern analytical chemistry. The most relevant matrices to be analyzed for this purpose are plasma or blood, due to providing a good correlation between their concentration and pharmacological effects. One of the major tools in the pharmacokinetic studies is the combination of high-performance liquid chromatography and mass spectrometry (HPLC-MS). This simple and selective method has been used for the determination of drugs belonging to different medicinal classes in various matrices. The main aim of this investigation was to apply a fast and sensitive extraction technique using electrochemically prepared a new polymeric coatings as sorbents for solid phase micro-extraction (SPME).

**Method:** The new model of the Heart-and-Lung Machine (HLM) was operated with heparinised blood maintained at 37°C as described [1]. The HLM model consisted of a centrifugal pump, a reservoir, a flow meter and side ports. To assess the influence of blood flow velocity on polymeric SPME coatings, the flow rates were varied in the range 50-500 ml/min at two different concentrations of selected drugs. For evaluation of the linearity of extraction, the concentration ranges were chosen according to the pharmacokinetics observed in patients. Sampling was accomplished in parallel either from flowing medium by introducing the SPME fibers into the re-circulating blood via side port (5 min) – *on line* – or by immersion into aliquots under stagnant conditions (10 min) – *off line*.

**Preliminary Data:** Polypyrrole (PPy), polythiophene (PTh) and poly(3-alikilothiopenes) SPME coatings were used and evaluated their ability to extract selected antibiotic drugs. SPME fibers are manufactured electrochemically by coupling the homemade electropolymerization system to a new generation potentiostat/galvanostat connected to a three-electrode array. Their physico-chemical properties, mechanical and chemical stabilities are characterized by scanning electron microscopy and Fourier transform infrared spectrometer. The SPME coatings were evaluated by analyzing clinically relevant antibiotic drugs used in bacterial therapy. Important factors in the optimization of SPME efficiency such as extraction time, temperature, pH of the matrix, influence of anticoagulants on sorption mechanisms, and desorption conditions are discussed. Mass spectrometric parameters were optimized for target compound in positive ion mode over the *m/z* 100-500 range. Quantitation was done using multiple reaction monitoring (MRM) mode to monitor precursor ion at $[M + H]^+$ to product ion transition of *m/z* 366 → 349 for amoxicillin (AMOX), 456 → 396 for cefotaxime (CEF), 332 → 288 for ciprofloxacin (CIP), 1621 → 811 for daptomycin (DAPTO), 307 → 220 for fluconazole (FLU), 478 → 322 for gentamycin (GEN), 425 → 377 for clindamycin (CLI), 338 → 296 for linezolid (LIN), 172 → 128 for metronidazole (MET), and 402 → 384 for moxifloxacin (MOXI). Validation data for accuracy and precision for intra- and inter-day were good and satisfied FDA's guidance: CV between 0.24% and 11.66% and accuracy between 93.8% and 108.7% for all compounds. Developed method can be used for the quantitative analysis of selected biologically active compounds, and provide a potential application to study the metabolism and pharmacokinetics of other drugs from different medical classes from the biological matrices. The results demonstrate the potential of *in vivo* SPME as a useful sample preparation tool for chromatographic based metabolomics drug monitoring in the biomedical application from patients receiving therapeutic dosages [1, 2].

**Novel Aspects:** Determination of selected antibiotic drugs from biological samples was performed with use of polymeric SPME fibers coatings and LC-$MS^n$.

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# Negative ion tandem mass spectrometry of prenylated fungal metabolites and their derivatives

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Meroterpenoids represent natural products of combined biosynthetic origin [1]. The class of meroterpenoid quinones occurs widespread in various living organisms including macromycetes and plays an important role in several life processes. A group of structurally related 2,5-dihydroxy-1,4-benzoquinones with prenyl side chains has been isolated from different fungal species belonging to the Boletales s.l. (Basidiomycetes) such as boviquinone-3 and boviquinone-4 [2]. A special benzoquinone is tridentoquinone, an unique red-colored ansaquinone which is apparently related to the linear boviquinone-4 [3]. These metabolites were investigated by liquid chromatography electrospray tandem mass spectrometry under negative ionization. Their fragmentation behavior was studied in comparison to the structurally related prenylated phenols.

**Method:** The negative ESI ion trap (IT) mass spectra were recorded on a LCQ Deca XP MAX (Finnigan) coupled with a Waters ACQUITY UPLC system (BEH C8 1.7 µm, 1.0×100 mm, gradient system: water/acetonitrile each containing 0.2% acetic acid) and equipped with a photodiode array detector (Waters). The ESI tandem MS experiments were performed in negative ion mode by selection of the ions of interest and using a normalized collision energy of 45% (activation Q: 0.250; activation time: 30.0 m/sec). The most abundant ions in each IT-$MS^2$ spectrum derived from the $[M-H]^-$ ions were subjected to a further $MS^n$ analysis. The compounds analyzed were kindly provided by Prof. Dr. Dr. h.c. Wolfgang Steglich and co-workers (Ludwig-Maximilians-University, Munich).

**Preliminary Data:** The results obtained by negative ion electrospray tandem mass spectrometry can be summarized as following: 1) Prenylated benzoquinones and phenols exhibit a characteristic fragmentation pattern in negative ion electrospray tandem mass spectrometry. 2) The mass spectral decomposition of both prenylated phenols and boviquinones under negative ion ESI conditions is mainly characterized by typical successive losses of the isoprene units. This is in agreement with their corresponding EI mass spectra [4, 5]. 3) The loss of the isoprenoid side chain of the boviquinones leads to a common radical anion comprising the quinoid moiety. 4) In contrast to the linear boviquinone-4, the cyclic tridentoquinone shows a quite different fragmentation pattern. 5) In case of the prenylated phenols, the loss of the complete isoprenoid side chain gives also information about the substitution pattern of the benzyl type moiety. In conclusion, in meroterpenoids the loss of the whole side chain as well as the characteristic neutral loss of a single isoprene unit provides useful information for their structural features.

**Novel Aspects:** Characterization of prenylated fungal metabolites and their derivatives by liquid chromatography negative ion electrospray tandem mass spectrometry.

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# Circular Dichroism Laser Mass Spectrometry: Report of Recent Progress and Outlook to Future Projects

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Six years ago, we started to develop a new method which combines circular dichroism and mass spectrometry. This was possible by using resonance enhanced multiphoton ionization with circularly polarized laser light as an ion source. We demonstrated the feasibility and reliability of the new method at the example of the chiral molecule 3-Methyl-cyclopantanone. Furthermore, we could study new features such as cumulative circular dichroism (two resonances are involved as in a (1 + 1 + 1)-multiphoton-ionization), circular dichroism of nonlinear spectroscopy (i.e. two-photon absorption) and chiral effects in consecutive absorption of molecular ions.

**Method:** Recently, we succeeded to improve the sensitivity for relative differences of the extinction coefficient e substantially [1] and De/e = 2 $(e_L - e_R)/(e_L + e_R)$ in the sub-promille range is now measurable. Thus, circular dichroism laser mass spectrometry (CD-LAMS) now is applicable to a considerable number of chiral molecules.

**Preliminary Data:** In the first part of the talk, the principle of CD-LAMS and the new developments will be explained. These include a twin-peak ion source of a time-of-flight mass analyzer, reversal of polarization by reflection of light and simultaneous ionization of achiral reference molecules. In the second part, the application of CD-LAMS to a list of chiral molecules will be presented. In some cases, comparison with conventional CD-spectroscopy in the gas phase revealed very good agreement. In most cases, only CD-values in the solution are available, which show comparable tendencies (e.g. sign of circular dichroism or dependence on the wavelength of the exciting light). In addition, recent results seem to prove, that CD-LAMS with short laser pulses is able to reveal fast structural changes to achiral isomers upon excitation or ionization. In the last part of this talk, an outlook will be given where the new method may be usefully applied. One such possibility is the use of CD-LAMS as a chirality sensor in fundamental studies of asymmetric catalysis on surfaces and nanoparticles. These studies are performed in ultra-high vacuum systems where all other types of chirality monitoring cannot be used. Another intriguing new development would be the combination of CD-LAMS with laser desorption of neutral molecules allowing access to large involatile molecules and biomolecules.

**Novel Aspects:** Improvement of the sensitivity for relative differences of the extinction coefficient. New possibilities to apply circular dichroism for chiral recognition.

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# Identification of psychoactive substances by LC-MS/MS-TOF after closing down 1378 smart shops in Poland

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Designer Drugs problem emerged in EU several years ago and appears in different countries with variable intensity and with a general trend to increase. Poland had the fastest growing market for new psychoactive substances in the EU with hundreds of hospitalizations resulting. In October 2010 the Polish Chief Sanitary Inspectorate undertook the following actions against designer drugs: the products containing psychoactive substances were immediately removed from the market and all sites manufacturing and distributing these substances were closed down. Over 12000 samples from smart shops were collected and delivered to six scientific institutes in Poland for investigations.

**Method:** In National Medicines Institute we have analyzed over 4000 various designer drugs and herbal highs products. The LC-MS/MS-TOF (MicrOTOF-QII, Bruker Daltonik) was applied for identification of samples' components in the first part of this study. MS/MS-fragmentation patterns have been used to elucidate the structures of unknown substances. The advantages of LC-MS/MS-TOF include its high specificity, sensitivity and ability to identify the unknowns even in small amounts; especially the use of TOF analyzer, which allows accurate mass measurements and hence the assessment of empirical formulas of unknown molecules is very useful in unknown products analysis. All new psychoactive substances have been confirmed by NMR analysis.

**Preliminary Data:** About 90 compounds (55 psychoactive) were identified including: substituted cathinones, phenethylamines, synthetic cannabinoids, phenylpiperazines, tryptamines, pharmaceuticals and other. The main problem with the designer drug market is its flexibility which allows to overcome legal regulations imposed by law. If distribution of a given compound becomes forbidden the market reacts instantaneously by introducing a homologue with alike psychoactive properties.

**Novel Aspects:** New psychoactive substances identified in this study have been reported to EMCDDA (European Monitoring Centre for Drugs and Drug Addiction).

# 9-aminoacridine: a powerful MALDI matrix – but with pecularities

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The selection of the most suitable matrix is a crucial step in MALDI MS [1] and particularly important if smaller molecules (such as (phospho)lipids) are to be analyzed because interferences between matrix and analyte signals have to be minimized. 9-aminoacridine (9-AA) has been suggested as a powerful matrix for lipid analysis [2] providing important advantages: (a) 9-AA offers a higher sensitivity in comparison to matrices such as 2,5-dihydroxybenzoic acid (DHB), (b) it favours the generation of $H^+$ adducts and suppresses the generation of alkali adducts, (c) the background is moderate and (d) 9-AA is particularly useful for the negative ion mode. Here, some disadvantages of 9-AA (related to the analysis of spermatozoa lipids) will be demonstrated.

**Method:** All chemicals, solvents, lipids and both matrices were obtained in the highest commercially available purity from Sigma-Aldrich (Taufkirchen, Germany) and used as supplied. Phospholipid standards were from AVANTI Polar Lipids (Alabaster, AL, USA). DHB was used as 0 5 M solution in methanol, while 9-AA was used in a concentration of 10 mg/ml in isopropanol/acetonitrile (60/40, v/v)) [2]. Lipid extracts (obtained according to the Bligh & Dyer method) from spermatozoa of different species were investigated as physiologically relevant samples. All lipid standards and lipid extracts were pre-mixed with the matrix (1:1, v/v) prior to deposition onto the MALDI target. Positive and negative ion spectra were recorded on an Autoflex workstation (Bruker Daltonics, Bremen) in the reflector mode.

**Preliminary Data:** All tissues or cells contain a large variety of lipids that can be identified in the organic extracts or in thin tissue slices by MS imaging. Besides zwitterionic (phosphatidylcholine (PC), phosphatidylethanolamine (PE) or sphingomyelin (SM)) or acidic phospholipids (PL) such as phosphatidylinositol (PI) there are also neutral (apolar) lipids (such as cholesterol or triacylglycerols). Whereas the latter are basically not detectable in the presence of 9-AA, all lipid classes can be identified in the presence of DHB if the positive and negative ion spectra are compared. However, when DHB is used to analyze a mixture of lipids, the detection of PE is normally impossible because the PE signals are suppressed by PC (positive ion mode) or PI (negative ion mode). This problem can be overcome if 9-AA is used because this matrix allows the detection of PE as negative ion. Unfortunately, unequivocal identification of PE is impossible because PC is also detectable as negative ion in the presence of 9-AA.Many diseases are accompanied by inflammation and, thus, the generation of "reactive oxygen species" (ROS) such as HOCl or hydroxyl radicals [3]. This leads to the generation of a variety of lipid oxidation products such as peroxides and lysophospholipids (LPL). In addition to diacyl PL, there are also alkenyl-acyl PL that are commonly termed "plasmalogens". Plasmalogens are particular abundant in stem cells and animal spermatozoa of several species [4]. The alkenyl ether linkage is particularly sensitive to oxidation and both, LPL and formyl-LPL, are generated under conditions of oxidative stress. These formyl-LPL are exclusively detectable with DHB but not with 9-AA as matrix. Although we have so far no convincing explanation for this discrepancy, it is concluded that caution is needed if the composition of complex lipid mixtures is determined from mass spectra recorded in the presence of 9-AA.

**Novel Aspects:** Significant differences between DHB and 9-AA were obtained regarding the detection of certain (phospho)lipids and their oxidation products.

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# Detailed mechanistic studies of a multifunctional catalyst in a one-pot reaction by Electrospray mass spectrometry

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In the last few years, organocatalysis has emerged as a new catalytic methods based on metal-free organic molecules. In many cases, these small compounds give rise to extremely high enantioselectivity. Usually the reactions can be performed under an aerobic atmosphere with non-anhydrous solvents. The catalysts can be easily synthesized in both enantiomerically pure forms and are often more stable than enzymes or other bioorganic catalysts. A new concept of multifunctional organocatalyst has been introduced based on catalytical peptide moieties [1]. This work includes a multi-reaction sequence ideally catalyzed by only one multifunctional catalyst in a one-pot synthesis. Herein, we present a mechanistic study of this one-pot cascade reaction with a multifunctional organocatalystby ESI-MS, and ESI-MS/MS.

**Method:** The mechanistic studies of different one-pot cascade reactions: desymmetrization / oxidation, epoxidation / epoxide-opening / desymmetrization systems with multifunctional organocatalyst was accomplished by ESI-MS using a Thermo TSQ Quantum Ultra AM triple quadrupole. Additional measurements were completed using a LTQ-FT-ICR MS system (Thermo Fischer Scientific FT-ICR MS 12 T) to make accurate mass measurements for signal interpretation. The first example, an acylation of meso-1,2-cyclohexane diol, was carried out with Acetic acid anhydride and the corresponding multifunctional catalyst A in dry toluene, followed with the oxidation step using m-CPBA as cooxidant. The second example is epoxidation of alkenes using the multifunctional catalyst B (5% mol) and hydrogenperoxide, N,N'-Diisopropylcarbodiimid followed with epoxide-opining and mono-acylation of the corresponding alcohol with Acetic acid anhydride.

**Preliminary Data:** The focus of this study was the investigation of organocatalytic reaction using a multifunctional catalyst. The catalytic reactions observed were. To achieve this goal reaction intermediates and their reactivity towards different substrates and the catalyst in each reaction step, especially with such a tough oxidation condition was investigated. One important point was the influence of the temperature on the reaction because unexpectedly the oxidation reaction appeared slower and showed a lower yield at room temperature comparing to 10 °C or even 0 °C which showed the best results. The reaction was fully studied, the important intermediates were detected and structurally confirmed by using MS/MS experiments and especially the reason for the temperature effect was determined. All relevant data will be shown and a proposed mechanistic cycle will be reported.

**Novel Aspects:** Development and application of electrospray mass spectrometry for detailed mechanistic studies of a multifunctional catalyst in a one-pot reaction.

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# Characterization of surgical aerosols – towards mass spectrometry-guided surgery

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Surgical aerosols, originating from disintegration of biological tissues using an electrosurgical instrument, were investigated by simultaneous measurements with a compact single-particle mass spectrometer LAMPAS 3, with a high resolution Orbitrap mass spectrometer and with an optical particle counter (OPC) [1]. Neutral and charged gas-phase biomolecules and aerosol particles are produced by electrosurgical treatment of porcine liver and kidney to a large extent. REIMS (Rapid Evaporative Ionization Mass Spectrometry) is a method for direct analysis of biomolecules under atmospheric pressure, applicable for the differentiation of tissue types and the identification of tumors, while single-particle mass spectrometry allows to investigate particulate matter.

**Method:** Evaporation of biological tissues was achieved by using standard electrosurgical equipment (Radiosurg 2200, Meyer-Haake, Germany). Chemical analysis of evaporated tissue was performed by bipolar single-particle time-of-flight mass spectrometry employing an atmospheric pressure inlet, and by an LTQ Orbitrap Discovery MS (Thermo-Scientific GmbH, Bremen, Germany). A commercially available optical particle counter (OPC type 1.109, Grimm GmbH, Germany) was operated simultaneously. Particles were detected and size-determined by two 532 nm laser beams, and ionized by a nitrogen laser beam (337 nm wavelength), that can be attenuated for reduction of fragmentation.

**Preliminary Data:** Rapid Evaporative Ionization Mass Spectrometry yielded tissue-type characteristic spectra, both of particulate and molecular matter, particularly in the negative ion mode. Orbitrap spectra featured high mass accuracies and were dominated by phospholipid signals and thermal decomposition products of phospholipids. For tissue discrimination and allocation, mass spectra were processed by Principal Component Analysis (PCA) and by Cluster Analysis in case of LAMPAS spectra. Aerosol particle numbers and particle number distributions were determined by an optical particle counter and by the LAMPAS 3 instrument in parallel. Combination of these methods (REIMS, LAMPAS and OPC) are expected to provide a comprehensive analysis of surgical aerosols, necessary for further refining mass spectrometry-guided surgery [2].

**Novel Aspects:** Analysis of particulate matter in Rapid evaporative ionization mass spectrometry.

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# Chemical Phosphorylation of Histidine Residues in Proteins and Peptides Assists in the Analysis of Acid-labile Phosphorylation Sites

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Mass spectrometry (MS) has been heavily used for the investigation of protein phosphorylation, but most studies dealt only with the hydroxyamino acids. This fact has mainly been attributed to the sensitivity of phosphoramidates and acylphosphates to low pH. Phosphohistidine, for instance, is estimated to be 10-100-fold more abundant in nature than phosphotyrosine, but its analysis is still largely based on traditional biochemical methods. We showed that this modification is not as fragile as commonly assumed and that His-phosphorylated peptides can be investigated by RP-LC-MS using acidic solvents [1]. In order to further elucidate their recovery after proteomic procedures such as gel electrophoretic protein separation, potassium phosphoramidate (PPA) was explored as chemical agent to generate model substances.

**Method:** PPA was produced via ammonium phosphoramidate [2-4]. In order to fully phosphorylate His-residues in proteins, they were dissolved in 1 mol/L aqueous PPA solution. The large excess of PPA was required because some of its activity was expected to be lost by hydrolysis. For softer reaction conditions, the PPA concentration was lowered by an order of magnitude in addition to the use of shorter incubation times. Samples stored at −80°C and thawed immediately before use. For MS experiments, samples were diluted with water. Modified protein was subjected to tryptic digestion in solution or following separation by 1D-PAGE. MS experiments were performed using Q-TOF Premier coupled to Acquity nanoUPLC (Waters Corp., Manchester, UK).

**Preliminary Data:** PPA is a mild phosphorylating reagent which does not modify the hydroxyamino acids. Its use does generate 1,3-pHis but mostly with low yield. 3-pHis is preferentially formed being the more stable isomer. Horse myoglobin having eleven His-residues was highly phosphorylated by that agent showing a distribution of modified protein forms with four phosphate-carrying His-residues in the most abundant species. All of the His-residues reacted, albeit to different degrees. Since myoglobin is a heme-binding protein it could additionally be demonstrated that synthetic phosphorylation may retain protein folding targeting only accessible His-residues. The phosphorylation procedure was extended to other standard proteins such as insulin, β-casein and cytochrome C. All of them could be phosphorylated on their His-residues and the corresponding peptides were detected in protein digest mixtures and background of a digest of *Escherichia coli* lysate. Intact protein forms could be separated by chromatographic methods. The unseparated reaction mixture was however used to test recovery of phosphorylated peptides after gel electrophoresis. As expected, lengthy procedures at low pH such as regular Coomassie-staining reduced recovery, but they can be circumvented by the use of much shorter silver-staining at higher pH. In summary, the use of PPA is a tool for reproducible protein modification delivering suitable peptide and protein standard compounds for method development and validation.

**Novel Aspects:** Use of chemical phosphorylation in proteomics method development for analysis of proteins phosphorylated on histidine residues.

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# Systematic study of copper and platinum complexes of peptide-heterocycle conjugates

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The application of electrospray mass spectrometry to the analysis of coordination compounds has a long history [1]. ESI-MS became also a method of choice in studying peptide-metal interactions, due to high resolution and sensitivity [2]. To increase the affinity for metal ions, peptides could be modified by introduction of heterocyclic fragments, containing 2,2`-bipyridyl motif [3]. Such conjugates combine the complexing properties of heteroaromatic compounds with physicochemical and structural features of peptide component. In our work we study the influence of the character and number of heterocyclic ring systems on coordinating properties of conjugates, as well as the conditions required for complex formation and ESI-MS analysis.

**Method:** Peptide scaffolds were synthesized on solid support using standard Fmoc procedure. Heterocyclic modifications were formed on-resin after the peptide assembly was completed, using general methods for respective ring systems. After cleavage from the support, the products were purified by preparative HPLC and used for complexation studies. Complexes formed on the resin were analyzed without purification. Interactions of conjugates with Cu(II) and Pt(II) ions were investigated using buffered water solutions as well as water-organic (methanol, acetonitrile) mixtures. The MS experiments were performed on micrOTOF-Q and Apex-Qe Ultra 7T mass spectrometers (Bruker Daltonics, Germany) equipped with standard ESI source. The stability of complex ions was analyzed using collision induced fragmentation (ESI-MS/MS). CE experiments were conducted according to standard procedures [4].

**Preliminary Data:** Several series of conjugates, containing quinoxaline, phenazine, benzimidazole and benzoxazole fragments, were synthesized and their affinity towards copper and platinum ions was investigated using ESI-MS. We analyzed the presence and composition of complex ions, as well as their fragmentation patterns in CID experiments. Capillary electrophoresis with UV detection was used as independent method of investigating metal ion binding [4], allowing for observation of novel components, including neutral species. Due to recent developments in solid-phase synthesis, it is possible to analyze the complexes pre-formed on-resin using LC-MS method. In order to investigate the relations between the results of MS experiments and the actual status of compounds in solution, we used two approaches. The first was based on comparing ligands in the same experimental setup (concentration, solvent, MS parameters), the diversification included character of heterocycle, number of heterocyclic moieties in one conjugate and length of peptide chain. The second compared MS spectra recorded for mixtures varying in pH, solvent composition (water, water with methanol or acetonitrile) and incubation time. With exactly the same parameters of MS experiment, the differences in MS spectra represent the changes occurring in solution or during electrospray. For a set of conjugates and control peptides, the differences in MS spectra therefore exemplify complexing properties. We conclude that the introduction of heterocyclic motif increases the metal ion affinity of the conjugate, moreover, for two modifications attached to one peptide a cooperation is observed, including stabilization of Cu(I) complexes. The results allow us to establish experimental conditions for screening compounds for metal ion affinity with all advantages of MS – minute sample consumption, sensitivity and structural information.

**Novel Aspects:** Systematic analysis of ESI-MS studies on metal ion affinity of peptide-heterocycle conjugates in context of structural diversity and experimental conditions.

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# Affintiy – Mass Spectrometry-based Cord Blood Serum Profiling Leads to an IUGR Proteome Signature

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Intrauterine Growth Restriction (IUGR) affects about 3 to 8% of all pregnancies. It is defined as a condition in that the fetus does not reach its genetically given growth potential, resulting in low birth weight. IUGR is an important cause of perinatal morbidity and mortality, thus contributing substantially to medically indicated preterm birth. The pathogenesis of IUGR still remains unclear [1]. Analysis of complex gestation-related pathophysiological conditions like IUGR requires methods that can cope with the complexity of the samples. We here describe mass spectrometric profiling of cord serum proteins to identify disease-related protein alterations that may become useful for diagnostics and perhaps even prognostics of gestation-associated diseases.

**Method:** Cord blood samples from 30 newborns (15 normal pregnancy and 15 IUGR samples) were taken post-natally from the umbilical cord vein. Serum was prepared using standardized protocols. Samples were processed using the Profiling Kit100 MB-HIC8. After elution, protein solutions were spotted directly onto a stainless steel MALDI target and mixed with ferulic acid in duplicate. Protein mixtures were analyzed with a Reflex III MALDI ToF mass spectrometer equipped with the SCOUT source and delayed extraction and operated in linear positive ion mode using an acceleration voltage of 20kV. Spectra were recorded in a mass range from 4-25 kDa, accumulating 900 shots per spectrum. Spectra evaluation was performed with the ClinProTools 2.2 software and in-house biostatistic analysis tools [2, 3].

**Preliminary Data:** Protein mixtures of cord blood samples were subjected to affinity fractionation in order to remove highly abundant proteins, such as albumin and immunoglobulins. After affintiy enrichment, MALDI mass spectra showed more than 60 protein ion signals between *m/z* 4000 and 25,000. The six best differentiating ion signals were at *m/z* 8205, 8766, 13,945, 15,129, 15,308, and 16,001, forming a proteome signature to distinguish between IUGR and controls. Apolipoprotein C-$III_0$ (*m/z* 8766, lacking glycosylation) was found more abundant in the IUGR samples, irrespective of gestational age. To assign ion signals of the proteome signature to specific proteins, cord blood samples were subjected to SDS PAGE followed by PMF. We identified apolipoprotein C-III, together with other apolipoproteins (apo A-I, A-IV), fetuin A, as well as others. Identification of Apo C-III was confirmed by MS/MS analysis. The MS/MS spectrum of the precursor ion at m/z 1196.61 showed a characteristic fragment ion signal at *m/z* 638.37 that was formed by a D-G cleavage together with other specific fragment ion signals confirming the amino acid sequence of peptide 41-51 from apolipoprotein C-III. Furthermore, the Apo C-III concentration in serum was measured by ELISA. Apo C-III belongs to HDL and is involved in triglyceride metabolism, which itself is discussed to be of importance in IUGR pathogenesis [4]. Our results show that protein abundance differences between IUGR and control cord blood samples can be determined reliably using an affinity-enrichment procedure combined with MALDI MS profiling. Mass spectrometry-based multiparametric analysis of cord blood samples is capable of differentiating individual samples from the IUGR group from those of the control group with high confidence. We suggested apolipoprotein C-$III_0$ as a potential key-marker of the IUGR proteome signature, and, as shown, that subtle alterations in glycosylation need to be considered for our understanding of the pathomechanisms in IUGR.

**Novel Aspects:** Multiparametric MS data analysis to identify a proteome signature of IUGR, providing a novel approach for IUGR pathogenesis investigations.

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# High-resolution LC-MS Analysis Reveals Novel Cellular Substrates of Clostridium difficile Toxins

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Clostridium difficile toxins A (TcdA) and toxin B (TcdB) are the major cause of hospital-acquired diarrhea. Both toxins are glucosyltransferases which glucosylate small Rho GTPases in intestinal cells of the host [1]. In detailed studies, the proteins RhoA, RhoB, RhoC, RhoG, Cdc42 and Rac1 have been identified as targets of both toxins. However, the glucosylation was analyzed in vitro with purified toxins and GTPases, neglecting differences from the in vivo situation. To analyze the intracellular glucosylation of small GTPases, protein extracts of colonic cells (Caco-2) treated with TcdA and TcdB were analyzed by high-resolution LC-MS analysis.

**Method:** Caco-2 cells were treated with recombinantly expressed and purified active TcdA or inactive TcdA for 24 hours. Protein extracts were separated by 1D-SDS-PAGE followed by nano-RSLC-LTQ-Orbitrap-MS analysis of tryptic peptides. For the quantification of proteins the SILAC method [2] was applied. Protein extracts of untreated cells were used as a global internal standard to identify the glucosylation pattern of small GTPases and proteomic changes in TcdA- vs inactive mutant TcdA-treated cells. In a follow-up study, the substrate spectra of additional wild-type TcdA and TcdB were determined by a targeted approach in which only the SDS-PAGE fraction of the small GTPases was analysed with a tailored LC-MS method. All MS data sets were processed with MaxQuant [3].

**Preliminary Data:** In our SILAC-based proteome analysis known and novel cellular targets of TcdA were confidently identified and the degree of glucosylation quantified. Glucosylation of RhoA, RhoC, and RhoG was about 90% and Rap1 as well as Rap2 were glucosylated to the same extent. Additionally a glucosylation of Ras and Ral was detected. As the sequence of the glucosylation-specific peptides is identical in two isoforms regarding Ral(A/B) and in three isoforms regarding (H/K/N)Ras, a precise and individual quantification was not possible however. This is the first time that the glucosylation of the GTPases by clostridial toxins in intact cells has been directly identified and helps answer so far unresolved questions. The GTPases Rap1 and 2 are involved in E-cadherin mediated cell-cell adhesion [4]. Thus, their complete glucosylation and inactivation by TcdA and TcdB nicely explains the loss of intercellular junctions and adhesions. The substrate spectrum extended by Rap, Ras and Ral, that was detected for recombinant TcdA was also found in protein extracts treated with wild-type TcdA and TcdB. However this targeted label-free approach did not yield quantitative data of the degree of glucosylation. The glucosylation analysis of the GTPases by gel electrophoresis and mass spectrometry presented here takes about as much time as a regular western-blot analysis. However it yields more meaningful data as it can be used for whole cell protein extracts. Additionally the addition of an isotope labelled global standard can be used to accurately quantify the degree of glucosylation of the substrate GTPases. With this method at hand, further experiments will be conducted to analyze the specificity of other CGTs in colonic cells.

**Novel Aspects:** High-resolution LC-MS was used to identify novel substrates of C. dfificile TcdA and TcdB that allow explanation of cellular effects.

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# Analysis of colorectal adenocarcinoma samples with Desorption Electrospray Ionization and multivariate statistical methods

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Desorption Electrospray Ionization (DESI) is an ambient ionization method first described in 2004 [1]. Ionization is achieved by directing a pneumatically assisted solvent spray onto the surface of interest, analytes are dissolved in the droplets and carried into the mass spectrometer. DESI is also applied for Mass Spectrometry Imaging where the surface of the sample is scanned in continuous lines. The spatial resolution is about 150 µm which is rough compared with other imaging techniques like MALDI or SIMS. It's a very simple and rapid method especially for the detection of lipid species.It has been shown previously, that the lipid composition of tissue species is unique and that the composition of lipids can be used to characterize samples [2].

**Method:** Colorectal adenocarcinoma samples were delivered as sections and stored at -85°C. H&E staining was carried out using an adjacent section to the measured one. The samples were analyzed in the mass range from *m/z* 600 to *m/z* 1000. To provide exact mass measurements, an LTQ Orbitrap Discovery from Thermo Fisher Scientific was used. Solvent was provided by a nanoLC instrument to ensure a stable solvent flow. The 3D-imaging stage from Newmark Systems Inc. is motorized and triggered by software to guarantee a constant scan speed throughout the measurement. The raw data of the line scans is converted to an imzML file using home-built software. The images are displayed in greyscale using the DataCube Explorer by Fom Amolf.

**Preliminary Data:** An ex-vivo tissue sample set of eighty primary colorectal adenocarcinoma and colorectal adenocarcinoma liver metastasis was analyzed in negative ion mode. The spatial distribution of phospholipids was determined by DESI Imaging Mass Spectrometry (IMS) with pre-set spatial resolution of 100 µm. The phospholipids detected in tissue samples comprise complex phospho- and sphingolipids including phosphatidyl-inositols, phophatidyl-ethanolamines, phosphatidyl-serines, phosphatidyl-ethanolamine plasmalogens, phosphatidic acids, phosphatidyl-glycerols, ceramides, sphingolipids and sulfatides among others. Lipids were identified based on exact mass measurements with the Orbitrap instrument and MS/MS fragmentation spectra. All histological tissue types found in the sample set were identified on haematoxylin-eosin stained sections. The different histological types of tissue (connective tissue, smooth muscle, healthy mucosa, healthy liver parenchyma, and adenocarcinoma) yielded characteristic phospholipid spectra. Although histologically specific markers were found for each individual section, no global markers were identified for the entire sample set. An alternative data analysis approach comprising multivariate statistical methods was also pursued. The full spectral data was analyzed with Principal component analysis (PCA) and Linear Discriminant Analysis (LDA). Phospholipid pattern of specific tissue types was found to be characteristic and reproducible for individual tissue types. Based on these experiments a reference spectral library was constructed, containing histologically specific data. The classification of unknown samples was carried out pixel-by-pixel, resulting in an exact and user-independent tissue identification method. In addition to the tissue identification experiments, KRAS status of the samples was correlated with the mass spectrometric data. Using the phospholipid pattern analysis with PCA and LDA, the separation of wild-type samples and KRAS mutant samples was achieved and the results are in agreement with the findings of genetic assays.

**Novel Aspects:** User independent pixel-by-pixel identification of tissue sections based on Principal Component Analysis and Linear Discriminant Analysis.

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# Software for 3D MALDI imaging data: Visualization and 3D spatial segmentation

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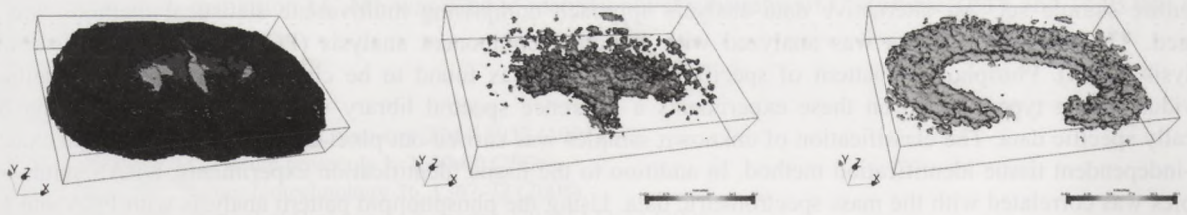
3D imaging has a significant impact on many challenges of life sciences. 3D matrix-assisted laser desorption/ionization imaging mass spectrometry (MALDI-IMS) is an emerging label-free biochemical analytical technique visualizing the distribution of hundreds of molecular compounds in 3D by providing a MALDI mass spectrum for each spatial point of a 3D sample. Currently, 3D MALDI-IMS cannot tap its full potential due to the lack of efficient computational methods for building, processing, and visualizing large and complex 3D IMS data. In this talk we present a collection of new efficient computational methods which enable creation, analysis, and interpretation of a 3D MALDI-IMS dataset.

**Method:** We present our software SCiLS Data Explorer for visualization and analysis of 3D MALDI-imaging data. It takes several 2D MALDI imaging datasets as an input, each dataset corresponding to a consecutive slice. Then, it creates a 3D cloud of measurement points in the 3D Cartesian space with coordinates (x, y, z), with a spectrum assigned to each point. For mining large 3D MALDI-IMS data, we propose using the spatial segmentation approach which is well-known in 2D MALDI-IMS [1]. In order to reduce significant spectrum-to-spectrum variation, we propose to use edge-preserving 3D image denoising prior to segmentation [2]. For segmentation, we developed a new efficient clustering method, called as bisecting k-means, which is suitable for hierarchical clustering of a large 3D MALDI-IMS dataset.

**Preliminary Data:** For demonstration of the software we have measured the central part of a mouse kidney, using 33 serial sections of 3,5 µm thickness after the PAXgene tissue fixation and paraffin embedding. Individual serial sections were measured using 2D MALDI-IMS following the standard protocols with the high spatial resolution of 50 µm. Altogether, 545,869 mass spectra were acquired that corresponds to approximately 50 GB of data. Our computational pipeline allowed us to process such large dataset and to reveal the 3D kidney anatomical structure based on mass spectrometry data only. Automated analysis revealed discovered *m/z*-values co-localized with the major anatomical regions, i.e. those corresponding to molecular compounds specific to anatomical regions.



**Novel Aspects:** Our Software, SCiLS Data Explorer, enables efficient and user-friendly computational algorithms specifically developed for 3D MALDI-imaging.

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# Spatial Proteomics: A new LC-MS/MS Tissue Imaging Workflow Providing Protein Distributions and their Identities in Tissue

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MALDI-Imaging of proteins in tissue sections represents a powerful new approach to biomarker discovery and histopathological research. However, the lack of direct identification strategies continues to be an obstacle preventing its broader use in Proteomics studies. Initial studies that utilized *in situ* digestion followed by MALDI-MS/MS analysis typically provided 5-50 peptide IDs of only 1-5 high abundant proteins.

**Method:** Here we introduce a novel proteomics technology that combines the spatial information with the routine identification of proteins from tissue sections. Highly resolved protein digests are generated by applying trypsin onto two subsequent tissue sections by supersonic nebulization. One of the sections is then analyzed by MALDI imaging mass spectrometry. Peptides are extracted from the other section and submitted to routine LC-MS/MS analysis using MALDI-TOF/TOF. The identified peptide list is then matched to the image and the co-localization of 2 or more tryptic peptides confirm their protein association.

**Preliminary Data:** We analyzed rat organs using the new Spatial Proteomics approach yielding peptide distributions at the 50-100 µm level. In brain, more than 100 peptides were identified and more than 20 proteins localized without the need for MS/MS analysis directly from the tissue. The intensity, co-localization of 2 or more peptides and the degeneracy of molecular weight of peptide-to-protein mapping were used as primary validation tools beyond significant mascot scores from peptide identification. As an extension of the established top-down imaging strategy, this bottom-up Spatial Proteomics approach may facilitate the identification and simultaneous localization of a much greater number proteins than it was previously possible.

**Novel Aspects:** A new LC-MS/MS Tissue Imaging Workflow providing Protein Distributions and their Identities in Tissue.

# Ion yields in UV-MALDI mass spectrometry as a function of laser wavelength and physico-chemical properties of classical and halogen-substituted matrices

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The laser wavelength constitutes a key parameter in UV-MALDI mass spectrometry. Optimal analytical results are only achieved at a sufficiently high optical absorption of the matrix. A second key element is a low proton/cation affinity (PA/CA) of the matrix that enables efficient protonation/cationization of analyte compounds and can be achieved by incorporation of electron withdrawing halogens. However, the accompanied blue-shift of the absorption band may necessitate adjustment of the laser wavelength to values below that of the standard $N_2$/Nd:YAG lasers (337/355 nm). Here, we studied the wavelength dependence of matrix and analyte ion yields between 280-355 nm using two classical matrices (DHB, HCCA) and five halogen-substituted cinnamic acid derivatives with PAs differing by as much as 60 kJ/mol.

**Method:** Experiments were performed with an orthogonal TOF mass spectrometer equipped with a modified oMALDI2™ ion source (AB Sciex) and, in some experiments, an axial Reflex-III TOF-MS (Bruker). A Nd:YAG-laser pumped dye laser (FL2001, Lambda Physik) emitting pulses of ~6 ns duration was used for desorption/ionization. The laser wavelength was varied in 5 nm-steps with pulse energies between 1.5-23 µJ, thereby generating data cubes consisting of 93 signal intensity points recorded per matrix. The laser spot size was carefully kept constant at ~200×400 µm$^2$. Proton and sodium cation affinities were calculated at the time-consuming MP2(Full)/6-311+G(*2d,2p*) level of theory without frozen core. Halogen-substituted matrices were synthesized as described previously [1]. Peptides and oligosaccharides were utilized as test compounds.

**Preliminary Data:** The following matrices were investigated: 2,5-dihydroxybenzoic acid (DHB); a-cyano-4-hydroxycinnamic acid (HCCA); 4-chloro-a-cyanocinnamic acid (ClCCA); a-cyano-2,4-difluorocinnamic acid (DiFCCA); a-cyano-2,4,6-trifluorocinnamic acid (TriFCCA); a-cyano-4-trifluoromethylcinnamic acid ($F_3$CCCA); a-cyano-2,3,4,5,6-pentafluorocinnamic acid (PentaFCCA). Absorption spectra were recorded in solution and in the solid state using an integrating sphere. Highest molecular analyte ion signals $[M + H]^+$ were obtained at the solid state peak absorption of the matrices and extending to longer wavelengths. A much stronger decline of molecular analyte ion signals was observed at the low-wavelength shoulder of the matrix-specific absorption band, resulting from increased ion fragmentation. In contrast, the total ion counts appear to follow the solid state absorption curve closely, i.e., similar total ion counts are obtained for wavelength-fluence combinations at which the same volume energy is deposited into the surface layer. Difference plots of spectra with and without analyte allowed the identification of ranges of relative proton transfer yields from matrix to analyte. In regions of high molecular peptide intensities, up to 60% of all matrix charges are consumed for analyte protonation. These values increase up to 100% for low pulse energies and longer wavelengths (matrix suppression effect). Optimal use of multiple halogenated matrices required adjustment of the laser wavelength to values below the standard ones of 337/355 nm. For example, DiFCCA [2], which provides the best MALDI performance (lowest LOD), is optimally employed at 305 nm. Poor crystallization and increased formation of matrix clusters currently hinder effective use of the two most reactive compounds, F3CCCA and PentaFCCA, exhibiting extremely low PAs of 790 and 771 kJ/mol. Its correspondingly low sodium cation affinity, however, makes PentaFCCA ideally suited as a potentially new matrix for the MALDI-MS analysis of oligosaccharides (which is not as dependent on co-crystallization constraints). $[M+Na]^+$ ion signal strengths of oligosaccharides desorbed with PentaFCCA were comparable to those obtained using the standard DHB matrix.

**Novel Aspects:** First study of the wavelength dependence in UV-MALDI mass spectrometry covering a wide wavelength range and classical and novel matrices.

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# Water Ice as a Matrix for the Analysis of Glycosaminoglycans in Infrared Matrix-assisted Laser Desorption/Ionization Mass Spectrometry

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Glycosaminoglycans (GAGs) are a class of complex glycans that form linear chains of diversely substituted disaccharide building blocks. Heparin oligosaccharides comprise a backbone of 4GlcAβ1-4GlcNα1-subunits which can be N-sulfated/acetylated and O-sulfated at C2 position of the GlcA and at C6 position of the GlcN residue [1]. To explore the chemical heterogeneity GAGs are usually enzymatically digested to a lower degree of polymerization (dp) and subsequently analyzed by mass spectrometry. However, MS analysis of GAGs is limited by the lability of the sulfate groups complicating a detailed structural characterization [2]. Here, we demonstrate the use of water ice as an extremely "soft" matrix allowing the infrared laser desorption/ionization of intact GAG molecules with only minor extent of fragmentation/loss of sulfate groups.

**Method:** Mass spectra were recorded with a modified Q-Star Pulsar I (AB Sciex) orthogonal TOF mass spectrometer in negative ion mode. Samples were prepared on an infrasilTM glass slide, frozen in liquid N2, and mounted on an in-house-built cool stage, based on cooling with liquid N2 and counterheating with a Peltier element. Temperature of the sample plate was adjusted to ~−80°C sufficient to prevent sublimation in the vacuum of the MALDI source (~1.5 mbar). For IR-MALDI an Er:YAG laser ($\lambda$ = 2.94 μm, $\tau$ ~150 ns) was used. Various heparin-derived disaccharides were analyzed. The results obtained with the ice matrix were compared with those obtained by use of glycerol as standard matrix for IR-MALDI-MS. Selected ions were submitted to $MS^2$ analyses for structural elucidation.

**Preliminary Data:** In this study, water ice was used as a new and especially "soft" matrix for the analysis of labile sulfated carbohydrates by infrared matrix-assisted laser desorption/ionization (IR-MALDI) mass spectrometry. The neg. ion mode mass spectra were dominated by intact deprotonated GAG species and showed only minor extent of fragmentation (i.e., loss of the sulfate group) of the labile compounds. Depending on the degree of sulfation the formation of highly charged ions (doubly- and even triply-charged ions) was observed which is very uncommon for the MALDI-MS analysis of low molecular mass biomolecules. When deprotonated GAG-derived molecules were submitted to low-energy CID tandem-MS analysis, loss of sulfate groups represented the main fragmentation processes, accompanied by cleavage of the glycosidic bonds as well as cross-ring cleavages. Since the SO3-groups were eliminated prior to the latter fragmentations no information on the position of the sulfate groups could be obtained from these experiments. However, fragmentations giving rise to improved structural information were obtained from sodium adducts of GAG analytes also present in the IR-MALDI mass spectra. These ionic species appear to exhibit a remarkable stability of the sulfate substituents allowing the formation of fragment ions that arise from either cross-ring cleavages or rupture of the glycosidic bonds and providing an unambiguous assignment of the sulfation sites. In contrast, when glycerol was employed as matrix at room-temperature a higher fraction of analyte ions decomposed by loss of labile sulfate groups. In addition, extensive formation of matrix-adducts as well as a more intense chemical background complicates the use of glycerol matrix.

**Novel Aspects:** IR-MALDI-MS with a water ice matrix constitutes a "soft" and powerful method for the analysis of labile glycosaminoglycans.

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# DART-TOF-MS: A time-saver in analytical chemistry

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A wide range of different techniques that can be summed up under "ambient ionization mass spectrometry" has been developed and some of them even commercialized throughout the last years. The most prominent representatives of this group are desorption electrospray ionization (DESI) developed by Cooks [1] and direct analysis in real time (DART) first presented by Cody et al. [2]. Major fields of application of these techniques are the direct analysis of solids with numerous reports, many of them related to forensic analysis or pharmaceutical analysis, but also imaging of surfaces (such as tissue cuts or TLC plates) is possible. In the present paper we would like to present applications of DART slightly beyond its accepted usage.

**Method:** Measurements were carried out on a JMS T100 (AccuTOF-LC-plus) time-of-flight mass spectrometer(TOF-MS) (JEOL Ltd, Tokyo, Japan) with a DART ion source from IonSense, Inc. (Saugus, MA, USA). For stir bar sorptive extraction (SBSE) Twister stir bars from Gerstel (2 cm length coated with a 0.5 mm layer of polydimethylsiloxane) were employed. For the coupling of HPLC to DART-TOF-MS a lab made interface described in detail in a previous publication was used [3].

**Preliminary Data:** SBSE is frequently used for the extraction of contaminants from aqueous matrices. Subsequently the stir bars are anaylzed by GC-MS after thermodesorption. A common drawback of this approach is that its time consuming and a lot of hours are wasted analyzing not-contaminated samples. Due to its ability for fast identification of compounds that are absorbed on surfaces or that have migrated slightly into the PDMS, DART-TOF-MS is a perfect tool for the rapid (semi-quantitative) screening of stir bars [4]. Thereby environmental water samples can be categorized in contaminated ones, requiring further quantitative analysis by GC or LC and non contaminated ones without need of further testing. Another not routine usage of DART-TOF-MS is its application as a detector in HPLC. DART has a relatively low tendency towards ion suppression particularly when compared with electrospray ionization (ESI), a fact that can be exploited in two ways; first the use of non-ESI compatible eluents in LC [3] and second, the analysis of samples with complex matrices often accompanied by unwanted ion suppression effects [5]. HPLC-DART-TOF-MS was used for solving a problem common in in pharmaceutical industries. Validated, filed methods (usually employed with UV detection) sometimes have to be combined with MS to allow the identification of unknown compounds. DART tolerates the use of non MS-compatible eluents like phosphate buffer even at concentrations above 100 mM, helping to avoid the necessity of developing an ESI compatible method, often in use for a single run only. Secondly, DART ionization helped to facilitate the analysis of samples with complex matrices, as often found in environmental analysis, food analysis or the analysis of biological fluids. Substantially reduced ion suppression (compared to ESI and APCI) allowed the direct analysis of sewage treatment plant effluents, river water and biological fluids without pre-treatment and with only minimal chromatographic separation.

**Novel Aspects:** Two non-routine applications of DART, HPLC-DART-MS and DART-MS for screening in SBSE help to save valuable time in analytical laboratories.

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# ToF-SIMS studies of the surface properties of Co/SiO2 catalyst for Fischer – Tropsch synthesis

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Literature data exhibits that supported cobalt is one of the most popular catalysts for Fischer-Tropsch synthesis [1]. However, it appears that a choice of the optimal conditions of the preparation and treatment of the catalyst is crucial for its catalytic activity in the process mentioned above. Especially, that the synthesis of highly active and selective catalyst demands a compromise between cobalt dispersion on the catalyst support surface and the reducibility of the cobalt oxides [2]. Therefore, knowledge about changes in the surface composition occurred during subsequent steps of the catalyst treatment is necessary for the development of more effective catalytic systems.

**Method:** The characterization of the catalyst surface can be performed using various analytical methods (i.e. XRD, XPS, TEM, SEM-EDS, FTIR, etc.). Nevertheless, in spite of many advantages these methods have also considerable limitations. For this reason we decided to apply also time-of-flight secondary ion mass spectrometry (ToF-SIMS). It is known that ToF-SIMS can give information from the upper layer of the investigated material with high mass resolution. Moreover, it is possible to obtain data about not only elemental composition of the catalyst, but also neighborhood of metal atoms on its surface. On the other hand, difficulties that arise during the collection of spectra are mainly connected with matrix effect, fragmentation reactions and quantification problems [3-5].

**Preliminary Data:** The main goal of this work is to determine the influence of the preparation method and treatment conditions of Co/SiO2 catalyst on its surface composition, cobalt oxidation degree and metal dispersion. Furthermore, the investigations are focused on the formation of different phases of cobalt oxides, cobalt silicate and decomposition of cobalt nitrate – catalyst precursor. The results of ToF-SIMS measurements allowed to select the optimal conditions of the synthesis of the catalysts and gain knowledge about the factors determining catalytic activity of the studied materials.

**Novel Aspects:** Application of ToF-SIMS to the studies of phase composition of cobalt supported catalyst.

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# Sources of inaccuracy of isotopic ratio measurements by MC ICPMS: Comparison of wet and dry plasma conditions

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The plasma source mass spectrometers are considered to be less accurate in recovering the true isotopic ratios in comparison to the more traditional TIMS instruments. Several different causes have been mentioned, however, inaccuracy of the mass bias correction seems the most significant. Studies of isotopic fractionation in the ICP source brought some authors to the conclusion, that the instrumental mass bias does not strictly follow the exponential law (e.g. Belshaw et al., 1998, Vance and Thirlwall 2002). Secondary normalisation procedures have been introduced, which help to improve both precision and accuracy. Additionally, Thirlwall and Anczkiewicz (2004) showed that problems related to secular drift among the normalized ratios can significantly be diminished by applying multidynamic rather than static mode of measurements.

**Method:** Isotopic ratio measurements of commonly used interlaboratory standards of Sr (SRM987), Nd (JNd-1), Yb (ICPMS – Merck) and Hf (JMC 475) were conducted by MC ICPMS *Neptune*. Wet and dry plasma conditions were applied with the use of the Stable Introduction System of Thermo (SIS) and the membrane dessolvating nebuliser AridusII by CETAC. Both systems were tuned to maximum sensitivity while keeping oxide level between 3.0-3.5% for SIS, and <0.5% for AridusII. Isotopic ratios were collected in static and multidynamic modes. All ratios were corrected for the collector gains, baseline (measured as "on-peak zeroes"), isobaric interferences, and instrumental mass bias. Precision is given at 2SD (standard deviations) level and the data outside ±2SD were rejected.

**Preliminary Data:** Static measurements of isotopic composition of Sr, Nd, Yb and Hf collected over 3 years period are presented. Accuracy was tested by comparison with the commonly accepted TIMS values, except for Hf, whose accuracy was verified by comparison with Hf multidynamic ratios, which were demonstrated to be less affected by secular drifts, and thus are more reliable (Thirlwall and Anczkiewicz, 2004). Both accuracy and precision of majority of the isotopic ratios obtained in this study are comparable to the reported TIMS values. However, in general, better precision and accuracy are achieved using dry plasma conditions. Isotopic ratios of Nd show systematic departure from the recommended values, proportional to the average mass difference between the normalized and the normalizing ratio, which was also observed by Vance and Thirlwall (2002) and Thirlwall and Anczkiewicz (2004) for different mass spectrometers. Again, the differences are much larger for the wet, than for the dry plasma. Additionally, for the wet plasma the departure of isotopic ratios from the recommended values correlates with the magnitude of deviation from the exponential mass bias fractionation curve. Inaccuracy of measurements using dry plasma could be partially related to inappropriate gas flow tuning of the membrane dessolvating nebuliser. Series of experiments conducted with the extreme gas settings, show that miss-tuned gas flow may introduce significant non-exponential mass bias component, which in turn leads to a large departure of the isotopic ratios from the "true" values. However, application of a secondary normalisation procedure introduced by Thirlwall (1991) results in highly accurate $^{143}Nd/^{144}Nd$ value and improves its external precision from 27 to 16 ppm (2RSD). Such secondary normalizations still seem to be inherent feature of MC ICPMS measurements, at least, in applications requiring top precision.

**Novel Aspects:** Demonstration of long term reproducibility of all isotopic ratios of Sr, Nd, Yb and Hf using wet and dry plasma.

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# Determination of Total Mercury Content in Wood Materials Using ICP-MS in Comparison to ICP-OES with Mercury Cold Vapor Technique

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Mercury is a well known transition metal; versatile used in various industry branches and at the same time the subject of controversial discussions about its effects to organisms. Mercury poisoning may cause severe damages of liver, kidney, brain, and central nervous system [1]. As a result of environmental pollution mercury may entering the human food chain [2, 3]. Due to wood preservation agents containing mercury, which were used since 1832 up to the middle of the 20th century [4], waste wood samples such as railroad ties or telegraph poles must be disposed on a save way. In this study, a mass spectrometry based method for sensitive mercury determination in wood samples was evaluated and compared with classic spectroscopic methods.

**Method:** In the literature a variety of techniques and methods for mercury determination is described. In recent years, multi element methods will gain a higher importance [5]. In this study, the total mercury amount in wood samples was determined with ICP-MS and ICP-OES using mercury cold vapor technique. The sample preparation was performed using microwave digestion with various types of microwave vessels and acid compositions. Certified wood reference material was used for the determination of repeatability, within-laboratory precision, method stability, measurement precision, recovery as well as the limit of detection and determination. Furthermore, a multitude of real samples such as natural wood samples from the local environment and treated wood such as railroad ties, telegraph poles and construction timber were analyzed.

**Preliminary Data:** The preparation of wood samples was performed using microwave digestion. Two vessel types (XP1500- and Xpress-vessels from CEM, Germany), nitric acid and aqua regia were used in the digestion procedure. For the determination of the repeat accuracy, up to 39 microwave digestions of certified reference material with a mercury content of 0.60 ± 0.14 mg/kg were performed and measured. Following coefficients of variation (CV) were determined: 8.69% (XP1500-vessels) and 8.35% (Xpress-vessels) by using aqua regia and 10.88% (XP1500-vessels) and 12.93% (Xpress-vessels) by using nitric acid. The recovery was calculated (according to measurement average and mean content of reference material) with the following results: 101.67% (aqua regia) and 96.67% (nitric acid) by using XP1500-vessels and 93.33% by using both acid compositions and Xpress-vessels. Further experiments were performed using XP1500-vessels and aqua regia. The within-laboratory precision was determined with three sample preparations and measurements at five consecutive days. The CV showed values of 0.94%-5.61%. The measurement precision was determined with ten measurements of the same sample and the resulting CV was 1.25%. The method stability was investigated with three samples at five days. After sample preparation each sample was split in five parts and one of them immediately measured. The other parts were frozen, at following four days thawed and measured. The values of CV were in the range 1.64%-3.21%. The experiments show the stability and the robustness of the sample preparation and measurement method. Finally, real samples were measured. The limit of detection (LOD) of the ICP-MS method was performed with ten procedural blank samples and was about 5.735 ng/L (2.294 ng/g wood material). The LOD of the ICP-OES cold vapor method was calculated by device software and was about 3.493 ng/L (1.397 ng/g). In contrast to ICP-OES cold vapor technique, ICP-MS is a multi element method and enables a sensitive mercury determination.

**Novel Aspects:** Rapid determination of mercury and further environmentally relevant element in wood samples; low detection limit; comparison of different measurement techniques.

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# In-vitro investigation of possible intracellular transformations of ruthenium-based anticancer drug-protein adducts by CE-ICP-MS and ESI-MS

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It was unequivocally confirmed for one of the most promising anticancer drug candidates, indazolium *trans*-[tetrachlorobis(1*H*-indazole)ruthenate(III)], that protein-bound form(s) is its major appearance in the bloodstream after intravenous administration [1]. More specifically, transferrin, involved in iron transport, is believed to be responsible for the drug delivery into cancer cell [2]. Inside the cell, the metal–transferrin adduct is thought to undergo decomposition due to an acidic milieu, resulting in a release of the Ru(III) moiety [3]. It is also hypothesized that in the hypoxic tumor tissue, the liberation of the metal is accompanied by the formation of reactive Ru(II) species, which more rapidly coordinate biomolecules [4].

**Method:** The influence of cytosol complexing and reducing agents on the stability of metallodrug adducts with main serum transport proteins, albumin, apo-transferrin and holo-transferrin, was investigated by capillary electrophoresis with inductively coupled plasma MS (CE-ICP-MS, Agilent Technologies) and electrospray ionization MS (ESI-MS, Agilent Technologies) under abnormal conditions of cancer cell cytosol. While it has early been proved that alterations in metal speciation can occur directly in the capillary, i.e. during electrophoresis [3, 5], in this study the preferences was given to an off-line mode. An ultrafiltration step was therefore implemented to separate the adduct from low-molecular-mass transformation products in the presence of citric acid, ascorbic acid, and glutathione. The ultrafiltrate was then analyzed by either analytical method.

**Preliminary Data:** The effect of physiological concentrations of cytosol components on the stability of protein–drug adducts was examined, considering an additional signal(s), emerging in CE-ICP-MS electropherograms as a sign of formation of a new metal species. All tested compounds were found not to enforce compositional changes of adducts but that of holo-transferrin, forming a second peak under the action of citric acid, ascorbic acid, and glutathione. It is important to note that this finding was clearly seen in electropherograms recorded only at a *m/z* of iron that requires further elucidation. When the same reactions were tracked by ESI-MS, new signals were observed in ultrafiltrates (at *m/z* 671, 655, and 778.5), corresponding to mixed-ligand conjugates in which the parent drug is coordinated by a cytosol component. Along with this, the fragmentation behavior of conjugates was different to that of the drug molecule, with the chlorido (and auxiliary) ligands leaving before indazolium. This proves that cytosol components may play an important role in releasing the ruthenium functionality from the protein adduct. Further ESI MS trials, possibly with coupling to CE, would allow us to explain the nature of additional Ru species found out by CE ICP MS. This work has been supported by the European Union in the framework of European Social Fund through the Joint UW and WUT International PhD Programme (Magdalena Matczuk) – "Towards Advanced Functional Materials and Novel Devices" realized by Warsaw University of Technology.

**Novel Aspects:** Presented for the first time that cytosol components with reducing/complexing properties affect the integrity of ruthenium-based drug-protein adducts.

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# Binding abilities of copper to phospholipides

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Cell membranes as well as membranes of inner organelles in cells are built from phospholipides [1]. Phospolipides are also used for the preparation of artificial membranes, which are mostly stabilized using multiply charged lanthanide ions [2]. Also other charged cations are able to create complexes with phospholipides. The formation of these complexes might influence the membranes' properties such as permeability, impedance, and wettability.

**Method:** Electrospray-ionization mass spectrometry (ESI-MS) was used to probe the ability of copper (an essential element for majority of living organisms) to create complexes with lecithin (L; dipalmitoylphosphatidylcholine), which represents a representative phospolipide with sum formula $C_{40}H_{80}NO_8P$ and molecular mass of 734 g $mol^{-1}$.

**Preliminary Data:** In the ESI mass spectrum are shown copper complexes with L, which can be divided into two main groups; note that all listed *m/z* values refer to the light isotopes ($^{1}H$, $^{12}C$, $^{35}Cl$, $^{32}P$, $^{16}O$ and $^{63}Cu$): (i) dicationic complexes: $[Cu(L)_2]^{2+}$ (*m/z* 765), $[Cu(L)_3]^{2+}$ (*m/z* 1132), $[Cu(L)_4]^{2+}$ (*m/z* 1498) and $[Cu(L)_5]^{2+}$ (*m/z* 1865); (ii) monocationic complexes with $Cl^-$ originated from the metal salt precursor: $[CuCl(L)]^+$ (*m/z* 831) and $[CuCl(L)_2]^+$ (*m/z* 1565). Lecithin itself is known as a zwitterion and has high affinity to protons ($H^+$), which allows creating very stable complexes with anions [2]. The presence of protonated L was also proved, $[LHL]^+$ (*m/z* 734) $[L_2H]^+$ (*m/z* 1467) were found. Under ESI conditions, copper is easily reduced from Cu(II) to Cu(I) [3], both species are often present in complexes with various ligands [4]. However the complexes with Cu(I) were of low abundance in the spectra, e.g. $[Cu(L)]^+$ (*m/z* 796).

**Novel Aspects:** This study helps estimating: (i) desirability of copper to stabilize artificial membranes, (ii) potential risk of copper binding on biomembranes.

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# A multi-reflection time-of-flight mass separator for high-resolution beam purification at ISOLTRAP

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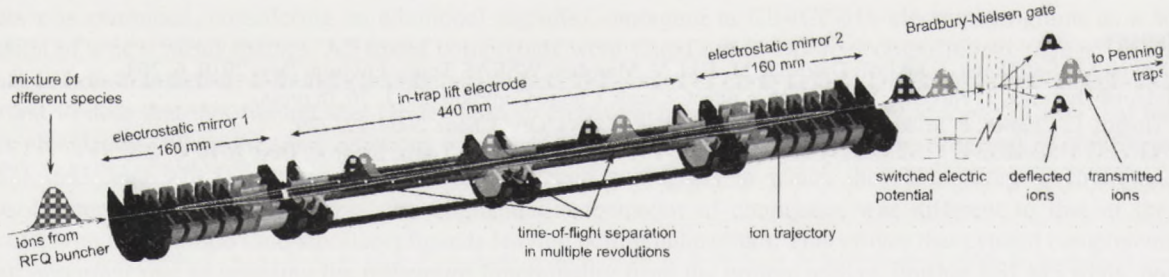
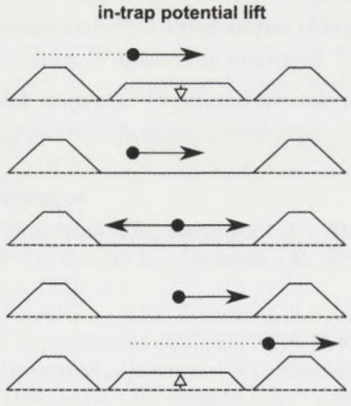
High-precision Penning-trap mass measurements of short-lived nuclei with half-lives even below 100 ms are performed with ISOLTRAP at ISOLDE/CERN routinely reaching a relative mass uncertainty of $10^{-8}$ [1]. The nuclides investigated range from light – such as $^{17}$Ne – to heavy ones – such as $^{233}$Fr. The mass data are important for, e.g., nuclear-structure studies concerning shell closures and residual interactions. Valuable input has also been provided for the modeling of neutron and proton rapid-capture processes during the nucleosynthesis in stellar environments. An important pre-requisite of precision mass measurements is the preparation of purely isobaric ion ensembles. To this end a multi-reflection time-of-flight mass separator (MR-TOF MS) developed at Greifswald [2] has recently been implemented at the ISOLTRAP setup.




**Materials and Methods:** The MR-TOF MS consists of two electrostatic ion-optical mirrors, between which the isobaric ion ensemble oscillates, and a Bradbury-Nielsen gate, see sectional illustration above. After the ions of interest are separated from contaminant species of different mass-over-charge ratios m/q, they are transmitted to the Penning traps whereas the contaminants are deflected and lost. A mass resolving power of R = 200.000 and a contaminant reduction of four orders of magnitude have been achieved. A pulsed drift tube between the ion mirrors facilitates ion capture and ejection (see illustration to the right) and provides a simple but efficient control of the MR-ToF time focus [3].

**Results:** The performance of the combined setup (including an RFQ ion buncher, the MR-ToF MS and the two Penning traps) in both off-line testsas well as in first applications with radioactive ion beams will be presented. Furthermore, the physics case and recent result of mass measurements of neutron-rich zinc will be shown.

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# Mass Spectrometric Analysis of Spatial Metabolite Distribution Pattern during Barley Grain Development

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Investigation of plant tissues with MALDI-MSI is quite recent and protocol optimization is needed as plant organs comprise complex tissue types. We are interested in the elucidation of components involved in barley grain development. An understanding of processes affecting agronomical traits such as final grain weight, seed quality and stress tolerance is of outmost importance, as barley provides one of the staple foods. We present an MALDI-MSI approach to the analysis of metabolite patterns. A number of candidate *m/z* values showing high tissue and developmental specificity were revealed and examples will be shown. Challenging aspects regarding spatial resolution, sensitivity and identification of compounds will be discussed. Finally, approaches for quantification of metabolites by LC- and GC-MS and for relative quantification of metabolites by DESI-MS will be presented.

**Method:** MALDI *imaging* MS was carried out using longitudinal as well as cross sections of barley grains from different developmental stages. Using DHB as matrix for small mass range analysis gave best results regarding application strategy, sensitivity and matrix derived background [1]. Fragment pattern analysis of selected m/z values, database searches and literature data were used for classification of candidate substrates. Current approaches for identification of unknowns and relative quantification of compounds include dissection of barley grains and fractionated extraction of substrates regarding their chemical properties. Subsequent targeted measurements are currently carried out by LC-MS and GC-MS.

**Preliminary Data:** With MALDI MSI approach characteristic abundances and distributions of several compounds for prestorage phase and storage phase of barley grain development were observed. First analytical efforts allowed for assignment of a number of the candidate *m/z* values to various metabolite classes, such as sugars, phospholipids and chlorophyll, directly from tissue sections. Our results revealed particular metabolite distributions indicative for nutrient transport into the developing endosperm and for grain hardness (e.g. dough and germination properties).

**Novel Aspects:** Application of MALDI MSI to analysis of metabolite patterns during barley seed development and MS-based compund identification from dissected tissues.

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# Expanding role of in vitro simulation of cytochrome P450 metabolism for drugs of abuse

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Currently, drug addiction is a cause of severe medical, social and economical problems. Besides the price, which is paid by the abusers themselves, the whole society bears the costs related to medical treatment, crimes, epidemics and infections (HIV, hepatitis). Our knowledge on the biological background of drug addiction is constantly increasing, but still many mechanisms are not examined. Nowadays, narcotic metabolites are generally studied using liver cells extracted from animals. Cytochrome P450 that is present mainly in microsomes is responsible for oxidation of the majority of drugs [1]. It was also shown, that metabolism of popular medicines may be successfully simulated by electrochemical oxidation under appropriate conditions [2]. The presented experiments feature precise comparison of these two entirely distinct techniques.

**Method:** Firstly, in this project validation of the incubation of narcotics with rat liver microsomes was performed. Incubation time was optimized for maximally efficient formation of metabolites and examination of the metabolism processes. The produced metabolites were analyzed and identified with the LC-MS/MS system in the MRM mode. Secondly, the flow electrochemical cell with three electrodes (Antec, the Netherlands) was used. Four different types of working electrodes were applied: Glassy Carbon (GC), Magic Diamond (MD), gold and platinum. Potential applied at working electrode and composition of the solvent were optimized for the best conditions for oxidation process. All products were directly analyzed by mass spectrometry with electrospray ionization (ESI) and CID (collision induced dissociation, MS/MS) fragmentation.

**Preliminary Data:** The obtained results for cocaine oxidation for variety of solvents on a Magic Diamond working electrode show that it is possible to create one of the most important metabolite of cocaine present in human body – norcocaine. This metabolite, serving as an evidence of drug taking, can also be produced by incubation of cocaine with rat liver microsomes, as the compound in a larger group of obtained metabolites. The EC system provides a new platform for drugs screening of the phase I metabolism. The metabolites can be directly analyzed by MS or collected and separated by liquid chromatography. In this experiment it was shown that using appropriate conditions it is possible to transform cocaine into pure norcocaine in a one single step of electrochemical reaction. Comparison between these two methods shows that experiments with microsomes are much more laborious (including microsomes preparation from rat liver and incubation with examined drug) than electrochemical simulation, but not all real-existing metabolites in human body can be obtained in a single electrochemical experiment. Therefore, both methodologies should be considered as complementary.

**Novel Aspects:** Comparison of metabolism examination with microsomes and latest application of electrochemistry in drug conversion simulated in electrochemical cell.

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# Software for Pathway Informed Metabolomics

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The historical approach to metabolomics research is to analyze samples in an un-targeted fashion, use software to find compounds in the data and then attempt to identify the found compounds. This approach is good for discovering new compounds but in practice it has yielded many unknown compounds that are difficult and slow to identify. If the primary purpose of the researcher is to perform translational biology, a more direct approach to the problem can be taken. In this case the data is still collected in an un-targeted fashion but now the data is mined using known biological pathways, the results are statistically analyzed and the results are projected onto biological pathways to better understand the biological system under study.

**Method:** We have developed three software tools to analyze data using pathways as the basis. The first tool is a software program that can read publicly available pathway databases, such as KEGG, and allow the user to select pathway(s) of interest based on name, common compound or related reaction. When extracting the pathway database, the compound information such as name, empirical formula, identifier and structure are captured. Duplicate entries and non-metabolites are stripped from the pathway database and added to a compound database. This yields a database of compounds of a set of pathways that are of interest to the researcher.

**Preliminary Data:** The second piece of software developed for this analysis is a mass spectrometer data feature extraction routine. This software works by taking an entry in the previously created database and calculates the ion to extract from the mass spectral data. It does this by taking user settable parameters such as charge state and adduct ion and calculates all the permitted combinations of ions to extract by combining it with the empirical formula in the database. This process is repeated for all the entries in the compound database and a list of results is created. Accurate mass alone is not a positive identification but in a biological context it is often enough for a preliminary compound assignment. The last piece of the software then takes the result of the list of found compounds and searches a pathway database for matches. A number of steps must be performed to do this. The pathway database and the compound database can have different identifiers if the sources were not the same. A mapping tool aligns the two databases. This is done by mapping the chemical identifier in the compound database to an identifier in the pathway database by use of a look up file. The next step is to score each pathway based on the number of metabolites present in the compound list. A pathway list is created with the number of entities found in each pathway. The user can then filter the pathway list to look at only the most interesting pathways. By selecting a pathway in the pathway list, the user can visualize the acquired data projected onto that pathway. The software allows for zooming in or out for closer inspection of the pathway.

# Development of a spectral interconversion algorithm for desorption ionization techniques

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The identification of biological tissues using a combination of surgical instruments and mass spectrometers is a potential improvement for intraoperative diagnostics. Rapid Evaporative Ionization Mass Spectrometry (REIMS) [1, 2] combines surgical instruments with on-line in-vivo, in-situ tissue identification. Desorption Electrospray Ionization (DESI) [3] and Laser Desorption Ionization (LDI) provide the possibility of creating huge amounts of histologically defined mass spectrometric data sets. Mass spectrometric patterns of REIMS and DESI or LDI are considerably different for a direct comparison using Principal Component Analysis (PCA). Therefore, in order to eliminate ionization-dependent features, a conversion algorithm has to be applied which transforms raw mass spectra to a universal data platform.

**Method:** Porcine kidney cortex, kidney medulla, brain grey matter and liver tissue were used as model system. Prior to DESI analysis, the tissues were sectioned while in the case of LDI and REIMS, bulk samples were analyzed. The tissue was analyzed in negative ion mode using an LTQ Orbitrap Discovery high resolution mass spectrometer manufactured by Thermo Fisher Scientific. Additional HPLC and *nano*-ESI experiments were carried out for identification of individual mass spectrometric signals. The obtained mass spectra from DESI, LDI and REIMS were processed by means of the interconversion algorithm. Algorithm comprises calibration, identification of lipids based on accurate mass, selection of specific set of lipids and applying scaling factors.

**Preliminary Data:** The transformation of a mass spectrum to a lipid spectrum by the developed interconversion algorithm includes several steps. At first the mass spectrum is re-calibrated offline in order to decrease the mass deviation. The second step is a comparison of all *m/z* values of this mass spectrum to a compound database to obtain a list of matched species. The database contains exact masses of several hundred species, including different ionic species like $[M\text{-}H]^-$ or $[M\text{-}NH_4]^-$, which were found in tissue extracts by HPLC and nano-ESI experiments. The following step is the selection of a compound set which consists of compounds that are present in the mass spectra of both compared desorption methods. In the fourth step scaling factors are applied to correct for the different response factors of compound classes using different ionization methods. Finally, the resulting chemical spectra are compared using PCA. The interconversion algorithm allows the identification of tissue analyzed by REIMS, based on data obtained by LDI and DESI. While in case of LDI there is a single compound set and scaling factor set which is applied to data of every tissue type (and which results in overlap of the same tissue types in PCA space, while different tissue types are separated) in case of DESI it is necessary to define different compound sets and scaling factors for each individual tissue type. The interconversion algorithm gives sufficiently good results for data derived by LDI. The mass spectra obtained by DESI are considerably different from those of REIMS, which makes it more complicated to create comparable chemical spectra, however preliminary results indicate the feasibility of the approach even in this case.

**Novel Aspects:** Identification of biological tissue with surgical instruments based on data derived by common imaging mass spectrometry methods.

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# Quantification of Phospholipids in Biological Samples Using µLC-FTICR-MS

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Phospholipids are the main components of cellular membranes and play important roles in the regulation of membrane trafficking and intracellular signaling. Due to their heterogeneity and the low lipid amounts found in isolated material, lipid identification and quantification in biological samples is difficult. We have developed a method for accurate identification and quantification of phospholipids in biological samples

**Method:** Lipids in biological samples were extracted using the Bligh & Dyer method and separated by head groups using normal phase *micro*-HPLC. Online detection was performed using a high resolution hybrid Apex-Qe FT-MS system (Bruker Daltonics, Bremen, Germany) equipped with a 7 Tesla actively shielded superconducting magnet and an Apollo Dual ESI/ MALDI ion source. Mass spectra were summed up according to the respective Rf values of the lipid groups of interest and analyzed using LipID [1] allowing the verification of the head groups and the overall fatty acid composition including the degree of saturation.

**Preliminary Data:** The developed can be used for the simultaneous profiling of PC, PE, PS, CL, PG, SM, Cer, PI, TAG and their corresponding lyso species in biological samples. Isobaric PC and PE species were baseline-separated using the LC system. For quantification deuterated internal standards were used. The assay linearity was typically greater than two orders of magnitude and correlation coefficients were >0.99. Typical limits of detection (LOD, defined as S/N = 3) for most compounds were below 0.02 µM. The limits of quantitation (LOQ, defined as S/N = 10) were in the range from 0.1 to 0.5 µM. The average precisions for all investigated analytes throughout the linear dynamic range were better than 3%. Applications of the developed method for lipid profiling in biological samples will be given. The financial support by the DFG (SFB TR22 Z01 and DFG LI-448/4) is gratefully acknowledged.

**Novel Aspects:** New methodology for the determination of lipids in biological isolates.

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# Development and validation of a LC-MS/MS method for routine determination of aldosterone and its application in pediatric endocrinology

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Aldosterone is the most important circulating mineralocorticoid. The serum concentration of aldosterone is in the picomolar range. Therefore reliable assays for the detection of aldosterone are essentiell for the clinical evaluation of different disorders like hypertension, pseudohypoaldosteronism, hypoaldosteronism and hyperaldosteronism. Currently, the correct and sensitive measurement is hampered by various preanalytical and method specific difficulties with immunoassays.

**Method:** We established an assay for measuring aldosterone in small sample volume. Combined with our previous Multi-Steroidassay [1] (Kulle et al. 2012) we determine simultaneously aldosterone and progesterone, deoxycorticosterone, corticosterone, 17-hydroxyprogesterone, 11-deoxycortisol, 21-deoxycortisol, cortisol, cortisone, androstenedione, testosterone and dihydrotestosterone. 0.1 mL plasma was extracted by solid phase extraction (SPE) and analyzed using an UPLC-MS/MS in MRM mode. Aldosterone was measured in negative ESI mode. We compared our assay with RIA and ELISA assays for aldosterone (DRG-Diagnostics).

**Preliminary Data:** The calibration curve was linear and reproducible in the range of 50-2000 pmol/L aldosterone. The limit of detection was 10 pmol/L and the limit of quantification was 30 pmol/L. The coefficients of determination were 0.87 for RIA vs LCMSMS and 0.81 for ELISA vs LCMSMS. The aldosterone concentration was about 1.3 times higher analyzed by nonextractive RIA and 20% lower analyzed by ELISA compared to LC-MS/MS. With this validated assay preliminary reference ranges for children aged 0-18 years were measured. As an example for its clinical application plasma from patients with pseudohypoaldosteronism was measured revealing 15-fold to 45-fold multiples of the median of the aldosterone reference value. The detection limit and the sensitivity of this assay allow measuring aldosterone simultaneously with 11 other steroid hormones in one injection.

**Novel Aspects:** The assay is rapid and reliable for clinical routine analysis. LC-MS/MS overcomes antibody related problems of the established immunoassays.

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# Strategies of protease substrate identification by mass spectrometry

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Proteases play a pivotal role in regulation of the activity of enzymes and other proteins or of controlling signalling pathways. The establishment of the substrate repertoire provides better understanding of the function of proteases. To find the substrates of a defined protease, it is essential to investigate the substrate repertoire in vivo, as "just because it can, does not mean it does". Moreover target cleavage can be the result of complex proteolytic cascade, which is especially interesting to understand the function of the protease in a biological context. Here we describe several mass spectrometry-based strategies, their advantages and limitations exemplified with substrate search for the human protease HtrA1.

**Method:** First, we compared the protein pattern of a placenta protein extract incubated in the absence and presence of the protease HtrA1 by two-dimensional-gel-electrophoresis (2DE) and subsequent LC-MS analysis of the tryptic peptides of the up- or down regulated proteins. Second we applied differential stable isotope labelling by amino acid in cell culture approach (SILAC) followed by SDS-PAGE, tryptic digestion of the protein bands and LC-MS/MS. Endometrial cancer cells (Hec1A) were SILAC-labelled, the cell lysate collected and incubated in the presence of the protease HtrA1. As a negative control we used the cell lysate of an unlabelled set of cells incubated without HtrA1. The N-terminal Edman sequencing and mass spectrometric top-down sequencing were used for cleavage site identification of the protease HtrA1 on the substrate β-casein.

**Preliminary Data:** Several candidate substrates were identified by the 2DE experiments and the SILAC approach. Among the placenta proteins we found 12 potential substrates. Proteins were considered to be candidate substrates when the spots that correspond to the full-length product disappear or were significantly reduce. The SILAC approach reveals 24 candidate substrates with significant ratio between SILAC-labelled (protease treated) and unlabelled sample (control sample) smaller then 0,5. From these 24 proteins only 4 were also identified as potential substrate in the placenta proteins. This is not surprisingly, as the protein pattern in cancer cells and placenta cells vary. The candidate substrates found with the 2DE approach include proteins of the cytoskeleton, proteins of the disulfide-isomerase family, several heat shock protein and a protease inhibitor, while the candidate substrates of the SILAC strategy include proteins of the cytoskeleton, heat shock protein and several proteins responsible for the protein biosynthesis. In conclusion, proteomics strategies to identify potential substrates are powerful and provide a wealth of new information about the substrates of defined proteases. Based on the results of this work we suggest a role of HtrA1 in the protease and protease-inhibitor balance in placentation and cancer cell proliferation.

**Novel Aspects:** Comparison of different mass spectrometry based strategies for the protease substrate search of the human protease HtrA1.

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# Mass spectrometry in studies of protein denaturation

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Mass spectrometry analysis coupled with a hydrogen/deuterium exchange (HXMS) is one of the most powerful techniques for probing structure and conformational dynamics of proteins. We combined HXMS with a high pressure-induced denaturation to monitor stability of an amyloidogenic protein human cystatin C (hCC). It is postulated that aggregation of hCC proceed through propagated 3D domain-swapping process, which was evidenced as a mechanism of hCC dimerization [1, 2]. To check implications of greater or decreased stability of the hinge region (QIV57AG), linking the swapping subdomains, for dimerization and aggregation propensity of hCC we constructed and studied mutants with $Val^{57}$ residue replaced by preferred in a β-turn region Asp or Asn residue, or by Pro, recognized as a residue able to destabilize loop structures.

**Method:** All MS analyses were performed on an Apex-Qe 7T FTICR spectrometer. High-pressure denaturation experiments were carried out according to the protocol established in our previous studies [2].

**Preliminary Data:** Results obtained in the pressure-induced denaturation studies of the wild-type and mutated human cystatin C, allow to conclude: 1) single-point mutation in the L1 region can alter compactness of cystatin C molecule and stability of its structure, therefore stabilization of this hinge region may be beneficial in preventing the dimerization and oligomerization of amyloidogenic human cystatin C; 2) stability of Val57Asn mutant is similar to the wild-type hCC, whereas Val57Asp is slightly more prone to unfolding induced by pressure application. The residue with ionic side chain is not able to stabilize the L1 region and the whole protein structure as efficiently as polar but not charged Asn residue; 3) conformation of refolded after pressure relief Val57Asp and Val57Asn mutants is less flexible than conformation of the wild-type hCC 4) Val57Pro mutant is quite unstable and its unfolding starts at lower pressure values than unfolding of any other studied hCC variant. A smaller number of the unexchangeable deuterons trapped in this protein interior strongly suggests 'open' conformation what is the proof of the previously postulated hCC dimerization mechanism.

**Novel Aspects:** Our results indicate that pressure can bring valuable and supplementary to other denaturing agents information about denaturation of mutated proteins.

**Acknowledgements:** The work was supported by grant of Polish Ministry of Science and Higher Education No. 1264/B/H03/2009/37.

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# Acid-catalyzed 18O-labeling – a new tool for peptide and protein quantitation

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Enzyme-catalyzed $^{18}O$-labeling is a smart method that allows the incorporation of stable isotopes at the peptide level without introducing a chemical tag. Trypsin can be used to catalyze the exchange of the C-terminal oxygen atoms. As two $^{18}O$ atoms are incorporated at the C-terminus of the peptides, the mass difference between the light and the heavy peptides is 4 Da. For larger peptides this can cause overlapping isotopic patterns of the light and heavy peptides, which can hamper data analysis.Recently, the application of acid-catalyzed $^{18}O$-labeling was suggested [1, 2]. In contrast to enzyme-catalyzed $^{18}O$-labeling, where only theC-terminal carboxyl group is labeled, all carboxyl groups (e.g., at Asp, Glu, C-terminal carboxyl group) are labeled by acidic catalysis.

**Method:** HCl gas was generated in situ by dripping aqueous 37% HCl solution on anhydrous calcium chloride in a glass apparatus. HCl gaswas dried over phosphorus pentoxide, and then bubbled through 100 µL of $^{18}O$-labeled water. Peptide samples were dried in aSpeedVac and resuspended in 5-10µL of HCl-saturated $H^2{}_{18}O$. The samples were incubated at 15°C for 5 h and 30 min. Hydrochloric acid was subsequently removed by evaporation using a stream of dry nitrogen.Residues of hydrochloric acid were neutralized with 2 µL of a saturated solution of $NH_4HCO_3$ in $H_2{}^{18}O$. Samples were diluted, mixed and acidified with formic acid for subsequent LC-MS/MS analyses.

**Preliminary Data:** We have used the synthetic peptide [Glu1]-Fibrinopeptide B (Glu-Fib, EGVNDNEEGFFSAR) foroptimizing the conditions for acid-catalyzed $^{18}O$-labeling. We found hydrochloric acid (HCl) to be the best reagent for the acid-catalyzed $^{18}O$-labeling reaction. With HCl-saturated $H_2{}^{18}O$ we were able to achieve 97% $^{18}O$ incorporation at the carboxyl groups at 15°C after 5 h and 30 min. Side reactions, such as proteolysis or deamidation happen to a very minor degree under these conditions. For evaluating the overall performance of the acid-catalyzed $^{18}O$-labeling for the relative quantitation of peptides and proteins, we have used theDionex peptide mixture. This peptide mixture was split in a ratio of 1:1, and the two samples were incubated with HCl-saturated $H_2{}^{18}O$ or $H_2{}^{16}O$, respectively. After evaporation of the hydrochloric acid the two samples were resuspended, pooled and analyzed with nanoLC-MS/MS. The H/L ratios for all identified peptides were calculated from the LC-MS/MS raw data using Mascot Distiller. The calculated mean H/L ratio of all peptides was 1.01 ± 0.14. Finally, we applied our new protocol for acid-catalyzed $^{18}O$-labeling to a set of phosphopeptides. No side reactions at the phosphate group (e.g. elimination) were observed, andthe oxygen atoms at the phosphate group were not exchanged under the applied conditions. The acid-catalyzed $^{18}O$-labeling turned out to be compatible with common phosphopeptide enrichment methods (e.g. $TiO_2$). Acid-catalyzed $^{18}O$-labeling can be applied for any peptide with at least one carboxyl group. Acid- catalyzed $^{18}O$-labeling does not introduce any chemical group, and thus does not alter any peptides property, such as hydrophobicity, ionization efficiency, or peptide fragmentation behavior. The acid-catalyzed $^{18}O$-labeling procedure is simple to perform without the need for any additional cleanup procedure. Finally, acid-catalyzed $^{18}O$-labeling is cost-efficient, especially when water with 97 atom % $^{18}O$ is used.

**Novel Aspects:** A new method for isotope labeling of peptides.

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# Specific detection of arginine-derived advanced glycation end-products and their localization in proteins

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Glycation (non-enzymatic glycosylation) is a common covalent protein modification [1]. On its early step lysine-bound aldoseamines2- and ketoamines3 were formed, while their dicarbonyl degradation products are involved in further reactions yielding advanced glycation end-products (AGEs). Reactive dicarbonyls glyoxal and methylglyoxal readily react with arginine residues forming Glarg [4] and methylglyoxal-derived hydroimidazolones [4] (MG-H 1, 2 and 3), respectively. These AGEs accumulate in uremia and diabetes patients [5] and can be considered as prospective biomarkers. Their formation in the presence of reducing sugars, however, is poorly characterized and the methods for specific and sensitive analysis on peptide level are still missing. Here we present kinetic studies of AGE-formation and new tandem mass spectrometry-based approaches for selective detection and reliable identification of these species.

**Method:** Synthetic peptides containing arginine residue in central position were synthesized on solid phase using Fmoc/tBu strategy and incubated in phosphate buffer in presence of D-glucose under conditions simulating food cooking (95°C) for 0, 15, 30, 60, 120 and 240 min using D-glucose-free incubations as controls. The resulting reaction mixtures were analyzed by RP-HPLC-ESI-QqTOF-MS and -MS/MS. AGE-peptides were identified by their fragmentation patterns, while quantification relied on integration of corresponding extracted ion chromatograms. The fragmentation patterns were compared with those of synthetic AGE-peptides. The analysis of in vitro glycated HSA was performed by RP-nanoUPLC-LTQ-Orbitrap- MS after tryptic digestion of the protein.

**Preliminary Data:** Studies on arginine-containing peptides incubated with glucose revealed eleven different peptide products. Five of these species were identified as Glarg, MG-H, two precursors of these products and carboxymethylarginine (CMA). The formation kinetics of the AGEs depended on several parameters including temperature, peptide sequence, and reactive oxygen species (ROS). In order to characterize modification-specific MS/MS-fragmentation patterns, Glarg- and MG-H-modified model peptides were analyzed. The modification sites could be unambiguously identified by abundant b- and y-ion series, which allowed the analysis of AGEs in the sequence of in vitro glycated human serum albumin (HSA). Thereby, 24 peptides containing arginine-related AGE moieties were identified, including 14 MG-H-, 6 Glarg-, 2 CMA- and 2 CEA-modified residues. The low mass range of the ESI-QqTOF-MS/MS spectra of Glarg- and MG-H-peptides was dominated by specific arginine-derived immonium ions at *m/z* 152.1 and 166.1, respectively. On the basis of these characteristic reporter ions very sensitive and specific precursor ion scans were developed to screen for prospective AGE biomarkers. This approach provided a sensitivity of 10 nmol/L for AGE peptides spiked into 1 mmol/L of tryptic protein digests.

**Novel Aspects:** Formation kinetics of arginine-derived AGEs was determined. Their fragmentation patterns were characterized and applied to identification of AGEs in proteins.

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# Determination of Catalase Expression by a Targeted UHPLC-MS/MS Based Proteome Analysis

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No proteome is static – diseases, environmental conditions or food constituents have a great influence on gene expression. In the field of molecular nutrition, the impact of food constituents on gene expression is analysed in order to evaluate their physiological and toxicological properties. Targeted mass spectrometry-based proteome analysis was suggested as a novel approach for gene expression analysis promising quantification of multiple proteins in parallel independent of the protein structure or antibody availability [1, 2]. In the present study, targeted proteome analysis was achieved by a UHPLC-ESI-MS/MS MRM coupled to OFFGEL electrophoresis and quantification by SILAC. The approach was applied to measure catalase expression in the lysate of macrophages treated with various food components. Catalase is a cytoprotective enzyme decomposing cell damaging endogenous and exogenous hydrogen peroxide [3].

**Method:** Catalase was partially hydrolyzed by trypsin and proteotypic peptides were pre-selected by in-silico digest of catalase using *PeptideMass* and *UniProt Knowledgebase*. Analysis of peptide specificity was performed by *PeptideMatch*. For UHPLC-MS/MS analysis of the peptide mixture, an Ultimate3000RS was coupled to an API4000QTRAP mass spectrometer equipped with an ESI-source. The separation was performed on an ACQUITY UPLC BEH300 C18 column. MRM transitions of proteotypic peptides were optimized by repeated measurement of trypsinized catalase. For the analysis of cell lysate, catalase was enriched by OFFGEL electrophoresis and subjected to filter aided sample preparation. Expression levels of catalase in dependence of nutritive cell stimulation was achieved by SILAC, using lysine-4,4,5,5-D4 and arginine-$^{13}C_6$,$^{15}N_4$ over seven passages for metabolic stable isotope labelling.

**Preliminary Data:** Application of bioinformatic tools revealed five proteotypic peptides of trypsinated catalase. UHPLC-MS/MS MRM analysis was performed using four specific transitions for each peptide. Four of these peptides revealed to be suitable for further analysis yielding consistent results for all transitions. Whereas pure catalase could be detected by this method with a detection limit of 0.05 µg/ml, cell lysate matrix resulted in a complete signal suppression. Therefore, enrichment of the intact catalase by OFFGEL electrophoresis was applied prior to mass spectrometry yielding a signal to noise ratio of 100 for catalase levels in unstimulated cells. Metabolic protein labelling of macrophages by SILAC led to a 99% complete labelling after seven passages. In contrast to label-free peptide quantification, SILAC provides maximal accuracy by compensating for variations in all experimental steps including cell lysis, fractionation, digestion and LC-MS/MS measurement. To check the recovery of labelled catalase a mixture of labelled and unlabelled cells in a ratio of 1:1 was prepared. The comparison of the signal intensities of light and heavy peptides yielded a factor of 1.02 with a coefficient of variation below 20%. The method was then applied to determine catalase expression in macrophages treated with different food components. The new method showed good correlation with established methods, like Western Blot and catalase activity assay. The UHPLC-MS/MS MRM method will now be expanded to further enzymes in order to achieve parallel expression analysis of an array of nutrient-controlled cytoprotective proteins.

**Novel Aspects:** Novel approach to measure gene expression of cytoprotective enzymes by targeted proteome analysis by UHPLC-MS/MS MRM of proteotypic peptides.

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# How to handle huge amount of mass spectrometric data? A new strategy with the Achroma software

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Mass spectrometry can be used for monitoring enzymatic conversion reactions in real time. In this project a setup of online coupling continuous flow enzymatic assays with mass spectrometric detection (de Boer et al., 2004) was used for a) screening single compounds or complex mixtures on their regulatory effect on enzymes, e.g. inhibitory effects and b) monitoring the regulation of enzymatic assays by assay compounds. The setup leads to a large amount of mass spectrometric data in short time. A new software strategy is presented for handling and interpretation huge amount of mass spectrometric data.

**Method:** A setup of online coupling continuous flow enzymatic assays with mass spectrometric detection [1] was used for a) screening single compounds or complex mixtures on their regulatory effect on enzymes, e.g. inhibitory effects and b) monitoring the regulation of enzymatic assays by assay compounds.

**Preliminary Data:** In this work data were generated in the above described online coupled setup. Evaluation and interpretation of the obtained data with the Achroma software show the capabilities and limitations of this new strategy.

**Novel Aspects:** New software strategy for handling and interpretation huge amount of mass spectrometric data.

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# A milestone in pharmaceutical trace level analysis in water – LC-MS/MS and LC-QTOF

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The growing use of pharmaceuticals worldwide has become an emerging problem, which has awakened concern among scientists. Consequently, there is a growing need for analytical methods, which enable their rapid and sensitive determination at trace levels. In light of these concerns, our focus was to develop a multi-residue procedure, based on LC-ESI-MS/MS, which is versatile, specific and selective, enabling pharmaceuticals detection in the low ng/L range [1]. However, matrix effects are one of the major drawback, which may result in suppression or enhancement of signals. Recently, the combination LC-ESI-QTOF is being employed [2] due to their ability to provide accurate mass measurements and structural information that is lost with MS/MS working with MRM mode.

**Method:** The method of pharmaceutical analysis is based on the simultaneous extraction of target compounds by SPE , using a hydrophilic-lipophilic balanced polymer followed by HPLC (Agilent 1200series) coupled with Q-Trap 4000 MS/MS from Applied Biosystems and Q-TOF 6530 from Agilent. Two selected reaction monitoring (MRM) transitions have been monitored per compound in order to fulfil the EC guidelines, as well as to ensure an accurate identification of pharmaceuticals in the samples.

**Preliminary Data:** The main advantages of the developed method, besides the selectivity and reliability of the results, is its high throughput. Results of the analysis of pharmaceuticals in waters and wastewaters of Tricity will be presented showing occurrence of specific pharmaceuticals at levels ranging from ng/L to µg/L.

**Novel Aspects:** Multi-residue analytical methodologies for multiple-class pharmaceuticals, are required tools to provide reliable knowledge about their fate in the environment.

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# Nontarget screening with LC-Q-TOF-MS to characterize input and fate of contaminants in surface waters

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To cope with the increasing number and variety of polar emerging contaminants in surface and groundwater, the identification of unknown compounds is an attractive but also challenging goal. LC-HRMS enables to perform screening approaches to detect a considerable number of known and unknown analytes in complex samples. The identification often is based on matches of the unknown compound in mass spectral libraries with accurate masses and fragmentation data. The limited availability of LC-MS libraries require search in chemical data bases (e.g. PubChem) combined with software tools to predict mass spectral fragmentation, e.g. MetFrag from the metabolomics field [1]. In this work we will show a nontarget approach to elucidate the input and fate of contaminants in surface water.

**Method:** Surface water samples have been sampled regulary over the period of one year from the river Neckar and the creeks Ammer and Goldersbach near Tübingen and preconcentrated 500 fold by solid-phase extraction on LiChrolut ENV+ and Isolute 101. LC-Q-TOF-MS measurements have been performed on an Agilent 1260 LC coupled to a 6540 QTOF instrument with enhanced mass resolution capability of m/Dm = 40 000. Data reduction to focus on the most relevant masses was done by statistical analysis (principal component analysis PCA; Mass Profiler Professional). MS libraries, the chemical database PubChem and in-silico fragmentation and structure correlation programs MetFrag (open license) and Molecular feature correlator (MSC; Agilent) have been used for further data analysis and compound identification.

**Preliminary Data:** A variety of compounds from pesticides, PPCPs and their metabolites could be found by target and nontarget screening approaches, e.g. in samples taken from the creek Ammer. Typical wastewater indicators have been found like the X-ray contrast media amidotrizoic acid, iomeprol and iopromide, or further pharmaceuticls like carbamazepine and diclofenac, but also the artificial sweeteners acesulfame and sucralose. Generally from the source of the creek Ammer to the mouth the number of compounds showed an increasing trend which could be attributed to a large extent to the input of wastewater treatment plant effluents. In the source region the herbicide metabolite desethyl atrazine indicates an historical background of atrazine pollution of the groundwater. Further intermittent findings of amidotrizoic acid in the source region could be traced back to the influence of storm water overflow basins. Further candidates were retrieved from the huge mass list of 10.000 features by PCA which allowed to select relevant candidates for wastewater input or for specific flow conditions of the creek like base flow or high tide. Several compounds could be identified by the application of PubChem search and mass fragmentation match using MSC and MetFrag, e.g. desethyl atrazine using the exact mass 187.063 and the fragment at m/z 145.0155. Since the search resulted in 10 and more proposed candidates further background information on possible contaminants or their metabolites was necessary to select the more plausible candidates. Identification generally required an authentical analytical standard or data from a MS library.

**Novel Aspects:** Nontarget screening for surface water contaminants with statistical analysis, computer-based fragmentation and library search.

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# Analysis of pharmaceuticals and personal care products in river water samples by UHPLC-TOF

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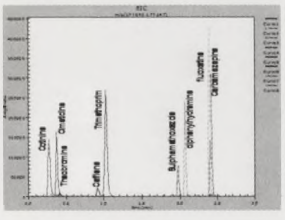
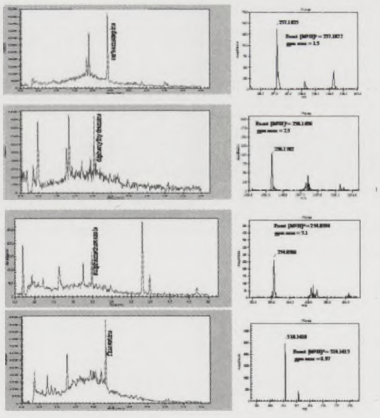
Identifying the presence of emerging pollutants in surface water samples is a growing area of concern in the environmental field [1, 2]. Many of these pollutants are introduced into the surface waters anthropogenically through municipal waste water. Among the emerging pollutants, pharmaceuticals and personal care products (PPCPs) have been detected at parts per million and parts per trillion concentrations in surface waters. We present a study of PPCPs in river water samples from north-eastern United States using UHPLC-TOF-MS for both targeted and non-targeted analytes. The time-of-flight (TOF) mass spectrometer provides full spectrum accurate mass data that can be used to analyze and identify an unlimited number of compounds, without prior knowledge of target analytes or when reference standards are not available.

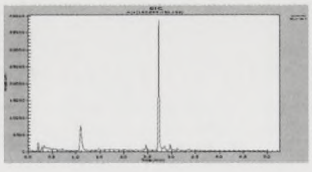
**Method:** River samples (400 ml) were collected in 1 L amber bottles. Prior to analysis, the samples were extracted through C-18 solid phase extraction (SPE) cartridges. Phenomenex StrataTM-X SPE cartridges (500 mg/6 mL) were used for extraction. The filtered river sample was loaded on the cartridges with or without a spike of standard PPCPs (100 ng each) and extracted through the cartridge at 5-6 mL/min. Pump: PerkinElmer FlexarTM FX-10 pump mobile phase A: Water containing 0.1% formic acid, mobile phase B: Acetonitrile containing 0.1% formic acid; gradient conditions: 10% B to 90% B in 5 min. Mass spectrometer: PerkinElmer AxIONTM 2 TOF.

**Preliminary Data:** In the present study the AxION 2 TOF MS was used to analyze nine targeted PPCPs and several unknown compounds present in the river water samples. The separation of the nine target analytes was achieved under 3 mins on column (Fig. 1). The SPE extraction procedure resulted in an estimated 75% or greater recovery of the analytes (based on 100 ppb standard spiked in MilliQ water). Using the AxION 2 TOF MS in the higher sensitivity TrapPulse mode, showed excellent sub ppb instrument detection limits for most analytes (detection limits for caffeine and theobromine were 4.0 and 8.0 ppb, respectively) and linearity over a very wide concentration range (0.5 to 500 ng/mL). The river sample data was further examined for unknown non-target analytes. A major chromatographic peak eluting at ~2.7 min (Fig. 3) was analyzed using AxION EC ID software. Accurate monoisotopic mass and isotope ratio information is used by AxION EC ID to search the PubChem database (or other data bases) for potential molecular formula matches and provide a ranked summary of the potential matches as well as suggestions for possible compound structures for a given elemental composition. In this example, the elemental composition, C12H17NO was listed with highest score (Fig. 4a) and the top candidate was identified as N, N-Diethyl-m-toluamide, (DEET) based on the number of active AIDS (Assay IDs) reported (Fig. 4b). DEET is a common ingredient in insect repellent and the accurate mass of the unknown compound was verified to be within 2 ppm mass error of the expected mass of DEET (Fig. 5). The presence of DEET in the river sample was further confirmed by retention time matching with the standard. Using a similar approach, acetaminophen, a commonly used anti-analgesic drug was also identified in the river water (data not shown).

**Novel Aspects:** The PerkinElmer AxION 2 TOF operated in the higher sensitivity TrapPulse mode resulted in instrument detection limits of < 1ppb.







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# Protein profiling using LC/MS method for identification of genetically modified foods

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The aim of presented research is to examine the applicability of the LC/MS method for the identification of genetically modified foods. Development of methods for testing this kind of food is important due to a widespread controversy over the lack of information about long-term changes that may occur after their ingestion. A large group of developed methods is based on the profiling of proteins in the extracts, where also the method of high performance liquid chromatography coupled with mass spectrometry, as well as electrophoretic methods coupled with mass spectrometry can be applied [1, 2].

**Method:** Proteins from corn samples were extracted within fractionated extraction using the following reagents: albumin fraction: H2O, globulin fraction: 0.5 M NaCl, zein fraction: 70% EtOH + 0.5% CH3COONa, zein-like glutelin fraction: 70% EtOH + CH3COONa 0.5% + 0.5% 2-ME, glutelin fraction: borate buffer (pH 10) + 0.6% 2-ME. The study was conducted with reverse phase column XDB-C18 (Agilent Technologies) 4.6 mm × 100 mm, 1.8 um. As the mobile phase 0.1% HCOOH (A) and acetonitrile (B) were used with a gradient elution: 0 min: 5% B, 10 min: 5% B, 30 min 100% B, 35 min 100% B. Liquid chromatograph was coupled with mass spectrometer Agilent Technologies 6220 with time of flight analyzer (TOF) and electrospray ionization.

**Preliminary Data:** Within 35 minutes of analysis it was possible to satisfactorily separate proteins from extracts of corn. In all the fractions from a few to several dozen proteins were found. Several of them have been identified based on literature data [2-5]. The protein content of protein for all fractions of reference materials of corn with 0% and 5% GM content was compared and some significant differences were found. It was also observed that some proteins may be only found in modified samples, hence they could be considered as markers of genetic modification.

**Novel Aspects:** It is the first such extensive comparison of protein profiles of genetically modified maize using LC/MS based of reference materials.

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# Heck coupling in the gas phase: Examination of the reaction mechanism by ion/molecule reactions and mass spectrometry

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The elucidation of complex reaction mechanisms is difficult but electrospray ionization (ESI) combined with tandem-MS proved to be a promising approach to study even labile and transient species out of complex reaction mixtures [1-3]. Using this strategy chemical reactions as the Pd-catalyzed *Heck* reaction can be online monitored by "fishing" ionic reactants, intermediates and products directly from solution [2]. Furthermore, ion/molecule reactions of selected ions with neutral substrate molecules executed in a tandem-MS instrument offer to follow the stepwise formation and to study intrinsic properties of reaction intermediates without the influence of solvent molecules [3]. To allow the detection of all formed species during the catalytic cycle charge tagged substrates can be applied, e.g. quaternary ammonium or phosphonium cations and sulfonate anions [4].

**Method:** The $MS^n$ experiments and gas phase ion/molecule reactions were performed on a modified Thermo Fisher LTQ Orbitrap XL (Bremen, Germany) hybrid instrument equipped with an ESI ion source, linear ion trap (LTQ), octapole collision cell (HCD cell) and an orbitrap mass analyzer [5]. Olefins for gas phase ion/molecule reactions were introduced via the nitrogen bath gas of the HCD cell using a stainless steel autoclave which was put between nitrogen source and gas inlet. The abundance of gas phase $[Ag(olefin)^n]^+$ ($n$ = 1, 2) complex ions formed by ion/molecule reactions of $Ag^+$ ions with $(olefin)_g$ was used as an indirect measure for tuning and verification of reproducible experimental conditions.

**Preliminary Data:** Firstly, the oxidative addition of (p-iodophenyl)-trimethylammonium iodide to bis-(dibenzylideneacetone) palladium ($Pd(dba)_2$) in the presence of tri(2-furyl)-phosphine (tfp) was examined in acetonitrile solution and the oxidative addition product ion was found with prominent abundance in the MS spectrum [4]. Under collision induced dissociation (CID) conditions using $N_2$/isoprene as collision gas the replacement of one tfp ligand with isoprene was observed after a delay time of some minutes [5]. When activating the palladium isoprene complex ion in a LTQ-$MS^3$ product ion experiment a fragment ion is delivered which matches the exact ion mass of the *Heck* coupling product of (p-iodophenyl)-trimethylammonium iodide with isoprene. We note that the reversed addition reaction i.e., the loss of isoprene from the palladium isoprene complex is not observed under collision activation conditions pointing towards the formation of the respective palladium isoprene σ-bonded complex ion. Collision activation of the *Heck* coupling product ion in a $MS^4$ product ion experiment shows the repeated loss of two methyl radicals. This remarkable fragmentation pattern i.e., the loss of a methyl radical from an even electron number precursor ion is very likely enabled by the substantial stabilization capability of the extended π-system. To further probe our structure assignment we undertook the *Heck* coupling of (p-iodophenyl)-trimethylammonium iodide with isoprene in solution. The ESI-MS analysis of the respective reaction mixture delivers an abundant ion representing the main reaction product which also exhibits the characteristic losses of two methyl radicals upon CID clearly supporting the assumptions presented above. Complete work-up of the solution phase *Heck* reaction including isolation, purification and conclusive structure elucidation of the product ion by NMR spectroscopy are currently underway.

**Novel Aspects:** Mechanistic study of the *Heck* reaction by $ESI\text{-}MS^n$, charge tagged substrates, gas phase ion/molecule reactions and accurate ion mass measurements

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# Investigation of Figopitant and its Metabolites in Rat Tissue by combining Whole Body Autoradiography with Liquid-Extraction-Surface-Analysis Mass Spectrometry (LESA-MS)

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Liquid extraction surface analysis mass spectrometry (LESA-MS) is a novel mass spectrometry based surface profiling technique [Kertez et al. 2009] that can be utilized in drug distribution and metabolism studies to provide complementary information to quantitative whole body autoradiography (QWBA) analyzing tissue sections. Potential advantages of the LESA technique are that no radiolabeled compound is required, no additional sample preparation is necessary, and increased sensitivity due to the microliter volume extraction combined with chip based nESI-MS. Here we apply LESA-MS and QWBA to study the distribution of figopitant and its metabolites in tissue sections of rats and compare our qualitative and quantitative findings with the classical tissue extraction, sample cleanup and HPLC analysis to evaluate this new technology.

**Method:** Orally with 5 mg/kg [14C]figopitant dosed rats were sacrificed at 4 hours post dose by chloroform inhalation. 30 µm thick whole body sagittal sections were collected following standard QWBA protocols. After QWBA analyses each tissue sample was analyzed using automated LESA with 1-2 µL of extraction solvent (50/50 ACN/water 0.1vol% formic acid). The solvent was deposited on the tissue surface forming a liquid micro junction, followed by 3 aspiration/dispense cycles for analyte extraction. After robotic transfer to a nano ESI Chip, a voltage of 1.4 kV was applied and nESI established. Two separate MS methods were designed for metabolite identification and metabolite quantification on the LTQ/Orbitrap MS. The classical tissue homogenization and extraction method with sample cleanup and HPLC analyses were analyzed by LC-RAD-nESI-MS.

**Preliminary Data:** LESA-MS of tissue sections showed that figopitant and the *N*-dealkylated metabolite M474 [1] were the predominant compound in all tissues. In addition, several metabolites formed by oxygenation, dealkylation, and a combination of oxygenation and dealkylation were identified. There was no qualitative difference between LESA analysis and HPLC analysis of extracted tissues, and all major metabolites identified in tissue extracts were also found with LESA. The relative abundance of metabolites by LESA-MS assessed based on average MS peak intensity. Concentrations of metabolites at the sample spots were calculated based on total radioactivity within these spots, which were obtained from QWBA. Analysis of the liver of the same animal by extraction and radiochromatography compared to LESA-based quantification were in good agreement. The most prevalent compounds (M474(1), M490(1) and figopitant) could be quantified by LESA-MS with accuracies between 82 and 110%. For low abundant metabolites, integration of chromatographic peaks is less accurate compared to higher abundant metabolites. Therefore, LESA-MS-based and radiometric quantification deviate more for lower abundant metabolites. In conclusion, the LESA technique was found to be a powerful tool as major metabolites in a variety of different tissues can be identified and relative quantitation of metabolites are achieved by MS. Furthermore, combination LESA with QWBA provide semiquantitation of absolute metabolite levels in tissue with a fast and robust method.

**Novel Aspects:** Combining Liquid Extraction Surface Analysis Mass Spectrometry (LESA-MS) with Whole Body Autoradiography.

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# Formation and fragmentation of sodiated amino acid cluster ions in the gas phase

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New cluster ions composed of sodiated amino acids have been abundantly produced by electrospray ionisation. The cluster composition follows the well-established pattern of $Cat^+(An^-Cat^+)_n$ for positively charged and $An^-(An^-Cat^+)_n$ for negatively charged ions with $An^-$ = amino acid anion, $Cat^+ = Na^+$ cation and $(An^-Cat^+)$ = ion pair. Salt-Clusters of this type are regularly observed for inorganic salts and the more recent ionic liquids and have been shown for the generation of clusters of all 20 naturally occurring amino acids.

**Method:** Collision-induced dissociation (CID) experiments of over 400 different cluster ions were conducted by ion trap tandem mass spectrometry.

**Preliminary Data:** For some amino acid salt clusters the well-established fragmentation behaviour of ion pair-loss ($An^-Cat^+$ = ANa) is accompanied by the release of the intact amino acid (AH). This reaction is unprecedented for salt-like clusters and involves $H^+/Na^+$-exchange within the cluster. Intracluster $H^+/Na^+$-exchange is only observed with salts of amino acids possessing sufficiently acidic H-Atoms in the side chain. The mechanism is corroborated by the study of mixed clusters incorporating the salts of both groups of amino acids. For salt clusters showing exclusively the loss of ion pairs, the loss of amino acids can be induced by doping with sodiated amino acids with acidic side chains.

**Novel Aspects:** CID causes the loss of the intact amino acid from salt-like sodium-amino acid cluster ions via $H^+/Na^+$-exchange.

# Increasing Importance of Electrochemistry/Mass Spectrometry in Life Science

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Electrochemistry (EC) in combination with mass spectrometry creates a powerful platform to simulate various oxidation and reduction processes in life sciences. Electrochemistry is a complementary technique to traditional in vivo or in vitro metabolism studies, and delivers the oxidative metabolic fingerprint of a (drug) molecule in a very short time [1]. Furthermore, protein/peptide cleavage, disulfide bonds reduction and covalent drug-protein binding can be performed in the electrochemical cell [2]. Mass spectrometry delivers selective and sensitive detection and allows for unambiguous identification of all products generated in the electrochemical cell. Additionally, automated data analysis by use of data bases (proteomics) or e.g., mass spectral trees (metabolomics) can shorten considerably the total time needed for the experiment.

**Method:** An analytical and preparative electrochemical cell (Antec, The Netherlands) equipped with a glassy carbon or conductive diamond (MD) working electrode were used for the oxidation of drugs. The cell potential was ramped to 2000 mV (glassy carbon) or to 3000 mV (MD). The outlet of the cell was connected to the electrospray source. Depending on the type of compounds typically 2-20 µM solutions were pumped through the electrochemical cell at a flow rate of 10-50 µL/min. For the formation of GSH adducts, 50 µM GSH solution was added after the cell. For reduction of disulfide bonds peptide or protein was dissolved in 1% formic acid/acetonitrile. The cell was operating in the pulse mode.

**Preliminary Data:** In this lecture a dedicated electrochemical system will be presented for fast screening of drugs to obtain their metabolic fingerprint. The easy and fast electrochemical conversion of Amodiaquine into its major phase I metabolites will be presented in both analytical and preparative EC cells. In a second step Glutathione (GSH) is added to the electrochemically generated metabolites to form the appropriate GSH-metabolite adducts, mimicking phase II reactions. All known adducts were successfully formed and identified with MS. Furthermore, we will show the application of on-line EC/MS using Electrochemistry up front MS for protein and peptide cleavage (as a promising new approach to enzymatic digestion). Electrochemical cleavage of proteins and peptides occurs very specifically at C-terminal of the Tyrosine and Tryptophan peptide bonds. Examples of oxidative cleavage will be presented. Disulfide bonds are one of the most important post-translational modifications for proteins. Therefore, a novel method for the reduction of the disulfide bonds in biologically active peptides and proteins based on electrochemistry (EC) will be presented. Investigation of drug-protein adducts by conventional techniques (microsomal incubation, in-vivo studies) are very laborious and time-consuming. With the application of EC, it is possible to activate proteins and drugs within seconds to undergo covalent drug-protein binding. The conjugation of ß-Lactalbumin with Amodiaquine metabolites will be shown. All these applications illustrate the tremendous power and broad applicability of electrochemistry as a tool to mimic nature's Redox reactions within a few seconds that can take weeks or months in real life.

**Novel Aspects:** Electrochemically assisted disulfide bonds reduction using special working electrode, drug oxidation using square wave pulse.

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# High-resolution time-of-flight mass spectrometry using folded flight path technology for profiling of steroids and steroid metabolites in urine

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Analysis of endogenous steroid levels has many important applications. These assays are usually performed on plasma samples in a targeted manner. The determination of steroid metabolites in other biological fluids is of variable clinical utility. High-resolution time-of-flight mass spectrometry (TOFMS) allows sensitive measurement of targeted steroid metabolites while simultaneously capturing a global profile of other analytes present in the matrix. This technology allows qualitatively and quantitatively useful data to be collected not just on the same instrument, but at the same time. The different qualitative and quantitative functions are accomplished post hoc with data analysis software, not by performing serial experiments or by compromising duty cycle in a single run.

**Method:** The urine samples were hydrolyzed followed by SPE extraction according to conventional practice. The methanolic extracts were analyzed by an UHPLC system interfaced to an high-resolution time-of-flight mass spectrometer using positive mode electrospray ionization. LECO's Citius$^{TM}$ LC-HRT high resolution time-of-flight mass spectrometer uses patented Folded Flight Path(FFP$^{TM}$) technology to allow flight paths as long as 40 m. Flow injection peaks were about 3 seconds wide; time-of-flight mass spectrometry maintains resolution approaching 100.000 even at high spectral acquisition rates. Pulsed in-source collision induced dissociation (isCID) was used to acquire alternating parent and product ion spectra that were recorded on separate data channels. Beside flow injection other direct analysis techniques including DESI and LESA were tested.

**Preliminary Data:** Endogenous steroid metabolites in urine samples were analyzed by liquid-chromatography/high-resolution time-of-flight mass spectrometry (LC-HRT). After normalization based on other analytes present in urine, time-course profiles of endogenous steroid metabolites correlated with known and induced biological variation. For example, variation in conjugated testosterone levels followed previously reported patterns in response to physical training. Collision-induced dissociation (CID) spectra of known analytes generated by LC-HRT were consistent with spectra previously reported from triple quadrupole and other MS-MS instruments and provided favorable database search results. These analytes included both endogenous steroids and some abused as dopants. CID spectra generated by direct analysis techniques including DESI and LESA also matched the spectra generated by LC-MS. The accurate mass measurement and accurate isotope ratio measurements included in the precursor and product ion spectra from the LC-HRT instrument provided a level of confidence in analyte assignment superior to triple quadrupole instrument data. All this information was obtained from a single chromatographic analysis, demonstrating several advantages of high resolution time-of-flight mass spectrometry.

**Novel Aspects:** Fast screening method using direct analysis techniques in combination with a high-resolution time-of-flight mass spectrometer.

# Maldi-tof analysis in the study of luminescent polyethers and polythioethers with the carbazolyl unit in the substituent for oled application

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In this work the MALDI-TOF technique is applied to determine the structure of star-shaped polyether prepared from i.e. ((1-(9-carbazolyl)-3-diphenylamino)propan-2-oxymethyl)oxirane (CAMO). We describe problems with the analysis of its MALDI-TOF spectra and propose how to solve them.

**Method:** MALDI-TOF (Matrix-Assisted Laser Desorption/Ionization Time-of-Flight) mass spectrometry is a tool and relative new technique for the analysis of synthetic polymers and biopolymers. It can be applied to identification of polymers and determination of the structure of their macromolecules. The latter can also lead to explain the mechanism of polymerization.

**Preliminary Data:** The method was used in the study of polyethers obtained from some monosubstituted oxiranes in the presence of cyclic oligo(potassium glycidoxide) as a macroinitiator activated 18-crown-6. It was proved that the polymers had usually star-shaped structure with a cyclic core and with mainly six arms. An exception to the principle was poly((9-carbazolyl)methyloxirane) which contained both the star-shaped and linear macromolecules and its molecular mass was particularly low and equal to about 1000. That was a result of the proton transfer reaction occurred during the anionic polymerization of (9-carbazolyl)methyloxirane.

**Novel Aspects:** The analysis can lead to explanation of the polymerization mechanism.

# Mechanistic and Analytical Features of Water Ice as a Matrix for IR-MALDI mass spectrometry

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There is an ongoing interest in using water as a matrix for IR-MALDI-MS because of its strong absorption band, centering at ~3 μm. In particular, water constitutes an endogenous matrix for direct analysis of biological tissue [1]. However, to prevent rapid evaporation/sublimation of (tissue) water, the employment of a cool-stage is generally required. Here, we describe a cool-stage for an oMALDI2 ion source (AB Sciex) and results from experiments revealing novel analytical features of the IR-MALDI process when using water ice as a matrix. For the first time, the analysis of a monoclonal antibody from a water ice matrix is demonstrated. The detection of non-covalently bound holo-myoglobin and heavily posttranslationally-modifed ribonuclease B furthermore demonstrate the softness of the method.

**Method:** Mass spectra were acquired with either a prototype orthogonal-TOF mass spectrometer [2, 3] or a QSTAR pulsar i (AB Sciex). The oMALDI2 ion sources of the instruments were amended with a cool-stage, based on liquid $N_2$ cooling and a Peltier element for counter heating [4], and IR-laser ports. $N_2$ at 1.5mbar was used as buffer gas. Sample temperaturewas adjusted to below -86°C throughout the experiments. Either a wavelength-tunable (2.71-3.14 μm) Nd:YAG-pumped KTP-OPO laser system (GWU Lasertechnik) emitting pulses of 6ns duration or an Er:YAG-laser (2.94 μm; ~150 ns) were employed. Several protein standards including ribonuclease B, holo-myoglobin, and an immunoglobulin G (IgG) antibody were investigated. All analytes were dissolved in pure water. For IR-MALDI-MS, small aliquots were frozen on InfrasilTM glass plates.

**Preliminary Data:** Freezing of water is accompanied by a red-shift of the peak absorption from 2.94 μm (RT) to ~3.10 μm (ice at ~86°C). To identify optimal laser wavelengths that provide the highest ion signals, we first scanned the IR-wavelength across the absorption bands of liquid water and water ice using the OPO laser and peptides as analyte. The best mass spectrometric performance was generally achieved at ~2.94 μm, i.e., at the peak absorption of liquid water. Together with the observation of a second smaller performance maximum at 3.10 μm, a fast change of the absorption characteristics is indicated during the 6 ns pulse duration. All further experiments were carried out at 2.94 μm. The limit of detection was determined to be ~400 fmol for substance P, which is a factor of ~40 above that obtained by UV-MALDI-o-TOF-MS analysis using 2,5-dihydroxybenzoic acid as a matrix. Similar detection limits of 600 fmol were obtained in the analysis of oligosaccharides. Notably, some peptides (e.g., substance P) are detected with high abundances of multiple charged ions. The particular softness of the method is exemplified by the analysis of ribonuclease B. Almost full glycosylation and phosphorylation patterns are observed in the mass spectra. In addition, the signals for both the holo- and apo-forms of myogolobin are detected in about equal abundances. Larger proteins like IGG, (MW ~150 000 u) could furthermore be analyzed from frozen water. Results from experiments with natural samples (e.g., strawberry fruit) showed the potential of the approach for IR-MALDI imaging of frozen water-rich tissue.

**Novel Aspects:** Employing a cool-stage for IR-MALDI-o-TOF mass spectrometry allows water ice to be used as matrix and provides new analytical features.

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# Charge-Tagged Peptides for Sensitive Detection by ESI-MS

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Electrospray ionization tandem mass spectrometry has become an essential tool for protein identification in proteomics and for analysis of large combinatorial libraries of peptides. However, the main problem during analysis of trace amounts of peptide mixtures is a limited sensitivity caused by the insufficient ionization efficiency of some peptides. One of the known methods to increase ionization efficiency of peptides is their derivatization to form fixed charge ionic species, employing quaternary ammonium salts (QAS) formation. The ideal charge tag should not fragment during analysis by tandem mass spectrometry, because it might complicate the mass spectrum and reduce the intensity of the other fragment ion peaks.

**Method:** QAS derivative (N,N,N-trialkylglycine) – was efficiently formed on solid support after the peptide synthesis. First, the Mtt-group was removed from the ε-amino group of lysine by 1% TFA in DCM. Iodoacetic acid was coupled to the amino group in the presence of diisopropylcarbodiimide. The iodine atom underwent nuclephilic substitution by appropriate tertiary amine [1]. The QAS-peptides could be removed from the resin by standard TFA procedure. For peptides with C-terminal Met residue orthogonal cyanogen bromide cleavage could be used resulting in peptides ending with homoserine lactone (HSL). The MS experiments were performed on an FT-ICR MS (Apex-Qe Ultra 7T, Bruker Daltonics, Germany) equipped with standard ESI source.

**Preliminary Data:** The aim of our work was to investigate a solid phase derivatization procedure for enhancing ionization efficiency. We designed and synthesized on solid support a series of peptide conjugates containing various QAS, including bicyclic tertiary amine such as 1,4-diazabicyclo[2.2.2]octane (DABCO) to form a new QAS-peptide derivative, which does not suffer from neutral losses during MS/MS experiments. The presence of almost exclusively y-type ion series facilitates the mass spectrum interpretation and sequence analysis. We have investigated the application of the developed procedure in analysis of one-bead-one-compound (OBOC) peptide libraries in the combinatorial chemistry. Even less than 10% of the peptide material obtained from one bead is sufficient to perform ESI-MS/MS analysis utilizing standard electrospray. The fixed charge tag at the C-terminus facilitates the MS/MS spectrum interpretation, since mainly y-type ions are observed in the MS/MS spectra, which provides reliable information about the peptide sequence. Application of our labeling methods for a training library of α chymotrypsin substrate revealed the utility of this derivatization for combinatorial chemistry. The isotopic exchange in model QAS-peptides was also investigated. Hydrogens at a-carbon atom in *N,N,N*-trialkylglycine residue can be easily exchanged by deuterons. The exchange reaction is base catalyzed but is dramatically slow in lower pH. Introduced deuterons are not back-exchanged in acidic aqueous solution and are therefore stable during LC-MS analysis. Increased ionization efficiency, provided by fixed positive charge on QAS group, as well as the deuterium labeling, enables analysis of trace amounts of peptides. The isotopically labeled QAS-peptides were useful in analysis of fragmentation pathways using the collision induced dissociation [2] and electron capture dissociation. We also elaborated a new method of coupling the *N,N,N*-trialkylamino-acid derivatives to peptides in solution.

**Novel Aspects:** The presented methodology offers a novel sensitive approach to analysis of trace amounts of peptides and other amines.

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# POSTERS

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# Cluster-induced desorption and ionization of biomolecules – influence of surface load and sample morphology on desorption efficiency

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Neutral cluster-induced desorption and ionization is a very soft method for desorption of oligopeptides from various surfaces [1]. Using a beam of neutral $SO_2$ clusters, the impacting cluster not only provides the energy for the desorption process but also serves as a transient matrix. As a consequence, desorption and ionization of oligopeptides was observed at low energies of the impacting cluster and without any fragmentation of the biomolecules.

**Method:** The $SO_2$ cluster beam was produced via supersonic expansion from a pulsed nozzle (f = 10 Hz, $t_{pulse}$ = 1000 µs, nominal orifice diameter 0.5 mm). With a gas mixture of 2% $SO_2$ in helium at a stagnation pressure of 15 bar, the cluster beam is characterized by a narrow velocity distribution around 1.6 km/s and a mean cluster size of about 2 to $3 \times 10^3$ molecules. The desorbed ions are transferred into the detection volume of a pulsed Wiley-McLaren-type time-of-flight (TOF) mass spectrometer via a biased grid placed 10 mm in front of the target.

**Preliminary Data:** Here we show that cluster-induced desorption and ionization can be efficiently applied for a wide range of surface concentrations and configurations, i.e. from µm-thick films of biomolecules down to surfaces prepared with submonolayer surface concentration of biomolecules. Highest signal intensity in the respective mass spectra was observed from thick films, indicating an efficient desorption mechanism from bulk-like material. In the submonolayer regime, the ion signal of the desorbed biomolecules was found to depend nonlinearly on surface concentration of the wet-chemically applied biomolecules. The behavior is traced back to the formation of multilayered islands of biomolecules on the surface, as observed by means of SEM and AFM, and a dominant contribution to the ion signal from these islands even at low coverage. With the current set-up, the lower detection limit is shown to be $10^{-13}$ mol [2].

**Novel Aspects:** Quantification of cluster-induced desorption and ionization as a soft method for desorption of oligopeptides for mass spectrometry.

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# Construction and first studies of a pulsed discharge nozzle for the production of resonance stabilised radicals

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In combustion and atmospheric reactions radicals are known for their wide range of reaction pathways. For an example poly aromatic hydrocarbons (PAH) play a crucial role in the soot and furthermore in the smog formation. The presence of PAHs in the atmosphere is widely known yet the formation process is still unknown. Resonance stabilised radicals (RSR) are believed to play an important role in the formation process of PAHs starting from benzene [1]. The production and detection of RSR under soot formation conditions should help to understand the involved reaction pathways. This work will show the incorporation of a pulsed discharged nozzle (PDN) into an existing ReTOF-MS with a REMPI source. As samples benzene, toluene and indene were used.

**Method:** The used apparatus consists of three parts. The sample is introduced over a pulsed nozzle (*Series 9 Valve, Parker Hannifin Corporation*). On top of the nozzle two electrodes are mounted separated by a peek spacer. The discharge is driven by an external power supply (*HCN 14-3 500, FuG Elektronik GmbH*) and pulsed by a push/pull transistor (*HTS 41-06 GSM, Behlke Power Electronics GmbH*). The resulting supersonic gas expansion is ionised by a two colour REMPI source consisting of a tunable dye laser (*Scanmate, Lambda Physics*) pumped by the 3rd harmonic of a Nd:YAG laser (*Surelite II, Continuum*) and the 4th harmonic of a Nd:YAG Laser (*Surelite I, Continuum*). The resulting ions are analysed in a ReTOF-MS (modified; *Bruker-Franzen Analytik GmbH*).

**Preliminary Data:** In this work three different measurements were carried out. At first it was necessary to establish the timing between the two used Nd:YAG lasers with different clock speeds and the delay for the PDN. In the used wavelength region from 250 nm to 270 nm obtained by the dye laser it was possible to obtain the 2c2p REMPI signal by the measurement of the toluene resonance signals. Furthermore it was possible to measure a 2c2p REMPI spectrum of indene in the wavelength region from 260.5 nm to 267.5 nm. The spectrum shows some unknown resonance signals. Secondly the influence of the discharge nozzle to the existing apparatus was analysed. Two major points can be outlined. A measured spectrum of the $6_0^1$ transition of benzene indicates an ineffective cooling of the supersonic expansion [2]. It is possible that the spatial extent of the PDN causes this observation. Also the duration of the discharge is crucial. In this work a high voltage pulse with several µs length indicated the best results. A too short high voltage pulse was not useful because no discrimination of the molecule signal was observed. A too long high voltage pulse was also unpreferred due to a continues flow of ions into the TOF. At last the influence of the discharge to the resulting REMPI spectra were analysed. It can be observed that the resonance signals of the incorporated samples vanish while the discharge take place.

**Novel Aspects:** A 2c2p REMPI spectrum of indene was recorded in the wavelength region from 260.5 nm to 267.5 nm.

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# A new high capacity MALDI target format for improved LC-MALDI analysis of complex proteomics samples

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High speed MALDI-TOF/TOF instruments are perfectly suited for LC-MALDI analysis of complex proteome samples. To fully explore the benefits offered by these mass spectrometers, target plates are required that can accommodate large spot numbers. Such targets allow the use of longer LC gradients enabling better separation and achieving improved depth of analysis due to more comprehensive MS/MS analysis. We describe a new pre-structured MALDI target that accomodates 1536 spots. For benchmarking, the target was applied to the LC-MALDI analysis of 500 ng E.coli trypsin digest using an extended LC gradient. The new MALDI plate allowed to acquire significantly more high quality MS/MS data per spot within a reduced time period, resulting in a clearly improved protein identification rate.

**Method:** 500 ng of E.coli cell lysate digested with trypsin was analyzed by LC-MALDI-TOF/TOF. The nanoLC system in use was equipped with a trap column (C18, I.D. 100 µm, 2 cm long, 5 µm particles) and a standard analytical column (C18, I.D. 75 µm, 15 cm long, 3 µm particles). LC separation was performed applying a 192 min gradient ranging from 2 to 40% acetonitrile. LC fractions were automatically deposited on a pre-structured MALDI target with a 1536 spot format. 1152 fractions, 10s each, were spotted. HCCA matrix was mixed with the LC eluate automatically. All MALDI-MS and MS/MS data were acquired at a laser repetition rate of 1000 Hz in positive ion mode.

**Preliminary Data:** For benchmarking of the new target format, 500 ng of E.coli trypsin digest was analyzed by LC-MALDI. All 1152 fractions collected from a 192 min gradient were deposited on one plate, which allowed the MALDI-MS and MS/MS acquisition to be completed within a single overnight run without the need of target exchange. The pre-structured target surface, characterized by hydrophilic sample spots surrounded by a hydrophobic surface, enables optimum spot alignment, which results in 100% spot finding efficiency of the laser and allows for complete utilization of the sample spot area during MALDI acquisition. Furthermore, the new target facilitates improved HCCA crystalization homogeneity and enhanced robustness of the matrix preparation spots against sample consumption by the laser. This enabled an increased number of MS/MS spectra to be acquired from individual target positions resulting in a significantly expanded depth of analysis.The complexity of the E.coli digest analyzed here required more than 30 MS/MS precursors to be fragmented from individual fraction spots. However, as the resulting MS/MS data reveals, highly significant peptide identifications were obtained even from those MS/MS spectra that had been acquired when a spot had already seen far more than 100.000 laser shots. As an example, the MS/MS spectrum, which was recorded at position 29 in the acquisition order of the fraction spot corresponding to a retention time 104.3 min, identified peptide HLWLEVK from Aspartyl-tRNA synthetase (E.coli). This clearly illustrates the outstanding MS/MS capacity offered by the new MALDI plate. Overall, LC-MALDI analysis of 500 ng E.coli digest when using the 1536 format target in combination with an expanded LC gradient resulted in 939 identified E.coli proteins, which represents a 20% increase compared to a previous approach based on a 2 hour gradient sampled onto two 384 format target plates.

**Novel Aspects:** A new pre-structured MALDI target that accomodates 1536 spots for faster analysis and increased numbers of identified proteins.

# Photodissociation mass spectra and mass-selected photodissociation spectroscopy of some alkyl iodide radical cations

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Mass Spectrometry, particular EI Mass Spectra, is characterised by numerus fragmentation processes of highly excited ions. Therefore gaining informations on excited electronic states of molecular ions is a contribution towards understanding dissociation dynamics, ionic structure and the appearence of mass spectra itself. Many techniques for ion spectroscopy exist [1] but the dissociative approach, used as photodissociation spectroscopy, also provides access to short lived or nonradiative electronic states. Ionisation is mainly achieved by laser ionization but electron ionisation has still its virtue when the investigated samples have no chromophoric groups useable for photon absorption. We will show the use of electron ionization and laser photodissociation spectroscopy applied to Iodoethane, Iodoethane-$d_5$, 1-Iodopropane, 2-Iodopropane, 1-Iodobutane and 2-Iodobutane.

**Method:** The photodissociation of homologous iodoalkane radical cations where studied using a home built Reflectron TOF Mass Spectrometer (Re-TOF-MS). The Re-TOF-MS is equipped with a combined electron ionisation/ photodissociation source. The two stage ion source consits of a sample inlet via a pulsed valve. Ion production is achieved in the first acceleration stage by a pulsed electron beam. The molecular ions are then extracted into the second stage of the source. Photodissociation is achieved with a pulsed nanosecond dye laser in the range of 13600-15900 $cm^{-1}$. The PD products are monitored without any interference from other ions by choosing an appropriate reflectron voltage.

**Preliminary Data:** PD mass spectra of the homologous iodoalkanes only showed ions of the alkyl moiety and no iodine and iodoalkyl ions where observable within the mentioned excitation energies. Thus monitoring the product ion abundance with respect to the laser wavelength, photodissociation spectra of molecular ions have been recorded. The mass selected (1+1)-photodissociation spectra of $C_2H_5I^+$. and its perdeuterated isotopomer ($C_2D_5I^+$) was studied within the A band of the molecular ions between 13500-15900 $cm^{-1}$. Within this wavelength region a real two photon absorption process could be verified by a power dependence study. A detailed interpretation of the $C_2H_5I^+$. and $C_2D_5I^+$ spectra where made on the information available for ground and excited neutral states as well as from the ground cationic states[2]. The A state origins were estimated at $T_0(C_2H_5I)$ = 13278±12 $cm^{-1}$ and $T_0(C_2D_5I)$ = 13363±12 $cm^{-1}$. The most intense progression is formed by the C-I stretching vibrations ($\nu_{10}$) with the harmonic vibration wavenumbers $\nu_{10}(C_2H_5I)$ = 155 $cm^{-1}$ and $\nu_{10}(C_2D_5I)$ = 145 $cm^{-1}$ The progression indicates a large geometry change along the C-I-coordinate accompanied by the $\sigma^*$(C-I)←$\sigma^*$(C-I) excitation. Furthermore we where able to measure the resonant photodissociation spectra of 1-$C_3H_7^+$ and 1-$C_4H_9^+$ Ascending the alkyl moiety increases predissociation of the excited ions and a red shift of the A band origin and only few resonances could be identified. Regarding 2-$C_3H_7^+$ and 2-$C_4H_9^+$ no resonances were observed in the spectra. There is a continous absorption with continously increasing fragment ion abundance leading to the conclusion that 2-Iodoalkyl ions dissociate in a repulsive excited state and the ionic structure of the 1- and 2-iodoalkanes do not interconvert [3].

**Novel Aspects:** Photodissociation spectroscopy of the excited ionic A state of $C_2H_5^+$, $C_2D_5^+$, 1-$C_3H_7^+$, 2-$C_3H_7$+, 1-$C_4H_9^+$ and 2-$C_4H_9^+$.

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# Spectroscopic properties of fisetin metal complexes

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Flavonoids are phenolic compounds widely distributed in plants with potential beneficial effects for human health. Flavonoids have long been recognized to possess anti-inflammatory, antiallergic, antioxidant and anticancer activities [1]. Their antioxidant activity are related with their ability to scavenge free radicals. Although, free radicals may have useful physiological functions, they can damage biomolecules when generated in excess, being therefore implicated in the etiology of several diseases and ageing. Another antioxidant mechanism of flavonoids may result from the interactions between transition metal ions and flavonoids to produce complexes that prevent the participation of these metal ions in free radical generating reactions [2]. The copper ion has a major role in the production of the very reactive hydroxyl radical through the Fenton and Haber-Weiss reactions.

**Method:** Fisetin (3,3',4',7-tetrahydroxyflavone) has been investigated for its ability to bind copper ion in different of pH values. Methanol solution of fisetin ($5\times10^{-5}$ M) was used. To evaluate the relevant interactions of copper with fisetin, combined spectroscopic (UV-Vis, ESI-MS), potentiometric and voltammetric approaches were used. The chelation sites, stoichiometry of copper-fisetin complex were defined. We used spectroscopic (UV-VIS, ESI-MS), potentiometric and voltammetric method.

**Preliminary Data:** Fisetin is a naturally occuring tetraxydroxy flavone molecule commonly found in fruits and vegetables. In the literature we found information about iron [4] and aluminium complexes [3]. Markovic at el. showed that fisetin is also active biological ligand which can act as a chelator of aluminium (III), decreasing its overload in the diet.

**Novel Aspects:** Novel complex fisetin-copper was not describe in the literature yet.

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# Liquid Injection Field Desorption/Ionization-Fourier Transform Ion Cyclotron Resonance Mass Spectrometry: Optimized RF-Hexapole Trapping Avoids Fragmentation of Labile Molecular Ions

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Field desorption mass spectrometry (FD-MS) is applicable to nonpolar, polar, and ionic compounds [1, 2]. Recently, we have devised a liquid injection field desorption/ionization (LIFDI) [3] and electrospray ionization (ESI) combination (LIFDI-ESI) ion source for a hybrid quadrupole Fourier transform ion cyclotron resonance (FT-ICR) mass spectrometer [4, 5]. The unique LIFDI-ESI source unites two complementary ionization methods on one instrument. The softness of LIFDI can only be exploited, if the intact molecular ions, $M^{+\bullet}$, are transferred from the field emitter through the mass analyzer, i.e., ion-activating events like collisions need to be suppressed. However, in hybrid quadrupole FT-ICR mass spectrometers, multiple ion-guiding and ion-trapping events occur prior to mass analysis. Here, we present refined ion trapping conditions that even allow to preserve alkane molecular ions.

**Method:** The experiments were performed on a Bruker Apex-Qe FT-ICR mass spectrometer equipped our custom-built LIFDI-ESI combination ion source and a 9.4 T superconducting magnet. It was operated to obtain LIFDI spectra of polystyrene 1050, of 2,3,4-tridodecyloxy-benzaldehyde, of sewing machine oil, and of docosane as well as a field ionization (FI) spectrum of pentafluoro-iodobenzene. The abundance of molecular ions, $M^{+\bullet}$, was optimized, in particular by variation of the trapping conditions inside the instrument's accumulation RF-hexapole ion trap. To preserve molecular ions, the standard gas argon, admitted for ion cooling as well as for CID, had to be replaced with helium. Furthermore, trapping potentials and DC bias of the hexapole rods were of outmost importance for the LIFDI spectra.

**Preliminary Data:** Earlier experiments have shown that ion–buffer gas collisions in the instrument's accumulation RF-hexapole ion trap were detrimental to the easy-to-fragment molecular ions of hydrocarbon species, whereas more robust even-electron ions were not affected [4]. Exchanging the instrument's standard supply of argon buffer gas for helium resulted in a remarkable improvement, because the loss of molecular ions by dissociation during transfer from the LIFDI source into the ICR cell was significantly reduced [5]. Together with further adjustments of potentials applied along the ion transfer path, some aromatic hydrocarbon species could thus be analyzed. Very fragile molecular ions, e.g., those of docosane, required even further refinement of the trapping conditions that are defined by a set of potentials applied to the hexapole rods and the axially attached trapping plates. The subtle balance between reasonably efficient trapping and low levels of collisional activation needs to be adjusted in an analyte-dependent manner. In particular, the DC offset of the rods turned out as an important parameter.

**Novel Aspects:** The first LIFDI-ESI combination ion source has been built and its refined operation is demonstrated on a FT-ICR mass spectrometer.

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# SRM quantitation of low ng/ml levels of calcyclin in serum: a candidate biomarker of pre-eclampsia

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Pre-eclampsia is a pregnancy-specific disease that complicates 2-8% of all pregnancies. It is associated with serious perinatal and maternal morbidity and mortality. We previously demonstrated that in pre-eclampsia placenta, calcyclin expression is significantly higher compared to controls. The sensitivity of this analysis was improved by Selective Reaction Monitoring (SRM) using a Xevo TQ-S mass spectrometer enabling the quantitation of calcyclin at low attomole levels in trophoblast cells. Next, a method was developed to quantify calcyclin in digested sera to evaluate calcyclin as a potential serum biomarker.

**Method:** An SRM assay was developed for quantitative measurements of calcyclin in serum using stable isotopic labeled peptides. We compared three sample preparation methods: digestion without any further sample treatment, digestion of affinity depleted serum, and off-line fractionation of digested sera with a Luna 5 µm, 150 × 2 mm SCX column. Samples were measured using a nanoACQUITY LC system equipped with a BEH130 C18, 1.7 µm, 75 µm × 250 mm column connected to a Xevo TQ-S triple quadrupole mass spectrometer. For all methods, linearity and limit of detection (LOD) of the assay were determined.

**Preliminary Data:** Endogenous levels of calcyclin were detectable in undepleted control sera but quantitation was not feasible due to high biological background and low signal intensity. For depleted and SCX fractionated serum samples, endogenous levels could be measured. We obtained LODs of 5 and 2.5 ng/ml serum, respectively, equivalent to 10 and 5 attomole of calcyclin peptides on column. Best results were obtained using SCX fractionation of the serum samples; quantitation could be performed for both selected peptides without interference. The average observed concentration of calcyclin in control serum samples was 35 ng/ml.

**Novel Aspects:** Developing, validating method, sufficiently sensitive and robust quantifying endogenous levels of calcyclin in serum and trophoblast cells of placental tissue.

# Strategies to Study DNA-Repair-Pathways of Cisplatin Based DNA Modifications

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The group of metal-containing cytotoxic drugs are most frequently applied in oncology. Especially Cisplatin, introduced in 1978, plays an important role in treatment of testicular and ovarian cancer, as well as, lymphomas and lung cancer. The mode of action of Cisplatin is based on the reaction with DNA nucleobases and the formation of interstrand and intrastrand crosslinks [1]. Due to these crosslinks replication is hindered and thus cells are going into apoptosis.

**Method:** The repair of DNA modifications is based on versatile and complex mechanisms. Nucleotide excision repair (NER) is one of these and requires the interaction of several proteins like Xeroderma pigmentosum A (XPA), XPC and TFIIH [2]. As a consequence the knock-out of one of these proteins could result in reduced repair in cancer cells and therefore to a higher response to Cisplatin treatment. Methods were established using calf-thymus DNA incubated *in vitro* with Cisplatin. After incubation, DNA was digested by three different enzymes. *In vivo* samples were studied from wild type mice as well as XPA and XPC knock-out mice. The formed adducts were separated by HPLC and identified by electrospray mass spectrometry. For quantification isotopic dilution ICP-MS analysis was applied.

**Preliminary Data:** Digested calf-thymus DNA showed a very complex adduct pattern, while the kinetics of adduct formation could be followed by variation of the Cisplatin incubation time. We show that more complex intrastrand adducts, e.g. G*XG*-cisPt$(NH_3)_2$ were formed over a longer period. In wild type mice, we could study the repair kinetics of Cisplatin adducts. Adduct formation was completed 24 h after Cisplatin treatment of mice. After 48 h the standard intrastrand adducts G*G*-cisPt$(NH_3)_2$ and A*G*-cisPt$(NH_3)_2$ were detected in lower abundance than the more complex adduct species. After 72 h nearly all adducts were removed by the repair mechanisms from the DNA. In repair deficient mice the adduct concentration was not reduced over time. Neither in XPC nor in XPA knock-out species an efficient repair could be observed.

**Novel Aspects:** Study and comparism of DNA-repair-pathways after Cisplatin treatment in wildtype and knock-out mice by HPLC/ESI-MS and HPLC/ICP-MS.

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# Study of collision cross sections of RAGE receptor domains using Ion Mobility Mass Spectrometry

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The Receptor for Advanced Glycation Endproducts (RAGE), a multi-ligand receptor, plays an important role in many human pathological conditions. RAGE can be used for diagnostic because many diseases are correlated with higher concentration of RAGE. Our project focused on biosensor for detection of RAGE activation for diagnostic and therapy monitoring. RAGE is composed of an extracellular part (V, C1 and C2 domains), a transmembrane and a cytosolic domains [1]. The aim of this research was to create the library of collision cross sections (CCS) of RAGE extracellular domains reflecting their stability in different pH and denaturing conditions. Variants of domains for the research: VC1 23-223 (VC1),V 23-143 (V143), C2 234-317 (C2) and mutants VC1 23-223 K43K44R104/A (VC1m), V 23-143 K43K44R104/A (V143m).

**Method:** CCS, a physical parameter strongly depending on ion shape and size, can be determined using Ion Mobility Mass Spectrometry (IMS). IMS combines classical mass spectrometry with separation of macromolecules by their shape. This challenging approach can provide information about mass, structure and homogeneity of the sample regarding for example oligomerization or denaturation [2, 3]. Drift times of RAGE domains were measured with IMS in this study and then CCSs values for them were calculated, basing on the protein calibration curves. Comparison of the CCSs values enables stating if oligomers of RAGE domains are formed and if mutation have influence on CCSs. Moreover, CCSs for C2, V143 and V143m were compared with regard to different pH and reducing conditions.

**Preliminary Data:** Library of collision cross sections of domains variants: VC1 23-223 (VC1),V 23-143 (V143), C2 234-317 (C2) and their mutants VC1 23-223 K43K44R104/A (VC1m), V 23-143 K43K44R104/A (V143m) was created and presented on a graph in dependence with charge. It was shown that after overexpression and purification monomer and dimer forms of C2 domain are present. Mutation K43K44R104/A, which prevents V143 and VC1 from binding with Aβ peptide, did not significantly changed CCSs of V143 and VC1. CCSs values of C2, V143 and V143m domains were compared among experiments in 3-8 or 9 pH range. No changes in CCSs values were observed for all the above mentioned domains. With IMS we can define if we work with native structures of RAGE domains, since there are significant differences in CCS values for native and denaturated forms of proteins.

**Novel Aspects:** Study proteins denaturation state using Ion Mobility Mass Specctrometry.

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# Trapped Ion Mobility Spectrometry – Mass Spectrometry (TIMS-MS) for the Analysis of Biomolecules

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A new type of mobility analyzer – the "Trapped Ion Mobility Spectrometry" (TIMS) analyzer is demonstrated. Whereas in a conventional IMS drift cell ions are pulled through a stationary gas, here ions are held stationary in a moving gas stream. The TIMS analyzer produces results similar to conventional drift cell analyzers in a physically smaller design (~10×) and at much lower operating potentials. Here, we demonstrate its use in the analysis of small molecules (e.g. drugs) and proteins.

**Method:** A TIMS funnel was incorporated into the first pumping region of a Bruker micrOTOF-Q. The TIMS funnel can operate in either "transmission mode" – i.e. as a normal funnel – or "TIMS mode" – wherein ions are trapped and subsequently mobility analyzed. In TIMS mode, the analyzer section of the TIMS funnel is first filled with ions. Further ions are prevented from entering by applying an attractive potential to the deflection plate. Ions are pushed downstream by gas flow and retained by an electrostatic field axially while an RF pseudo-potential confines the ions radially. Then the axial field is slowly reduced by ramping up the potential at the entrance of the analyzer section. By this ions elute according to their mobility – low mobility first.

**Preliminary Data:** Samples of small molecule isobars were prepared in 50:50 acetonitrile:water. Low concentration Agilent tune mix G1969-85000 was used to externally mobility calibrate the analyzer. A 1 M sample of ubiquitin prepared in 50:50 acentonitrile/water was mobility analyzed immediately following calibration. The results of the TIMS analysis of two sets of small molecule (drug) samples which produce isobaric ions and have the same molecular formula are shown. In both cases the isobaric ions were readily separated by mobility. Resolution here was estimated to be greater than 50. The analyzer can be calibrated by plotting known nobilities vs. 1/V (V = axial potential across analyzer at time of elution) Observed conformers of ubiquitin (Fig. 3C). Number of conformations (i.e. peaks) and relative motilities of ubiquitin, e.g. are demonstrated to be consistent with literature.

**Novel Aspects:** New type of mobility analyzer – the "Trapped Ion Mobility Spectrometry".

# UV-LDI-MS Analysis of Saturated and Unsaturated Cuticular Hydrocarbons from Insect Cuticle Using a Colloidal Silver Matrix

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Insects and other arthropods express species-specific blends of cuticular hydrocarbons (CHCs) on their surface that serve as pheromones and play an important role in behavior [1]. Recently, we demonstrated that ultraviolet laser desorption/ionization orthogonal time-of-flight mass spectrometry (UV-LDI-o-TOF-MS) enables the spatially-resolved analysis of CHCs from whole insects [2]. However, one limitation of this method is that alkanes cannot be detected. Here, we demonstrate that the UV-LDI-MS analysis of saturated CHCs is possible after spraying nm-sized colloidal silver particles onto the cuticle, giving rise to the laser desorption of silver adducts of both saturated and unsaturated CHCs [3]. The analysis of the CHC blend of the honey bee (*Apis mellifera carnica*) and of *Drosophila melanogaster* is demonstrated.

**Method:** Experiments were performed with an orthogonal (o-)TOF mass spectrometer equipped with a modified oMALDI 2$^{TM}$ ion source (AB Sciex). The ion source was operated at a back pressure of ~ 2 mbar, as described [4]. An $N_2$ laser, providing a laser spot size of ~200 × 400 µm$^2$, served for LDI. Intact male and female *Drosophila melanogaster* or dissected individual body parts (e.g., legs and wings) were mounted on glass slides. Only dissected body parts of worker bees were analyzed due to the large size. In addition, cuticular hexane extracts were prepared. Silver colloids (Purest Colloids) were sprayed onto the samples using an airbrush (Infinity, Harder & Steenbeck).

**Preliminary Data:** Colloidal silver was assessed as a matrix using synthetic hydrocarbon standards, cuticular extracts, and intact insects. Synthetic standards (e.g., hexacosane, tricosene) were prepared on glass substrates and subsequently covered with airbrushed colloids. Intense $[M+Ag]^+$ adducts of these HCs were generated, demonstrating the feasibility of the method. Next, cuticular extracts from *D. melanogaster* were prepared. Direct UV-LDI-MS analysis of untreated cuticle only shows the profiles of sodiated or potassiated mono- and polyenes and/or oxygenated species [1, 4]. In contrast, the profiles of endogenously expressed alkanes (e.g., tricosane, heptacosane) were detected as silver adducts from the colloid treated extract. Due to the mass difference of 2 u between $^{107}$Ag and $^{109}$Ag isotopes, ion signals of alkanes, monoenes and dienes partially overlap, complicating spectra interpretation. The genuine distribution can partially be retrieved by subtraction of isotope-derived signals. Next, we airbrushed whole flies with the colloids. Although sizable abundances of silver adducts of the major CHC compounds (e.g., hexacosadiene and nonacosadiene for female flies) were produced by UV-LDI, the intensities of alkane-derived signals were low compared to the data obtained from extract. These findings could be due to 1) the relatively low abundance of alkanes on the fly cuticle or 2) poor coverage of the hydrophobic cuticular surface with the colloids as indicated by the observation that only relatively low Ag signal intensities were also produced. In contrast, analysis of cuticle of honey bees showed much better coverage by the airbrushed silver colloids. Mass spectra recorded from these samples (or from cuticular bee extract) reflect the complex alkane blend of *A. mellifera* [5]. We were able to record spatially-resolved LDI-MS images from bee body parts (wings, legs).

**Novel Aspects:** UV-LDI-MS analysis of saturated and unsaturated HCs directly from insect cuticle and cuticular extracts.

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# 1c2p-REMPI of DHB isomers

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1c2p-REMPI-spectra of DHB isomers in a molecular beam were recorded. These results are important to understand proton transfer during MALDI and other ionization processes using DHB as reactant. It is known that different DHB isomers show varying efficiencies in proton transfer to analytes like small peptides in MALDI [1]. In MALDI-spectra there is a lack of analyte radical cations, where DHB-cation-peaks are observed strongly. A low ionization energy may explain this observance. Ionization energies of DHB isomers were calculated [2]. The results are found to be too high to explain the spectra. Via Photo Ionization Efficiency 2.5-DHB found to be ionized at 8,05 eV. This is more than twice the photon energy of nitrogen lasers, but three-photon ionization is improbable [2].

**Method:** In our study DHB was ablated by a CO2-laser and introduced into a molecular beam. The warmer areas of the beam were rejected by a skimmer. After passing by, the cold DHB isomers were ionized using a tunable dye (Scanmate, Lambda Physics) laser pumped by a Nd:YAG at 355 nm (GCR-230, Quanta-Ray). The resulting ions were accelerated by static fields into a modified ReTOF-analyzer (Bruker) and detected by a two-stage MCP. Spectra were recorded by a home built software [3], controlling an oscilloscope (LSA1000 Series, LeCroy). The oscilloscope was triggered by the previous laser pulse.

**Preliminary Data:** 2,5-DHB as important MALDI matrix performed well at 337 nm and 355 nm but yet very poorly using 260 nm or 266 nm nanoseconds laser pulses regarding the ionization process [1]. In the gas phase 0-0 transition was found at 357,69 nm. By reasons of numerous Franck-Condon active modes, there is a nearly continuous absorption up to 80 nm shorter wavelength of the 0-0 transition. Earlier results of our group showed better performance of 3, 4- and 3, 5- isomers protonating small peptides excited by laser wavelength close to 260 nm [4]. To investigate this behaviour in a more detailed manner, 1c2p-REMPI-spectra of DHB will be presented in this work.

**Novel Aspects:** 1c2p-REMPI-spectra of DHB isomers.

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# A TOPP Pipeline for Protein Identification

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In proteomics, processing and analysis of mass spectrometric data is a key step towards the identification of proteins. Many software tools for this task have been supplied by the manufacturers of mass spectrometers. However, the details of the underlying algorithms are usually not well documented. Therefore, the groups of Oliver Kohlbacher and Knut Reinert have been developing the OpenMS platform [1], an Open Source Software for LC/MS data management and analysis. For experimentalists with no software engineering background OpenMS comprises TOPP (The OpenMS Proteomics Pipeline) [2]. TOPP consists of several small applications that can be chained to create pipelines tailored to specific analysis tasks. Here we demonstrate the application of TOPP for the consensus protein identification in a two-dimensional separation MS approach.

**Method:** A tryptic digest of a human breast carcinoma cell culture was separated in the first dimension via an isoelectric focussing. In the second dimension the resulting fractions were separated and analyzed by LC-ESI-QTOF-MS/MS. Proteins were identified using TOPP and its graphical user interface TOPPAS. The identification was carried out with two different search engines (OMSSA, X!Tandem). Then identified peptides from both search engines were merged to compute a consensus identification. Decoy probabilities were estimated on peptide level. To ensure that only reliable peptides and proteins were identified an ID-Filter with a significance threshold of $q < 0.05$ was used.The identified proteins were validated by ProteinProphet™. Furthermore only proteins with two or more peptide hits were passed to the result table.

**Preliminary Data:** With the TOPP pipeline presenting above we identified 437 proteins and 5935 peptides. With this pipeline it was possible to analyze 195 LC-ESI-QTOF-MS/MS-runs in one single consensus identification run with the use of two different search engines. We achieved one single output file with the complete identification information of the whole two dimensional separation MS experiment. In addition to such an identification pipeline the OpenMS system offers the possibility to process raw data and to perform quantifications like iTRAQ or label free quantifications with a high degree of transparency in terms of the algorithms used. Therefore OpenMS provides experimentalists with no software engineering background with its graphical user interface TOPPAS the opportunity to create effective pipelines tailored for data processing as well as for peptide and protein identification.

**Novel Aspects:** We present a TOPP-Pipeline for peptide and protein identification, which can be used by experimentalists without a software engineering background.

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# Advantages of High Resolution Multi-Reflecting Time-of-Flight Mass Spectrometry for Rapid and Comprehensive Pesticide Screening in Food Abstracts

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High resolution mass spectrometry has grown appreciably in use in the recent past with availability of new instrumentation being a significant contributor to that growth. Recognition of the value of accurate mass data, ultra high resolving power, and accurate isotope abundance in compound identification and formula determination has become more prominent. Simultaneously, the need for accurate global analysis of regulated compounds in complex matrices has created pressure to apply better tools in the analysis of these compounds. A high resolution TOF on a novel Folded Flight Path ™ platform, serves as the framework for the analysis of diverse compounds of environmental interest. The ability to simultaneously detect compounds of interest (targeted) and survey other possible contaminants offers substantial opportunity in environmental and food safety efforts.

**Method:** Plant, vegetable and food extractions were performed by the method of Mol, et al (Anal. Chem. 80 (2008), p 9450). These extracts were spiked at various levels with a mixture of 210 compounds, covering the range of 0.3 through 300 ng/mL. Of the 210 compounds (pesticides, mycotoxins and other exogenous chemicals) 181 were amenable to analysis by positive mode electrospray ionization. Samples were diluted and analyzed by UHPLC system interfaced to an high-resolution time-of-flight mass spectrometer. The mass spectrometerwas set to a resolution mode of 50,000 at full mass range (50 to 2,500 m/z). Pulsed in-source collision induced dissociation (isCID) was used to acquire alternating parent and product ion spectra that were recorded on separate data channels.

**Preliminary Data:** The analysis of trace level analytes in complex matrices has been investigated using high performance time-of-flight mass spectrometry. Speed of acquisition and high performance capabilities have led to the detection and confident identification of over 200 analytes in complex food-based matrices including tomato, feed grain and egg. Analysis times below 3 minutes were achieved by providing full-spectrum data collection up to 200 spectra/sec (if needed) with no compromise to the quality of the analytical data.Sub-ppm mass accuracy and isotope abundance accuracy can be used to provide unambiguous molecular formula confirmation of targeted and non-targeted analytes.Resolution and mass accuracy also lend to a more robust deconvolution. Additional structural confirmation can be achieved using in-source CID.

**Novel Aspects:** High data acquisition speed does not influence the resolution and mass accuracy specifications of the system.

# Cycloreversion and other reactions of $[M+H]^+$ and $[M-H]^-$ ions of chlorins under the collision-induced dissociation conditions

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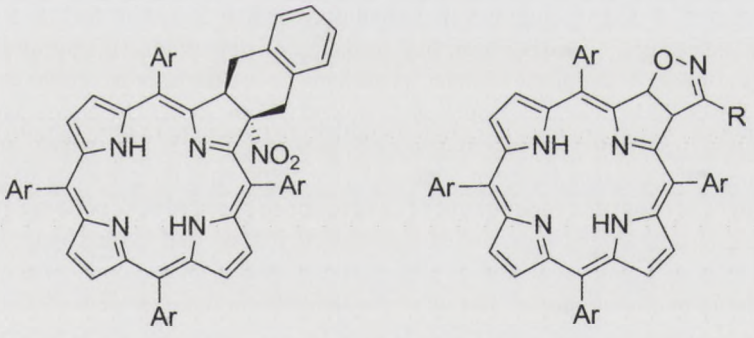
Chlorins and bacteriochlorins are tetrapyrrolic macrocycles that are observed in biologically important compounds [1]. For example chlorophylls and bacteriochlorophylls are magnesium complexes of chlorins and bacteriochlorins, respectively. They are the pigments responsible for photosynthesis. Chlorins may be considered as second generation photosensitizers in antitumor photodynamic therapy (PDT) [2] due to their characteristic strong absorption bands shifted to the red region of visible spectrum (630-780 nm).

**Method:** Two groups of chlorins (Scheme 1) were investigated by atmospheric pressure chemical ionization (APCI) mass spectrometry in positive and negative ion modes. The compounds were synthesised by S. Ostrowski group. Experiment were performed using 4000 Q-Trap (ABI Sciex).

**Preliminary Data:** The pseudomolecular ions $[M+H]^+$ and $[M-H]^-$ were undergone collision-induced dissociation (CID) analysis. Our results demonstrate the characteristic fragments of this class of compounds. Several characteristic differences between positive and negative ion CID mass spectra were shown and discussed. Fragmentation mechanisms are proposed based on the $MS^2$ and $MS^3$ experiments. The knowledge of the fragmentation pattern and key fragment ions will be useful for the characterization of novel members of this class of compounds.

**Novel Aspects:** The fragmentation reactions of recently synthesised chlorins were investigated.




Scheme 1

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# Ambient Hydrogen/Deuterium Exchange during ESI of Amino Acids and Peptides

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Hydrogen/deuterium exchange (HDX) combined with mass spectrometry is a well established method for the study of non-covalent interactions and for conformational investigations. Here the HDX is affected by the involved kinetic controlled accessibility of deuterium to binding-sites and the sterically or H-bond protected exchange sites. In all these examples an informative status of deuteration is conserved. The majority of these studies utilize in-solution or gas-phase exchange. Another line of HDX experiments is counting the number of exchangeable hydrogens to improve the analytical information of MS. The number of exchangeable hydrogen atoms is helpful, e.g. for the identification of unknown analytes, differentiation between structural isomers and de-novo sequencing of peptides.

**Method:** H/D exchange experiments were performed with an ion trap LCQ Deca Plus ESI mass spectrometer and deuterium oxide. $D_2O$ or $H_2O$ is used as solvent or as gas-phase reactant in the form of $H_2O$- or $D_2O$-saturated nitrogen. By permutation of reactants and introduction sites, four different exchange conditions were used. These are (i) deuterium free conditions, (ii) deuterium is present only in the gas-phase of the ESI chamber, (iii) deuterium is present only in the solvent combined with a $H_2O$-saturated gas-phase, and (iv) $D_2O$ is used as solvent with simultaneous presence of $D_2O$-saturated gas phase.

**Preliminary Data:** We demonstrate a method that replaces all exchangeable hydrogen atoms against deuterium under ambient conditions using electrospray ionization. This results in a virtually complete deuteration degree of >98% for amino acids and peptides. For a comprehensive description of the H/D exchange behavior we used four experimental set-ups. (i) Spectra were recorded under deuterium-free conditions as starting point for the exchange experiments, (ii) gas phase in-exchange of deuterium, (iii) gas phase out-exchange of deuterium, (iv) performing ESI-MS under full-deuterium conditions (liquid phase + gas phase). Under in-exchange conditions, most amino acids exhibit a high degree of HDX of > 85%, while for W the efficiency is somewhat reduced (about 75%). The amide hydrogen containing amino acids N, Q, and R were found to show the lowest exchange efficiencies (60-70%). Compared to amino acids, peptides in general exhibit lower deuteration degrees between 45% and 75% indicating the presence of internal hydrogen bonds. The spectra recorded under out-exchange conditions of deuterium in the ambient pressure region of the ESI source showed broadly identical exchange efficiencies, indicating reproducible in- and out-exchange conditions. ESI in a full-deuterium environment leads to amino acid and peptide spectra with a characteristic mass shift as expected from counting of the exchangeable hydrogen atoms. The achieved deuteration degrees were >98%. As a result the isotopic patterns of the fully exchanged species highly resemble those of the corresponding unexchanged species. The amount of exchangeable hydrogens is thus direct accessible via the mass difference.

**Novel Aspects:** Ambient deuteration of bio-molecules via ESI and conditions for complete deuteration.

# An Automated Data Analysis of Hydrogen/Deuterium Exchange Mass Spectrometry Using DynamX

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Hydrogen/deuterium exchange mass spectrometry (HDX MS) has proven to be a useful analytical method for the study of protein dynamics and changes to protein conformation. Recent improvements in LC-MS system have made HDX MS an indispensible tool for discovery and development of protein drugs. Conventionally, the HDX data are interpreted either manually or being processed with semi-automated tools to determine the deuterium uptake at peptide level. They are time-consuming processes due to the thousands of spectra involved for processing hundreds of peptides, multiple time-courses, replications in comparative analyses. In order to enhance the efficiency of data processing, a new HDX software tool, DynamX was used in this study.

**Method:** All samples were analysed using the Waters® SYNAPT™ G2 HDMS™ in combination with the nano-ACQUITY UPLC®with HDX Technology. Analytical column was an ACQUITY UPLC® BEH C18 1.7 µm 1.0 × 50 mm. The trap column was an ACQUITY VanGuard® Pre-column, BEH C18 1.7 µm 2.1 × 5 mm. Online pepsin digestion was performed using a 2.1 × 30 mm immobilized pepsin column (Applied Biosystems). MSE data were collected for all analyses. Undeuterated analyses were processed using ProteinLynx Global Server (PLGS) 2.5 with IdentityE informatics. DynamX was used to measure the deuterium uptake of each peptide as a function of deuterium exposure time.

**Preliminary Data:** The automated software generated a list of reproducible peptic peptides based on retention time, intensity, fragment ions, and mass accuracy. The amount of deuteration of the identified peptides was automatically determined and displayed in processed spectra view. The entire data processing was completed within minutes to an hour using DynamX. The deuterium uptake curves were automatically plotted in a function of time for comparative analysis using multiple batches of proteins. Finally the calculated results of relative deuteration and its difference were summarized in butterfly and difference charts for convenient data interpretation. In addition, DynamX was used to process the ion mobility data and successfully eliminated the interferences of co-eluting peptides separated by a mobility drift time. Therefore accurate deuterium measurement was achieved in significantly reduced time.

**Novel Aspects:** Automated Data Analysis of Hydrogen/Deuterium Exchange Mass Spectrometry, Ion Mobility, DynamX.

# Analysis of legal herbal highs by high resolution nano-LC-Q/TOF supported by accurate mass ion profiling by chemometric tools

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Increasing sell and use of so called legal highs including legal herbs, incenses and spices, obtained easily in headshops and via internet are becoming a big problem in many countries also in Poland. Similarly as in the case of designer drugs sold as a legal alternatives of drugs prohibited by the law many legal herbs are circulated as an alternative to prohibited by low plants of confirmed psychoactive activity and are consumed with the intent to induce 'high' at users. The control of designer drug and legal herb market is difficult. The high number of offered products of unknown composition makes the analytical task of product characterisation and classification as well as of the detection and identification of their ingredients very challenging.

**Method:** Samples of legal herbs, incenses, spices and cigars were extracted with methanol. Extracts were filtered and subjected to the analysis by liquid chromatography coupled to tandem mass spectrometry. A nano-flow liquid chromatograph (Agilent 1200) coupled to a high resolution tandem mass spectrometer (Agilent 6538 LC-Q/TOF) equipped with a chip-cube was used. Reverse-phase gradient separations were carried out and positive ion scans were acquired. Accurate mass profiles of examined samples were obtained and processed using MassHunter and Mass Profiler Professional software (Agilent). Principal component (PCA) and PLS discriminant analyses were performed using Simca (Umetrics).

**Preliminary Data:** High resolution positive ion mass spectra were obtained for legal highs by nano-LC-Q/ToF. To generate product accurate mass profiles of examined products mass profiling was carried out after retention time and ion mass alignment was performed, which allowed to eliminate instrument variability input from collected data. Accurate mass product fingerprints were obtained by recursive analysis. The fingerprints of herbal extracts were compared and pools of similar and different ions were identified. PCA and PLS DA analyses allowed the classification and discrimination of examined products. Attempts at the identification of compounds extracted from legal-herbs as well as of the herbs identity were made in order to determine product legality status. Accurate mass profiling by nano- LC-Q/ToF supported by chemometric tools appeared to be a promising method for the herbal highs classification and their legality status determination.

**Novel Aspects:** Accurate mass profiling by nano-LC-Q/ToF for the purpose of herbal highs classifications and legality determination was examined.

# Electrospray ionization mass spectrometric study of 2-phenyl-1, 3, 4-selanadiazole-palladium complexes

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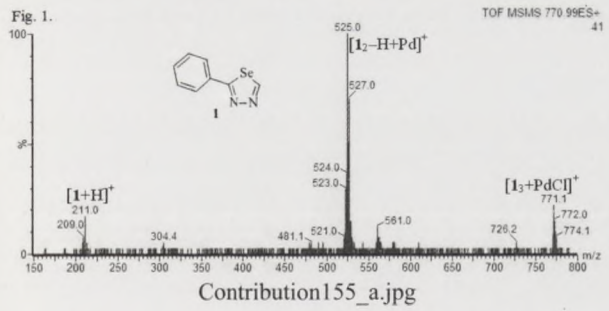
The compounds bearing 1,3,4-oxa- or 1,3,4-thiadiazole ring form stable complexes with a number of metal cations. Among metals taken for consideration, palladium(II) has attracted notable attention. The compounds bearing 1,3,4-selenadiazole ring, as expected, are much less common than 1,3,4-oxa- and 1,3,4-thiadiazoles. Here, we decided to perform the electrospray ionization mass spectrometric study of complexes of some 2-phenyl-1,3,4-selenadiazoles with palladium cation. Palladium chloride and palladium acetate were used as sources of palladium cations. For comparison, oxadiazolesand thiadiazole as well as nickel cation was also included in the study.

**Method:** The 2-phenyl-1,3,4-selenadiazoles were prepared by using Woollins' Reagent.The detailed description of the synthesis procedure will be published in a separate paper [1]. It has to be stressed that upon the synthesis of selenadiazoles, respective oxadiazoles were formed, as by-products, in notable amount (about 40%) as evidenced by NMR spectroscopy. The thiadiazole was prepared as described elsewhere [2]. In order to obtain ESI mass spectra the sample solutions were prepared in methanol. The ligand concentration was $10^{-5}$ mol/dm$^3$ and an excess of metal cations was used. The full scan and CID MS/MS ESI mass spectra were obtained.

**Preliminary Data:** The 2-phenyl-1,3,4-selena(thia)diazoles (**M**) form the complexes with palladium of ligand:metal stoichiometry 3:1, when palladium chloride is used (ions $[\mathbf{M}_3 + PdCl]^+$). The main mass spectrometric decomposition of ions $[\mathbf{M}_3 + PdCl]^+$ consist in the loss of **M** and HCl molecules, to form fragment ions $[\mathbf{M}_2 - H + Pd]^+$ [Fig. 1]. The fragment ions can be regarded as organometaallic species. The selenadiazoles are more prone to form the palladium complexes than thiadiazoles. Palladium acetate, in contrast to palladium chloride, is trimeric both in solid and in solution. The tendency of palladium acetate to form cluster species (containing more than one palladium ions) was also manifested in our studies. The ions containing a few palladium ions and only one ligand were detected. Unfortunately, the composition of detected ions was not rationalized, thus the peaks were assigned as $[\mathbf{M} + Pd_n + ?]^+$. Upon decomposition of ions $[M + Pd_n + ?]^+$, both "in-source" and MS/MS, the fragment ions $[M + Pd_2CH_3COO]^+$ were formed [Fig. 2]. Formally, the ion $[\mathbf{M} + Pd_2CH_3COO]^+$ contains two Pd atoms at +1 oxidation state. For the complexes obtained by using palladium chloride, upon gas phase fragmentation we observed the formation of organometallic species. For the complexes obtained by using palladium acetate instead of the formation of organometallic species the palladium reduction took place.

**Novel Aspects:** Palladium complexes of selenadiazoles.

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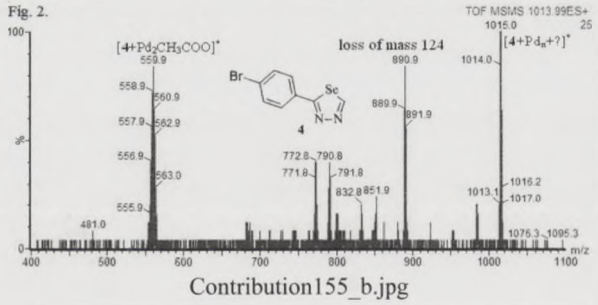


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# Studies on functionalised silsesquioxanes by ESI and APPI mass spectrometry techniques

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Polyhedral oligosilsesquioxanes of the general formula $(RSiO_{1.5})_n$ constitute a class of organosilicon compounds that currently have a strong impact on both catalytic research and materials science. Silsesquioxanes can have a cage like structure, which is most commonly cubes, hexagonal prisms, octagonal prisms [1], decagonal and dodecagonal prisms [2]. The vinyl group as substituent at the silsesquioxane core $(ViSiO_{1.5})_8$ is particularly interesting for producing hybrid materials as it can be easily functionalised by a variety of transformations. Effective and synthetically attractive methods for the functionalisation of molecular and macromolecular compounds with vinylsilicon functionality are silylative coupling and cross-metathesis. They have been developed by Bogdan Marciniec from Adam Mickiewicz University [3].

**Method:** In this report we compare the applicability of the elektron ionization (EI), electrospray (ESI) and Atmospheric Pressure Photoionization (APPI) ionization methods for recording mass spectra of the series of the functionalised silsesquioxanes using following ionization techniques: EI, ESI, APPI with or without a dopant. ESI mass spectra were recorded in methanol and acetonitrile.

**Preliminary Data:** Peaks corresponding to $[M + H]^+$ and $[M + Na]^+$ ions were observed in the spectra. APPI experiments were performed in polar and nonpolar solvents (acetonitrile, methanol and hexane) with or without a dopant. Acetone and toluene were used as dopants. Using this method the $[M + H]^+$ ions were obtained with substantially higher abundance compared to ESI. It was found that the best ionisation method for recording mass spectra of silsesquioxanes was an atmospheric pressure photoionization (APPI) procedure with or without a dopant, depending on the structure of the analysed compound. Several examples will be shown and discussed in the presentation.

**Novel Aspects:** Comparison of the efficiency of ionization techniques in silsesquioxanes MS analysis.

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# Interactions between diclofenac aromatic ring and copper cation as studied by electrospray ionization mass spectrometry

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Copper(II) complexes with non-steroidal anti-inflammatory drugs (NSAIDs) as ligands have attracted a lot of attention recently, as stressed in the review papers [1-3]. In each of the synthesized Cu-diclofenac complex, in solid state, the copper is coordinated by deprotonated diclofenac molecule through carboxylato oxygens. Taking into account the importance of cation-π interactions from pharmacological point of view [4], it is demonstrated here that cation-π interaction exist between diclofenac molecule and $Cu^{2+}$ cation.

**Method:** The full scan ESI mass spectra were obtained on a Waters/Micromass (Manchester, UK) ZQ2000 mass spectrometer (single quadrupole type instrument, Z-spray, software MassLynx V3.5). The CID MS/MS ESI mass spectra were obtained on a Waters/Micromass (Manchester, UK) Q-tof Premier mass spectrometer (software MassLynx V4.1, Manchester, UK).

**Preliminary Data:** The $Cu^{2+}$-π interaction may lead to the transfer of electron from aromatic moiety to the $Cu^{2+}$. Such process lead to the formation of $Cu^{+}$ (copper reduction) and formation of respective organic radical cation (oxidizing of organic molecule) [5]. In this way, organic radical cations are often formed under electrospray ionization (ESI) conditions, respective molecular ions $\mathbf{M}^{+}$ are clearly seen on ESI mass spectra. Among the copper salt used ($CuCl_2$, $Cu(ClO_4)_2$, $Cu(NO_3)_2$, $CuSO_4$, $Cu(CH_3COO)_2$) the most abundant diclofenac molecular ion $\mathbf{M}^{+}$ at m/z 295, was observed for solution containing diclofenac and $CuCl_2$ and was not observed at all for solution containing diclofenac and $Cu(CH_3COO)_2$. We also found that even small amount of water in methanol substantially increase the abundance of diclofenac molecular ions $\mathbf{M}^{+}$. It may be that ions $\mathbf{M}^{+}$·are formed from doubly charged ions $[M + Cu]^{2+}$ through the following charge separation reaction $[\mathbf{M} + Cu]^{2+} \rightarrow \mathbf{M}^{+}\cdot + Cu^{+}$.

**Novel Aspects:** Interactions between diclofenac aromatic ring and copper cation has not been earlier observed.

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# Stable aminomethanesulfonic acid clusters in the gas phase: An electrospray Mass Spectrometric and computational studies

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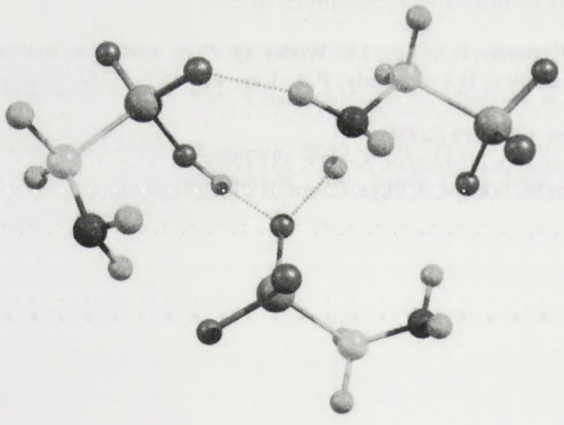
There is no information in the literature on the homo- and hetero-aggregation of aminosulfonic acids although these processes are postulated to play a role in the atmospheric chemistry, biological systems and technology. Sulfamic acid ($H_2NSO_2(OH)$) and aminomethanesulfonic acid (AMS) ($H_2N(CH_2)SO_2(OH)$), have been suggested as the possible precursors of atmospheric aerosols [1]; on the other side AMS [2], taurine ($H_2N(CH_2)_2SO_2(OH)$) and homotaurine ($H_2N(CH_2)_3SO_2(OH)$) show biological activity [3].

**Method:** All mass spectra were obtained by using a Bruker Micro TOF-Q spectrometer equipped with electrospray ionization source. The mass spectrometer was operated in the negative-ion mode. In MS/MS mode, the quadruple was used to select the precursor ions, which were fragmented in the collision cell. Ggenerated product ions were analyzed by the orthogonal reflectron TOF mass analyzer. The break-down curves were obtained by changing the collision energy.

**Preliminary Data:** The ESI-MS spectra demonstrated the formation of stable, under vacuum conditions, negatively mono- and double-charged AMS supramolecular clusters with an aggregation number up to 20, [$(AMS)_n^{-1}$ or $(AMS)_n(AMS)_m^{-2}$] (n = 1 - 10, n + m = 4 -20).The stabilities of the clusters have been studied by MS/MS experiments that demonstrated the sequentional losses of AMS monomer or multimer as neutral species.
The possible structures of AMS monomers, dimers and trimers, their energetic and abundance in 273 K and 473 K are discussed.

**Novel Aspects:** Self-assembling of aminomethanesulfonic acid in the gas phase asstudied by electrospray ionization mass spectrometry and DFT calculations.



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# ESI-MS, IMS and CID studies of the complexation of nickel(II) ions by phenylpyridines

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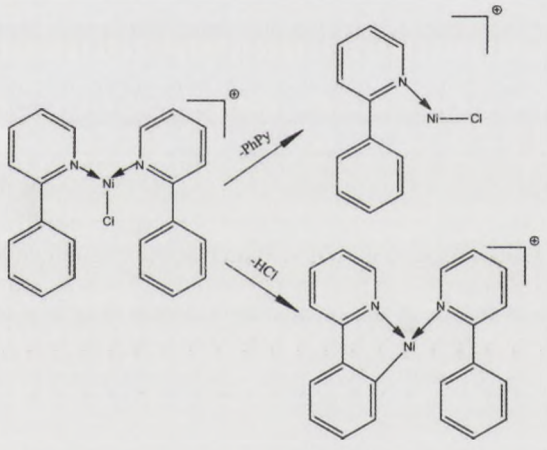
Due to the enormous importance of organometallic chemistry, it is essential to better understand the processes taking place in the reaction mixtures. Complexes of late transition metals with organic molecules are a particularly attractive field of study as they are encountered in different kinds of metal-mediated transformations [1].

**Method:** ESI-MS, ion mobility and CID experiments were performed for ions generated form dilute solutions of 2-, 3- and 4-phenyl pyridines and $NiCl_2$ in methanol. The experiments were carried out in the positive mode.

**Preliminary Data:** Electrospray ionization mass spectra show the generation of Ni(II) complexes with phenyl pyridines (both singly and doubly charged). Furthering this, ion mobilitiy studies of the complex at *m/z* 403 was chosen. In the case of 2-phenylpyridine, the mobilogram shows the appearance of two components peaks, indicating the presence of two different species, which were in parallel studies using quantum chemical calculations.CID measurements show the occurrence of C-H bond activation, which is most pronounced in the case of 2-phenyl pyridine.

**Novel Aspects:** Mass spectrometry accompanied with ion mobility is shown as a powerful technique for studying processes occurring in organometallic reactions.

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# High resolution MALDI imaging of tryptic peptides

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Mass spectrometry imaging (MSI) is a method of scanning a sample of interest and generating an 'image' of the intensity distribution of a specific analyte signal. Spatial distribution of biomolecules such as proteins can be used to identify potential markers for pathological processes. Unfortunately the direct detection and identification efficiency of proteins is limited. On-tissue digestion of proteins and detection of the resulting peptides can overcome some of these limitations. We improved tryptic peptide identification in MALDI MSI by measurements with high mass accuracy combined with a high mass resolution and complementary off-line LC-MS/MS analysis.

**Method:** A series of washing steps were applied to coronal mouse brain tissue sections (20 µm) for fixation and to remove salts and lipids. Subsequently trypsin solution was deposited (15 cycles à 2 µl) on tissue with a home-built spraying device [2]. 100 µl dihydroxybenzoic acid (30 mg/mL in acetone/$H_2O$, 1:1, v/v, with 0.5% TFA) were applied with the same device. A home-built atmospheric pressure MALDI imaging source [3] was attached to an orbital trapping mass spectrometer (LTQ Orbitrap and Exactive Orbitrap, respectively). Selected ion images were created with the home-built software 'Mirion'.

**Preliminary Data:** This study was targeted at the identification of proteins in MALDI MSI measurements using a dedicated digestion protocol. For optimization of the tryptic digestion a series of ethanol/water washing steps and a final step with acetic acid was used. The efficiency and necessity of each of these washing steps was evaluated. For the first measurements we used a LTQ Orbitrap mass spectrometer. Numerous peptides were identified by matching imaged mass peaks to peptides which were identified in complementary LC measurements of homogenized adjacent tissue sections. These results were based on accurate mass measurements (<3 ppm RMS) and provide a confident identification of tryptic peptides. Selected ion images of identified peptides were generated with a bin size (mass range used for image generation) of $\Delta m/z = 0.01$. Several tryptic peptides of myelin were detected. The distribution of myelin as detected by MALDI imaging was verified on the basis of histological staining of adjacent tissue sections. Myelin peptides were mainly distributed in the *corpus callosum* and the *thalamus*. In addition to myelin, numerous other tryptic peptides were detected with different spatial distributions. We also demonstrate the necessity of the high spatial resolution of 50 µm. Small histological structures, such as *hippocampus*, *fasciculus retroflexus and commissura posterior* were visualized in mouse brain sections. In the next step the protocol was adapted to the Orbitrap Exactive mass spectrometer. First measurements showed that in addition to the high mass accuracy (<3 ppm) we can achieve a higher mass resolution up to R = 100000 (@m/z = 200) which improves the specificity of the spatial distribution information by reducing interferences with neighboring peaks.

**Novel Aspects:** MALDI imaging measurements with high mass accuracy combined with high mass resolution.

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# Functionalized carbon nanotubes as the matrices for MALDI mass spectrometry

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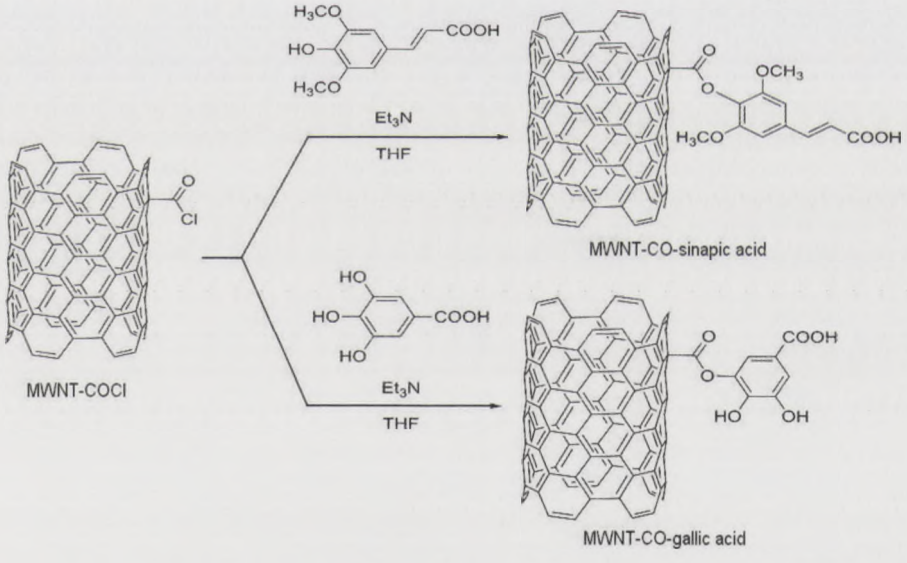
Carbon nanotubes were functionalized in order to improve their properties as the matrices in matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) analysis. Widely used matrices in MALDI method are compounds consisted of aromatic ring which allows absorption of the laser radiation and transfer of energy to the analyte as well as carboxylic groups which enable the analyte ionization. According to these features sinapic acid and gallic acid were used to modification of carbon nanotubes.

**Method:** Multi-walled carbon nanotubes containing carboxylic groups (MWNT-COOH) were treated with SOCl2 and then reacted with sinapic acid or gallic acid. MALDI-TOF-MS spectra were measured in the mass range 100-2000 m/z on Waters Q-TOF Premier mass spectrometer.

**Preliminary Data:** Formation of MWNT-CO-sinapic acid and MWNT-CO-gallic acid was confirmed by the FT-IR spectroscopy. Functionalized carbon nanotubes were used in the MALDI-Time of flight mass spectrometry analysis of cefaclor, a β-lactam anitbiotic representing the cephalosporin group. This orally administered drug has a broad range of antibacterial activity including both Gram-positive and Gram-negative organisms. Obtained MALDI spectra indicate the influence of modified carbon nanotubes on the matrix character. They will be discussed in details in the poster.

**Acknowledgement:** This work was supported by the Ministry of Science and Higher Education (Grant no. N N 204029636).




**Novel Aspects:** Application of the functionalized carbon nanotubes as the matrices for MALDI MS.

# Improving intact protein and top-down analysis by Orbitrap mass spectrometry

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The ultrahigh resolution of the Orbitrap Elite is one of the most important prerequisites for intact protein characterization and top-down analysis. Resolving power of intact protein mass measurements in Orbitrap analyzer is limited by pressure in the Orbitrap mass analyzer due to fast transient decay caused by collisions with residual gas. A standard Orbitrap Elite ETD instrument has been modified to accomplish high molecular weight ion characterization. Changes are made in the trapping scheme of the ions in the C-trap and a valve in the C-trap collision gas supply is used to reduce the pressure in the Orbitrap mass analyzer.

**Method:** All sample measurements were done on a modified Thermo Fisher Scientific Orbitrap Elite ETD instrument coupled to an LC. The modifications include a switching valve to control the pressure in the HCD collision cell and changes in the instrument control software. An intact monoclonal antibody in non-reduced and reduced condition was used for the instrument performance characterization. ProSight PC 2.0 and Protein Deconvolution 1.0 were used for data evaluation.

**Preliminary Data:** Reduction of gas load in the C-trap/HCD collision cell allows to reduce the pressure in the Orbitrap mass analyzer and hence reduce decay of transients of intact proteins. On the other hand, lowered pressure in the C-trap/HCD collision cell results in undesired reduction of trapping efficiency in the C-trap. This effect is compensated by trapping ions in HCD cell for all modes of operation with subsequent gentle transfer of cooled ions into the C-trap prior to injection into the Orbitrap analyzer. The reduced pressure in the Orbitrap mass analyzer leads to the detection of more discrete beats in the transient of high molecular weight ions, thus facilitating increase of upper mass limit of isotopic resolution in online analysis up to around 50 kDa. In addition, lower number of collisions during ejection from the C-trap was found to result in an increased signal even for the first beat. This increases signal-to-noise in spectra of heavier proteins such as antibodies and improves analysis of their glycosylation status. Top-down analysis using SID, CID, HCD, and ETD is achieved for the reduced antibodies.

**Novel Aspects:** Modifications to a standard iontrap-FTMS hybrid instrument for improved intact protein analysis and top-down analysis.

# Investigation of fragmentation routes of steroid ethers via MIKES and B2*E-linked-scan techniques

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Metastable ions have been studied using MIKE- and B2*E-linked scan techniques to investigate the fragmentation of estradiol- and estriolethers appearing in the EI-mass spectra. The aim was to find out how a fragment is built: during a normal elimination process, following a rearrangement or during another consecutive process. Using this spectral data it is possible to draw a "fragmentation tree" were fragmentation routes are shown.

**Method:** In this work a ZAB 2F build by Vaccuum Generators was used. Its sector fields are assembled in reversed Nier-Johnson geometry. The EI-ions were generated at 70 eV. To obtain the MIKE spectra a fragment ion was selected by the magnet and the E-field was scanned. In B2*E modus the B- and E-fields were scanned at a constant ratio to point out the precursor ions.

**Preliminary Data:** The MIKE spectrum of the Estriol 3 benzylether radical cation shows the following eliminations: -18, -28, -36, -41, -55, -59, -91, -109, -116, -127 and -181 Da. Using MIKES and B2*E-linked scans it is possible to check if these fragments are directly formed by elimination, rearrangements or in a consecutive elimination of two or more precursors. An example for this is the elimination of 109 Da from Estriol 3 benzylether radical cation. This is an elimination of the ether group as a radical and the elimination of a water molecule. Following a consecutive reaction mechanism beginning with the loss of water, the MIKE spectrum of the [M+.-18] should show an elimination of 91 Da which rather appears with quite low intensity. In addition the B2*E-linked spectrum of the [M+.-109] should show the [M+. 18] as precursor, it does not. Instead it shows the [M+. 91]. Looking at the MIKE spectrum of the [M+.-91] the loss of a water molecule is the most intensive elimination. This indicates that first the ether group is eliminated and adjacent the water molecule. Following this example the methyl-, ethyl-, propyl-, allyl-, and benzylethers of estradiol and estriol have been examinated and the fragmentation routes have been determined.

**Novel Aspects:** Fragmentation routes of steroid ethers have been investigated.

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# Investigation of highly-diluted DME flames by mass spectrometry

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The need for alternative and cleaner combustion processes attracts increasing attention in combustion research because of climatic and environmental influences and of limited fossil resources. Especially low-temperature combustion is a promising strategy to achieve increased efficiency, reducing $CO_2$ emission, while simultaneously decreasing $NO_x$ and soot formation. It is thus expected to contribute to cleaner engine development. Low-temperature combustion may show potentially destructive instabilities caused by dilution and exhaust gas recirculation. Also, detailed information on combustion chemistry in this regime is only recently becoming available.

**Method:** In this study, atmospheric-pressure highly-diluted (90% Ar) laminar dimethyl ether (DME)-oxygen flames were stabilized for the first time. Time-of-flight molecular-beam mass spectrometry (MBMS) with electron ionization (EI) was used to investigate and quantify chemical species profiles of reactants, intermediates, and products. MBMS in combination with a new radial movement system allowed for measuring axial and radial mass spectra in the flames. Thus, a fully two-dimensional concentration field was accessible. In a systematic approach, combustion parameters including stoichiometry, dilution, and gas preheating temperature were varied.

**Preliminary Data:** The results indicate that these flames at peak temperatures between 1400 and 1550 K may neither be dominated clearly by high-temperature nor low-temperature kinetics; they give access to an intermediate-temperature regime which may be important in practical systems and which can be used for kinetic investigations, such as the validation of relevant kinetic mechanisms.

**Novel Aspects:** Atmospheric-pressure highly-diluted (90% Ar) laminar dimethyl ether (DME)-oxygen flames were stabilized and analyzed by MBMS for the first time.

# Isotopically Resolved FTICR Mass Spectrometry of Proteins Above 100 kDa at 7 Tesla

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FT ICR continues to be the leading technique in mass resolution and mass measurement accuracy. Its application in the field of top-down proteomics and protein modification analyses via accurate mass measurements is demanding further increase of the resolution. Higher mass resolution needs higher magnetic fields or principally new measurement methods. The new type of FT ICR cell [1] we are presenting here is based on a Penning ion trap with shaped excitation and detection electrodes. This type of cell provides mass resolution that is practically only limited by the pressure in the ICR cell region.

**Method:** The new type of FT ICR measuring cell with profiled excitation, detection, and trapping electrodes for dynamic harmonization of the trapping field to remove the effect of ion cyclotron motion dephasing during signal detection has been experimentally characterized. Its performances have been compared with Bruker's "infinity" cell and an open cell with 4 compensation rings [2] on the same FT ICR mass spectrometer for peptides and proteins. We have compared mass resolution, mass accuracy and maximum achievable cyclotron radius for these three cells.

**Preliminary Data:** In the new dynamically harmonized cell (Fig. 1) axial quadratic distribution of the electric potential is achieved by shaping the excitation and detection electrodes. Very high resolution was routinely achieved for reserpine (resolution 6 000 000 at 609 Da, see Fig. 2) and BSA (resolution 1 500 000 at 66 kDa). Also higher mass proteins like Immunoglobulin G1 could be measured with isotopic resolution (see Fig. 3, resolution 500 000 at 147 kDa,) in a moderate magnetic field of 7 Tesla. Resolution values are pressure limited. The cell permits to work with cyclotron radius of up to 80% of cell radius. Experimental comparisons with Bruker's "infinity" cell and the open compensated cell have shown advantage of the new cell over both of them in resolution and mass accuracy. The resolution achieved clearly demonstrates the possibility to resolve isotopic clusters of proteins with masses above 100 kDa even on medium field magnets.

**Novel Aspects:** The new approach to FT ICR mass spectra measurement is invented. It should simplify identification of high mass protein modifications.

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# Low-temperature plasma probe for residual and petroleum crude oil analysis by FT-ICR-MS

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Low temperature plasma [1] (LTP) probe is an ionization technique based on dielectric barrier discharge [2], which is a series of micro-discharges that are induced due to the electrical breakdown of a gas present in a dielectric gap by applying an oscillating high voltage to electrodes that are on opposite sides of the dielectric gap and are themselves covered by a dielectric material [3]. LTP allows chemicals, ranging from pesticides to drugs of abuse, to be desorbed and ionized from sampling surfaces or the sample *per se* by exposing them to the plasma. Herein, is shown the application of LTP in the analysis of residual fuel oil and petroleum crude oil using high mass accuracy and high mass resolution mass spectrometry.

**Method:** The LTP probe is a glass tube that acts as dielectric barrier, a stainless steel rod as an inner grounded electrode placed coaxially to the glass tube and the outer electrode, which is a copper tape wrapped around the glass tube. The LTP probe is set to 10 kV and frequency of 3.4 kHz. Helium is the discharge gas set at a flow-rate of 1 L/min. A linear ion trap-Fourier transform ion cyclotron resonance-mass spectrometer with mass resolving power set to 400000 was used. Instrument parameters were set to capillary temperature of 100 °C, capillary voltage of +75 V and tube lens of +100 V. Crude oil standards were obtained from NIST. Teflon and glass were used as surfaces.

**Preliminary Data:** Mass spectra of the crude oil samples revealed signals from 100 to 300 Da. The chemical compositions of the most intense signals were assigned using Xcalibur. Kendrick plots [4, 5] in respect to the presence of one sulfur atom were generated and they show that NIST petroleum crude oil standards numbers 1582, 2721 and 2722 exhibited several signals in the mass spectra related to sulfur compounds. (Fig. 1) For example, the mass spectrum of petroleum crude oil 1582 (Fig. 2) show as most intense signals, the ions of m/z = 185.1357 and m/z = 187.1514, whose molecular formulas can be assigned as $C_{11}H_{21}S^+$ and $C_{11}H_{23}S^+$, respectively. Each sample has shown a different chemical profile. In order to show these differences, principal component analysis (PCA) was performed using mass spectral data acquired by LTP ionization as the data matrix for the PCA software. Each crude oil sample was analyzed five times. The 2D scores plot (Fig. 3) shows that repetitions of the same sample are grouped within the same region and each crude oil standard has its own cluster, therefore we could successfully differentiate the samples by PCA. LTP analysis of petroleum crude oil does not produce as many signals as spraying techniques like ESI or other types of desorption ionization methods. But LTP-MS exhibit the majority of its signals in the lower mass range of the spectrum which is normally not show by other techniques. Rather than competing, LTP adds to the vast range of analytical techniques used to characterize petroleum crude oil.

**Novel Aspects:** The first time that low-temperature plasma probe is used in the analysis of petroleum crude oil and residual fuel oil.

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# A mass spectrometric perspective into AlPO4 molecular sieve synthesis

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ESI-MS analysis on the formation of silicate-based zeolites provided insights into the progress of first particle formation. [1] Aluminium Phosphates ($AlPO_4$s) are another class of zeolites made up of an alternating network of Al (III) and P(V) oxides. These materials allow incorporation of heteroelements into their framework, making them highly attractive as solid catalysts. $AlPO_4$-18 in particular is made up of stacked layers of tilted hexagonal ring structures [2]. Unlike their silicaceous counterparts, the formation of $AlPO_4$s are in many cases, postulated to go through a layer to framework transformation. In this contribution, the formation of $AlPO_4$-18 from solution species was investigated with ESI-MS and first result about the intermediates formed are presented.

**Method:** A clear solution synthesis approach was adopted [3] to facilitate the analysis. Aluminium isopropoxide was dissolved in an alkaline solution containing tetraethyl ammonium hydroxide and allowed to stir for 1 h. Concentrated phosphoric acid was then added drop-wise and the final mixture was stirred for 2 h before being transferred into a stainless steel autoclave. The mixture was heated at 150°C and samples were obtained with time. Prior to analysis, the samples were all centrifuged. The supernatants were introduced into a Micromass ZMD 2000 quadrupole mass spectrometer by direct infusion and measured in negative mode. Solids obtained were analyzed by powder x-ray diffraction (XRD).

**Preliminary Data:** The reaction progresses with slow addition of phosphoric acid to the solution containing aluminium isopropoxide and by time-dependent analysis with ESI-MS, it is revealed that larger molecules are slowly being formed which indicates an oligomerizaton process. The species that are observed consist of Al (III) and P (V) tetrahedral oxo units and most species occur in their multiple dehydrated forms. It is observed that oligomers up to 11 members are present in the solution in a non-clustered but highly dehydrated manner. By considering their isotopic distributions, larger m/z species consist of clusters built up of anionic oligomers with multiple tetraethyl ammonium cations obtained with high intensities. Upon heating at 150°C, these species begin to decrease in intensities, suggesting their participation in the formation of solid $AlPO_4$-18. This was further substantiated by XRD confirmation of solid $AlPO_4$-18 isolated after 3.5 h heating. As dehydrated species could account for either water removal from linear molecule or a ring-closing reaction where water is eliminated, work is in progress to determine the structures of molecular species formed during $AlPO_4$-18 synthesis.

**Novel Aspects:** For the first time, the formation of $AlPO_4$ from solution was successfully studied with ESI-MS.

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# Activation Kinetics and Dimerization of Signaling Proteins Followed by Quantitative Mass Spectrometry

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Reversible protein phosphorylation is a key principle of intracellular signal transduction. Activation of signaling proteins is often achieved by phosphorylation of an activation motif. Signaling proteins from the STAT family are present in different isoforms and form dimeric phosphoprotein complexes, which are transported into the nucleus for their action as transcription factors. A powerful concept for a targeted, quantitative and site-specific phosphorylation analysis is the combination of immunoprecipitation (IP), 1D-PAGE, in-gel digestion, addition of a stable isotope labeled one-source ratio standard, and UPLC-MS analysis [1]. This concept allows measurements of phosphorylation degrees with RSD values below 3%. Our studies combine accurate phosphorylation degree measurements in combination with IPs using antibodies with known isoform specificity.

**Method:** BAF3-EpoR cells (mouse) were stimulated by variable amounts of Epo usually for 10 min or up to 4 h and immunoprecipitated under native conditions. IPs were performed using (i) a STAT5A-specific antibody, (ii) a STAT5B-specific antibody, or (iii) a generic antibody for both isoforms. Immunoprecipitated proteins were purified by 1D PAGE (Invitrogen). Gel bands for STAT5 were cut with wide safety margin to assure sampling of all STAT isoforms and their phosphorylated species. In-gel digestion was performed in the presence of stable isotope labeled one-source ratio standards of the homologous tryptic STAT5A and STAT5B peptides containing the activation motif. MS Analysis was performed by a UPLC (Waters) coupled to LTQ-Orbitrap XL system (Thermo).

**Preliminary Data:** We observed that stimulation of BAF3-EpoR cells with Epo resulted in strong STAT5 activation. After 10 min of Epo stimulation we observed a maximum phosphorylation degree around 80% at the activation motif of STAT5 (Tyr694 for STAT5A, Tyr699 for STAT5B), followed by a decline of the phosphorylation degree within the next hours. Murine STAT5A and STAT5B can be relatively quantified by MS on the basis of several unique homologous tryptic peptide pairs. A set of Epo-treated BAF3-EpoR samples was divided into three aliquots which were then treated with an (i) anti-STAT5A, (ii) anti-STAT5B, and a generic anti-STAT5 antibody. Using an isoform-unspecific IP, we observed that both isoforms were phosphorylated to approximately the same extent. However, the use of isoform-specific antibodies resulted in isoform-specific phosphorylation degrees. For instance, using the anti-STAT5A antibody for a sample with low total phosphorylation degree, the STAT5A status was also low, whereas for STAT5B a much higher phosphorylation degree was measured. This result indicates an effective dimerization of phospho-STAT5, and implies that STAT5B is only immunoprecipitated when present in a phospho-STAT5A/phospho-STAT5B complex. The lack of nonphosphorylated STAT5B leads to false-high phosphorylation degrees. The general conclusions from this observation are twofold. Firstly, phosphorylation degree studies connected with protein complex formations require antibodies that are completely isoform-specific or isoform-nonspecific. Secondly, this phenomenon can be used to characterize the composition of phosphorylation-induced protein complexes. The specific conclusion of our data is that dimerization of phospho-STAT5 proceeds via homo- and heterodimer formation between the A and B isoforms whereas nonphosphorylated STAT5 do not dimerize.

**Novel Aspects:** Recognition of the connection between immunoprecipitation by isoform-specific STAT5A/B antibodies, phosphorylation-induced STAT5 dimerization and STAT5 phosphorylation degree determination.

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# Analysis of low-resolution mass spectra of organic nickel derivatives by means of joint computer-aided chemometric methods

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Analysis of organometallic mass spectra unlike those of typical organic (i.e., containing C, H, O, N) is usually a difficult task, due to their modest representation in popular databases and extremely complex character caused by rich isotopic profiles for numerous metals, dehydrogenation phenomena and presence of damaged m/z signals as well as overlapping effects in their spectral bands. As a consequence, most of methods such as for example MS library search [1] or classification procedures [2] routinely useful for spectra of typical organic compounds are ineffective for those of organometallics. This work reports the results of mass spectra analysis of nickel organic derivatives by means of new promising procedure, which applied a combination of three chemometric methods.

**Method:** The computer-aided chemometric methods used jointly in presented research were: two well-known multivariate analyses of Principal Component (PCA) and Cluster (CA) as well as Band Composition Analysis (BCA) [3] applying isotope profiles. 96 spectra of nickel organic derivatives containing common substructure i.e. cyclopentadiene ring connected to Ni atom by coordination bond, were selected from NIST MS database (Edition 2008). Modeling of 58-70 m/z band (characteristic for 5 isotopes of $Ni^+$) by means of BCA gave several parameters for each spectrum. After PCA and CA done by computer program R [4] only 58 spectra indicating complex character of the band mentioned above were chosen.

**Preliminary Data:** As the primary variables for PCA the following parameters derived from BCA were used: theoretical variance ($s^2_{theor}$), main signal intensity ($I_m$), upper standard deviation ($\sigma_U$), fit factor of compared model and experimental bands ($\alpha$) and contributions ($x_i$) of their ions: $C_5H_8^+$, $C5H_7^+$, $C_5H_6^+$, $C_5H_5^+$, $C_5H_3^+$, $C_5H_2^+$, $Ni^+$. By means of PCA the values of all 11 principal components were calculated as well as variance percent in each of them. Also the values of PC1PC11 coordinates were generated for all spectra analyzed. PCA data treated with CA resulted in obtaining of hierarchical clustering dendrogram. Its preliminary visual inspection led to differentiation of at least three classes of organic nickel derivatives containing in their molecules the following substructures: (1) 1,2,3,4,5-pentamethylcyclopentadienyl, (2) two Ni atoms each connected to the substructure (1) and (3) at least two Ni atoms each connected to cyclopentadienyl. It is believed the present results will help to create mass spectra classifier enabling.

**Novel Aspects:** Elaboration of the new chemometric approach for elucidation of chemical structures of organometallics based on their low-resolution mass spectra. automatic elucidation of chemical structures of organometallic compounds.

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# Sensitivity comparison of ESI, APCI and APPI mass spectrometry techniques with reference to catalysts of olefin metathesis

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Olefin metathesis is one of the most important synthetic methods. It is used in total synthesis, to obtain complex macromolecules and polymers. Olefin metathesis allows the exchange of substituents between different olefins in the presence of transition metal complexes. All of these applications are possible by the continuing development of homogeneous catalysts [1]. In this report we present the results of our research on comparing of sensitivity of three atmospheric pressure ionization techniques (ESI, APCI and APPI) with reference to catalysts of olefin metathesis.

**Method:** A series of metathesis catalysts was used to perform this research. They differ in NHC carbene and in ligands at Ru atom. ESI, APCI and APPI with and without a dopant were examined.

**Preliminary Data:** The results that we obtained indicate that electrospray is more effective than the rest of used ionization techniques. Using electrospray we are able to detect olefin metathesis catalysts at the concentration one or even two orders of magnitude lower than using APCI or APPI.

**Novel Aspects:** Sensitivity comparison of ESI, APCI and APPI with reference to catalysts of olefin metathesis has not been studied so far.

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# Stereochemical effects in fragmentation of diastereoisomers of protected diethyl 1,2-diaminoalkylphosphonates. Electron ionization mass spectrometry and theoretical calculations

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Differentiation of stereoisomers on the basis of their mass spectra is possible due to the streochemical effects during fragmentation of ions in the gas-phase. In many cases differences in abundances or formation of specific fragment ions can be observed in common mass spectra especially when electron ionization (EI) has been applied as an ionization technique. It usually takes place in cyclic systems, where stereochemical details are retained in certain fragments ions because of the different accessibility of reacting groups. A useful and complementary tool for mass spectrometry studies is a computational chemistry, which can give additional insight into the MS-type fragmentation, providing decisive evidence to confirm the proposed mechanism, or even reveal unconsidered decomposition pathways.

**Method:** In our studies we focused our attention to diastereoisomeric 5-phenyl-(2-thioxo-imidazolidin-4-yl)phosphonic acid diethyl esters, which can be regarded as masked diethyl 1,2-diaminoalkylphosphonates, and their specific deuterium labeled analogues. All studied compounds have been analyzed by electron ionization mass spectrometry. In order to confirm the stereospecificity of particular fragmentation pathways we recorded product and precursor ion mass spectra using linked scan mode for metastable decompositions and collision induced dissociation (CID) products. Relative free energies of substrates, intermediates and products ions formed by the fragmentation were calculated using *ab initio* quantum mechanical theory. All calculations were performed using Gaussian 03 package. Optimized geometries were obtained by density functional theory (DFT) calculations using B3LYP level of theory with 6-31G* basis sets without symmetry constrains.

**Preliminary Data:** Our earlier studies have shown that *cis*- and *trans*-diastereoisomers of N-Boc diethyl 5-substituted (2-thioxoimidazolidin-4-yl) could be easily differentiated on the basis of their EI mass spectra [1]. The most important differences were observed for ions formed by loss or elimination of the diethoxyphosphoryl group. Elimination of diethyl phosphonate which is preferred for the *trans*-isomers, requires migration of hydrogen to the phosphoryl group followed by elimination. In presented studies we tried to indicate the most probable source of the hydrogen atom present in the diethyl phosphonate. EI fragmentation data of specific deuterium labeled *cis*- and *trans*-diastereoisomers and DFT calculations indicate that the migration of hydrogen proceeds by five-membered transition state. For isomer *trans* the least activation barrier for hydrogen migration is needed for hydrogen from position C5 and N3. We also performed quantum mechanical DFT calculations of many others possible fragmentation routes for radical cation of model diastereomeric pair which are characteristic mainly for the *cis*-isomer and were detected in standard or product ion mass spectra.

**Novel Aspects:** Elimination of diethyl phosphonate by five-membered proton transfer mechanism.

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# Straight-forward N-glycopeptide analysis by new software functionalities

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Protein glycosylation represents one of the most important post-translational modifications and is found in > 50% of eukaryotic proteins e.g. antibodies, receptors, hormones, etc. The glycan moieties are often either directly involved in the regulatory process or influence physicochemical properties of the glycoprotein. MALDI and LC-ESI provide analytical solutions for glycopeptides and glycans, but *are* still challenging due to the high variety of glycan structures at single glycosylation sites and the general lower ionization efficiency of glycopeptides. High sensitivity, good mass resolution and fast data acquisition are therefore essential. The data interpretation is usually done *de novo* and time-consuming. Software comprising classification of MS/MS spectra in glycosylated and non-glycosylated ones and characterization of glycan structures exhibits a timesaving alternative.

**Method:** The *N*-glycosylation pattern of the murine monoclonal antibody MOPC-21 was investigated as a model analyte for biopharmaceutical applications. It has one *N*-glycosylation at asparagine 294 and a highly heterogeneous complex-type N-glycan pattern as well as additional galactosylation and sialylation of the antennae and core-fucosylation. The tryptic digest of MOPC-21 was separated by an Acclaim PepMap C18 column on an Ultimate 3000 nanoRSLC (Dionex) which was coupled to the CaptiveSpray™ source of an amaZon speed ETD ion trap (Bruker). CID fragmentations was performed in autoMS(n) in Enhanced resolution mode for MS and UltraScan mode for MS/MS acquisition. LC MS/MS data were analyzed in the ProteinScape 3.0 software (Bruker), which was used for glycopeptide classification, identification, and visualization of the mass spectra.

**Preliminary Data:** *N*-glycopeptides of MOPC-21 were chromatographically separated in two groups – a main group at about 29.2 min and the second group around 33.6 min. Data processing was achieved using ProteinScape 3.0, as sophisticated bioinformatics platform. Specific low mass signals, so-called oxonium ions, and glycan fragment distances are used to classify MS/MS spectra as potential glycopeptide spectra. Furthermore, consecutive glycan fragment distances are used to determine the mass of the attached glycan and the peptide backbone for each glycopeptide. Classified *N*-glycopeptide spectra and corresponding glycan masses were then taken for glycan database search with the search engine GlycoQuest™. This resulted in several glycan structure proposals for each MS/MS spectrum. The current approach gives an overview over the glycan database search results obtained for MOPC-21 with ProteinScape. Several different glycan structures with and without core-fucosylation, with additional galactosylation and with attached *N*-glycolylneuraminic acid have been identified for the *N*-glycosylation site at asparagine 294 of the antibody in one LC-MS/MS run.

**Novel Aspects:** Software for automated classification of glycopeptides and glycan structure elucidation.

# A MALDI-TOF MS study of the fragmentation behaviour of cholesteryl esters and phosphatidylethanolamines

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During the last decades the interest in lipids has considerably increased because of their recently discovered physiological importance. Different analytical methods are available for lipid analysis, whereby mass spectrometric (MS) techniques are the most powerful tools. In particular, electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI) turned out very successful ionization methods. Beside these methods, matrix-assisted laser desorption and ionization time-of-flight MS is also increasingly used for the analysis of lipids. This method is simple, exhibits a fast performance and is rather robust against impurities. We will show here that the proton adducts of selected lipids such as phosphatidylethanolamines and cholesteryl esters are not stable enough to survive the flight path and lead to the generation of fragment ions.

**Method:** All chemicals and solvents were obtained in the highest commercially available purity from Sigma-Aldrich (Taufkirchen, Germany) and used as supplied. PL standards (PC 16:0/18:1 and PE 16:0/18:1) were purchased from AVANTI Polar Lipids (Alabaster, AL, USA) as 10 mg/ml chloroform solutions and used as supplied. Different concentrations (from 10 mM to 2 M) of HCl, NaOH, $NH_3$, $NH_4HCO_3$ and NaOAc were prepared in water. The selected samples (PE 16:0/18:1 or cholesteryl linoleate) were then mixed with the acid/base and 2,5-dihydroxybenzoic acid (DHB) as matrix in a ratio of 2:1:2. One microliter of the mixture was directly investigated by positive ion MALDI-TOF MS on an Autoflex workstation (Bruker Daltonics, Bremen) in the reflector mode.

**Preliminary Data:** It is well known that the positive ion MALDI mass spectra of the majority of lipids are characterized by the simultaneous generation of $H^+$ and $Na^+$ (or other alkali metal ion) adducts. It is commonly accepted that the $H^+$ adducts are generated by proton transfer from the (acidic) matrix (such as DHB) or auxiliary compounds such as trifluoroacetic acid. In contrast, $Na^+$ ions can be considered as "impurities" particularly from the used biological samples. If the positive ion mass spectrum of PE 16:0/18:1 is recorded, the peaks at m/z 718.5 and 740.5 correspond to the $H^+$ and Na+ adducts, while the additional peak at m/z 762.5 corresponds to $H^+/Na^+$ exchange [1]. An additional peak at m/z 577.5 indicates pronounced loss of the headgroup [2]. This fragment may be significantly enhanced if the spectrum is recorded in the presence of acids. In contrast, the intensity of both, the $H^+$ adduct and the fragment, are reduced if the spectrum is recorded in the presence of ammonium hydrogen carbonate, i.e. under alkaline conditions. This is a strong indication that the fragment is exclusively stemming from the $H^+$ adduct. A similiar mechanism holds for cholesteryl esters: All cholesteryl esters are exclusively detectable as sodium adducts. All cholesteryl esters are additionally accompanied by a peak (m/z 369.3) that corresponds to free cholesterol subsequent to the loss of water [3]. The addition of HCl enhances the yield of cholesterol and, of course, reduces the sodium adduct of the cholesteryl ester. This is a strong indication that exclusibely the $H^+$ adduct (that is not detectable) leads to fragmentations. Thus, the extent of fragmentation of lipids can be triggered by their environment.

**Novel Aspects:** Fragmentation of lipids can be strongly influenced by the addition of acid or base.

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# The isotopic exchange of oxygen as a tool for detection of the glycation sites in proteins

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The fragments of glycated proteins obtained by enzymatic hydrolysis could be considered as the biomarkers of the aging process and diabetes mellitus. Herein we propose a new method for the identification of peptide-derived Amadori products in the enzymatic digest of glycated proteins by the procedure combining the isotopic exchange with MS [1]. Our recent studies have proven that glycated peptides incubated with $H_2{}^{18}O$ exchanged selectively and almost quantitatively oxygen attached to the anomeric carbon atom which resulted in increase of molecular mass by 2 units per one glycation site [2]. The presented approach can be adapted for analysis of in vivo glycated proteins.

**Method:** The high temperature glycation of ubiquitin was performed according to Boratyński method [3, 4]. The enzymatic hydrolysis of glycated ubiquitin was performed according to the modified procedure described by us [5]. Model glycated protein and peptide-derived Amadori products obtained from the enzymatic hydrolysis of glycated ubiquitin was labeled by $H_2{}^{18}O$ under microwave activation using the procedure published recently [2]. All mass spectrometric experiments were performed on an Apex-Qe Ultra 7T instrument (Bruker Daltonic, Germany) equipped with a ESI source. The instrument was operated in the positive ion mode and calibrated with the Tunemix™ mixture (Bruker Daltonic, Germany). The obtained mass spectra were analyzed using a Biotools (Bruker Daltonic, Germany) software.

**Preliminary Data:** The isotopic exchange may allow selective detection of glycated peptides in enzymatic digest and consequently facilitate identification of glycation sites in the proteins. To evaluate the possibility of utilization of isotopic labeling for detection of glycation sites in the proteins we performed two experiments. In the first approach the sample of glycated ubiquitin was dissolved in $H_2{}^{18}O$ and incubated in microwave oven and then the enzymatic hydrolysis was performed. Obtained results suggests that glycated protein underwent ($^{16}O/^{18}O$) isotopic exchange and the number of exchanged oxygen atoms correlated with the number of glycation sites in the protein molecule (Fig. 1). It indicated that only one oxygen atom in hexose moiety is susceptible for isotopic exchange. In the second approach the glycated protein was subjected to enzymatic hydrolysis, and then the obtained peptides were incubated with $H_2{}^{18}O$ under microwave activation. This procedure does not require direct microwave treatment of the investigated protein. Therefore, the thermal stability of protein is not a limitation. In both cases the incubating was conducted also in $H_2{}^{16}O$ to compare the level of exchange of oxygen atom attached to the anomeric carbon. We found that only peptides containing hexose moieties are susceptible to isotopic exchange under experimental conditions and that the isotopic exchange of oxygen atoms in nonglycated peptides is not significant. Summing up the presented results indicate that the incorporation of $^{18}O$ is characteristic of peptide-derived Amadori products but not of peptides without the hexose moieties [1].

**Acknowledgements**: This work was supported in part by Grant No. N N204 180340 from the Ministry of Science and Higher Education (Poland).

**Novel Aspects:** Application of high resolution mass spectrometry combined with isotopic labeling for analysis of in vivo glycated proteins.

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# Time-dependent intensity changes of free fatty acids detected by MALDI-TOF MS in the presence of 1,8-Bis-(dimethylamino)-naphthalene

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Free fatty acids (FFAs) play important roles in the organism although their concentration must be relatively small due to their detergent properties [1]. FFAs are particularly important in the liver where they may be converted into triacylglycerols or oxidized as fuel. Additionally FFAs represent educts of the synthesis of phospholipids and mediators such as prostaglandins. However, FFA analysis is even nowadays normally based on GC/MS although this technique is laborious and time-consuming. As MALDI MS allows high-throughput screening, reliable FFA analysis by MALDI MS would represent a major methodological progress. Here, we will show that the recently suggested DMAN-(1,8-Bis-(dimethylamino)-naphthalene)-based method [2] is capable of providing quantitative data but requires an exactly defined time-regime.

**Method:** Stock solutions of different saturated and unsaturated FFAs (each 0.1 mg/ml) were prepared in chloroform and diluted 1:1 (*v/v*) with the DMAN matrix (10 mg/ml in ethanol). The FFA / matrix mixtures (about 0.25 µl) were subsequently applied onto a gold-coated standard aluminium MALDI target. Negative ion mass spectra were acquired on an Autoflex I MALDI-TOF mass spectrometer (Bruker Daltonics, Bremen, Germany) in the reflector mode. The system utilizes a pulsed 50 Hz nitrogen laser, emitting at 337 nm and the extraction voltage was 20 kV. The laser fluence was set about 10% over threshold and the pulse delay was between 20 and 100 ns. The high vacuum was of the order of $1'10^{-7}$ mbar.

**Preliminary Data:** DMAN is a powerful matrix that enables the sensitive detection of deprotonated FFAs without significant matrix background and reasonable sensitivity in comparison to earlier approaches [3]. Negative ion MALDI-TOF mass spectra of mixtures between 16:0 (palmitic acid), 18:2 (linoleic acid), 18:1 (oleic acid), 20:4 (arachidonic acid) and 22:6 (docosahexaenoic acid) of the same concentration (w/v) could be easily recorded. Although there are slight deviations, the peak intensities reflect the individual concentrations and, thus, there is no pronounced influence of the double bond contents of the individual FFAs. However, it was noted that the spectrum of the FFA/matrix mixture changes with time if the target is simply left in the MS device under high vacuum conditions: under these conditions the DMAN matrix is not stable but slowly evaporates. A loss of the matrix moiety is indeed accompanied by an increased FFA moiety. Unfortunately, the intensities of the individual FFAs are affected to a different extent: while the intensities of the highly unsaturated FFAs do not show major differences in the presence and the absence of the matrix, the intensities of the other FFAs and particularly that of palmitic acid (16:0) at m/z 255.2 is considerably reduced in the absence of DMAN. Although we do not yet have a convincing explanation of this result, the different absorptions of the individual FFAs at the laser wavelength (337 nm) may play a prominent role because these absorptions increase with the number of double bonds.Thus, it is not surprising that the highly unsaturated FFAs are less affected by the DMAN loss than the more saturated FFAs. It is concluded that a carefully controlled time-regime is necessary for quantitative investigations in order to overcome the time-dependent intensity changes of saturated FFAs.

**Novel Aspects:** One must adhere to a strict time-regime in order to obtain quantitative data of FFA in the presence of DMAN.

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# XtraQuant: New dissociative reagents for efficient relative quantitation of proteins by tandem mass spectrometry using selected reaction monitoring

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Quantitative proteomics is of great significance for the investigation of biological processes and is an appropriate tool to elucidate and evaluate these processes to draw conclusions about influencing factors [1]. In the field of the relative protein quantitation state of the art are reagents which use different labelled reporter ions – that are released in the tandem mass analysis – for quantitation (e.g. TMT or iTRAQ) [2]. Based on our successful research on CID-labile reagents for chemical cross-linking [3] we developed a set of new reagents suited for sensitive and effective quantification of peptides and proteins via MS/MS. The analytical procedure relies on constant neutral loss reactions upon CID which are used for quantification by selected reaction monitoring (SRM) [4].

**Method:** The ESI mass spectrometric analysis of the XtraQuant reaction products was done on a LTQ Orbitrap XL mass spectrometer (Thermo Fisher Scientific). The CID experiments were performed with helium as collision gas. Collision energies were chosen that rarely fragmentation of the peptide occurred, but clearly the fragmentation of the reagent, delivering the desired CNL. Quantification experiments were performed on a triple quadrupol mass spectrometer API 4000 QTrap (Applied Biosystem) coupled with a HPLC system. The quantification was archived by comparison of the characteristic constant neutral losses, generated by specific deuterium label.

**Preliminary Data:** We present an example for the sophisticated and resourceful synthesis of one of the new XtraQuant reagents with a yield of 58% over 5 steps. These reagents are created as activated *N*-hydroxy succinimide (NHS) esters for selective reaction with amine functionalities (lysine, ornithine and N-terminus) in proteins. Deuterium labelled derivatives are synthesized as well. Peptide identification and quantification is accomplished by SRM relying on defined mass shifted neutrals that are expelled upon CID originating from the defined and preferred dissociation of the XtraQuant reagents. We present first results of the application of XtraQuant reagents demonstrating the functioning of the analytical concept. The application of the new reagents for the relative quantitation will be shown on various model peptides. The results evidence convincingly that peptide and protein quantification with SRM is a promising concept adopting a strategy for proteomics that is well established in pharmaceutical industry for metabolite profiling [5]. Hence, it may serve as a new blueprint for substantial improvement of protein quantitation by tandem mass spectrometry.

**Novel Aspects:** New protein/peptide quantification reagents based on selected reaction monitoring (SRM).

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# Design and synthesis of superbasic matrices for low-mass MALDI-MS

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For analyzing large biomolecules like proteins, peptides and sugars, Matrix Assisted Laser Desorption/ionisation mass spectrometry (MALDI-MS) is a powerful technique as it requires relatively little sample preparation and allows a lot of samples to be analyzed in one run. However MALDI is not suited to study metabolites with $m/z < 600$ as conventional MALDI-matrices create a large number of interfering signals in the low mass region. An unconventional approach to this problem is Matrix Assisted Ionisation/Laser Desorption (MAILD) in which a strongly basic (or acidic) matrix is used to analyze weak acidic analytes e.g. fatty acids (or basic analytes like amines). The superbasic 1,8-bis(dimethylamino)-naphthalene [1] is an excellent matrix for this method giving mass-spectra without any matrix derived signals.

**Method:** Taking the structure of DMAN as starting point, several new matrices were synthesized. These were then screened for their potential as MAILD-matrices with stearic, palmitic and trifluoroacetic acid.

**Preliminary Data:** The aim of this study is to develop new matrices with even better ionization properties to not only improve the limit of detection but also to extend the scope of possible analytes towards weaker acidic analytes e.g. amino acids. Initial experiments show that the analyte signal intensity is not only influenced by the basicity of the matrix, but by other factors as well, e.g. reprotonation of the analyte in the gas phase.

**Novel Aspects:** Acid/base driven approach for MALDI-MS for detection of small molecules with potential for metabolomical studies of acidic analytes (fatty acids).

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# Influence of initial velocity of analyte ions on fragment ion yield in MALDI in-source decay mass spectrometry

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Matrix-assisted laser desorption/ionization in-source decay (MALDI-ISD) has been utilized as a tool for the top-down approach to protein identification. The hydrogen transfer from excited matrix molecule to carbonyl group of peptide backbone, leading to hydrogen-abundant peptide radical, initiates MALDI-ISD. Subsequently, the *c'* and *z'* ions are formed by the radical-induced cleavage at the N-$C_\alpha$ bonds [1]. However the MALDI-ISD process generates unexpected fragments, such as *a*, *y'* and w ions, which cannot be explained by the N-$C_\alpha$bond cleavage alone. In this study, the influence of initial velocity of analyte ions on fragment ion yieldin MALDI-ISD processes is described.

**Method:** Analyte peptides were dissolved in water at concentration of 20 pmol/µl. Matrices were dissolved in water/ acetonitrile (1;1, v/v) with 0.1% formic acid. The each 0.5 µl of matrix and analyte solutions were deposited on the MALDI plate and allowed to dry at room temperature. MALDI-ISD mass spectra were recorded using a time-of-flight mass spectrometer, UltraFlex II (Bruker Daltonics, Germany) equipped with freauency-tripled Nd:YAG laser (355 nm).

**Preliminary Data:** MALDI-ISD generates *c'* and *z'* ions by the radical-induced cleavage at the N-$C_\alpha$ bonds. The [z+matrix] ions were also generated by the recombination of *z*· fragments with matrix radicals, whereas the matrix adducts bound to *c'* ions were not observed. Therefore, MALDI-ISD processes may form the *c'*/*z*· fragment pair, and the *z*· radical fragments then gain a hydrogen atom or react with a matrix radical. In this study, we focus on the formation mechanism of *z'* and w ions, which are originate from *z*· radical. Their reactions competitively occur during MALDI-ISD processes. The hydrogen attachment reactions of *z*· radicals must be occurred by the collision between *z*· radicals and matrix molecules. Therefore, it is suggested that the ion yield of *z'* depends on the collision rate of analyte molecules in the MALDI plume. The collision rate of analyte molecules in MALDI plume, N, is proportional initial velocity of analytes, $v_{in}$, and mean free path, λ. $N = v_{in,} \times \lambda$. It is known that the vin, is dependent on the matrix [2, 3]. We compared the MALDI-ISD spectra and initial velocity for the four different matrices, 2,5-dihydroxyl benzoic acid (2,5-DHB), 1,5-diamino naphthalene (1,5-DAN), 2-aminobenzoic acid (2-AA) and 2-aminobenzamide (2-AB). The order of $v_{in}$ is 2,5-DHB > 2-AA > 2-AB > 1,5-DAN. The use of 1,5-DAN gave high ion yields of w ions, while the use of 2,5-DHB gave low ion yields of w ions. The order of ion yield of w ions is 1,5-DAN > 2-AB > 2-AA > 2,5-DHB, which is inversely proportional $v_{in}$. When the 1,5-DAN is used, the high abundance of w ions compared when using 2,5-DHB can be understood as resulting from low collision rate in MALDI plume. The results obtained here suggest that the side chain loss of *z*·radical fragments occur by uni-molecular dissociation process.

**Novel Aspects:** The ion yield of w ions in MALDI-ISD isinversely proportional initial velocity of analyte.

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# Discrimination of protein profiles in pancreatic islets of high fat diet vs. normal diet mice: A MALDI-Imaging study

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This study aims to investigate the applicability of MALDI imaging mass spectrometry to discriminate the protein profiles in pancreatic islets of mice fed with a high fat/high sucrose diet (HFD) in comparison to others undergone a normal diet (ND). MALDI-imaging is a well-suited technique to address this question as islets and the associated protein profiles can be determined simultaneously in pancreas sections with no need of tissue homogenization. Advanced statistical methods were applied for the detection of discriminating m/z-values in islet spectra from the two different classes. Our approach is the basis for further studies with the aim to enable the identification of differentially expressed proteins in type-2-diabetes in a mouse model.

**Method:** The pancreas of wild-type mice (HFD and ND) was immediately dissected and snap-frozen in liquid nitrogen cooled isopentane. Cryosections were mounted on indium-tin-oxide coated glass slides for MALDI-imaging. As a control, consecutive sections were mounted on glass slides for immunohistochemistry staining using an anti-insulin antibody. For MALDI-imaging, slides were coated with sinapic acid using the Bruker ImagePrep™ instrument. Spectra were acquired on the Bruker autoflex speed™ MALDI-TOF mass spectrometer with a lateral resolution of 75 µm and a mass range of 2000-20000 m/z. Islets regions were defined as areas where the insulin signal was higher than in 99% of all spectra. Spectra of islets were classified using DWT-SVM classification approach [1] with the double cross-validation evaluation.

**Preliminary Data:** The detection of islets in pancreas sections by MALDI-Imaging correlated very well with results from immunohistochemistry staining of insulin. Islet mean spectra from the HFD-mouse appeared to be similar to islet mean spectra from the normal diet control. However, some m/z values were overrepresented or underrepresented in the HFD model indicating changes in protein expression levels. This finding could be attributed to the putative development of diabetes in the HFD mouse. This study is the basis for further investigations to verify our results and to identify differentially expressed proteins in a type-2-diabetes mouse model.

**Novel Aspects:** Combination of MALDI-Imaging and Immunohistochemistry in mouse pancreas sections. Classification of spectra using the DWT-SVM approach.

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# Azahelicenes as MALDI matrixes for acidic analyte

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MALDI-MS is as a powerful tool in the analysis of large biomolecules like proteins, peptides and oligosaccharides. However, extending MALDI-MS towards small molecule (< 500 Da) analysis appers to be challenging, as conventional MALDI matrices produce large number of interfering peaks at low mass region. Development of 1,8- bis(dimethylamino)naphthalene (DMAN), a superbasic "Proton Sponges" as a novel matrix for the negative mode MALDI-MS analysis, obviated the problem of low mass region interferences and indicated the potential of MALDI-TOF technique [1]. As some helicenes are also displaying high "Proton Sponge" basicity [2], it would be interesting to study the effectiveness of those helicenes as novel MALDI matrices for the analysis of fatty acids and organic acids in wide range of samples.

**Method:** Twelve helicenes were screened interms of achieved sensitivity, ion-lessness and suitability for the analysis of anions. Weakly acidic low molecular weight fatty acids (stearic acid, palmitic acid) and absicic acid were used as analytes in the preliminary screening. Based on the preliminary observations, the promising candidate, 1,1'-diazapentahelicene was further tested to study its suitability to detect organic acids in fruit juices (apple juice, orange juice , pineapple juice) / wine and to determine its applicability to diverse compounds of natural origin or synthetic drugs.

**Preliminary Data:** Out of the tested helicenes, 1,1'-diazapentahelicene appeared as a potential candidate, which could be used to detect analytes at picomole concentrations in the negative mode MALDI-MS. Clear signals for acid anions were observed for the tested acids with suppression of matrix-related ions in the low mass region. Further more, clear signals for deprotonated organic acids were detected in the tested fruit juices and wine samples, confirming its suitability as a novel matrix.

**Novel Aspects:** Development of novel MALDI matrices facillitate small molecule analysis via MALDI-MS.

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# Whole Bodies Mass Spectrometry Imaging of Cuticular Lipids on Drosophila melanogaster Flies

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The insect cuticle has many functions owing to the several-micrometer-thick layer of wax on its surface [1]. In addition to the protection the cuticle offers, some of its lipids are involved in chemical communication [2]. To date, relatively flat samples (insect wings, leaves) have been imaged using MALDI or LDI-MSI [3]. There are several disadvantages to conducting 2-D MALDI-MS imaging on 3-D biological samples. Drying a sample causes its deformation and thus changes its morphology. Additionally, as on-line laser focus readjustment towards 3-D shape of the biological sample surface on MALDI-MS instrument during imaging is by now technically impossible, significant differences in signal intensities are inevitable. Additionally, non-conductive body surface induce charging and associated mass shift.

**Method:** Targets with either simple grooves or profiled holes designed to accurately accommodate the male and female bodies of flies were fabricated. Virgin *D. melanogaster* males and females were glued onto targets and LDI-MSI was performed using Maldi micro (Micromass, Manchester, UK) fitted with 337 nm laser. MALDI-MSI was performed after spraying by LiDHB matrix over the fly body. The mass data were refined and PCA-classified using in house software.

**Preliminary Data:** The spatial distribution of cuticular lipids on the surface of six-day-old separately reared *Drosophila melanogaster* flies has been visualized and studied using matrix-assisted laser desorption/ionization – time of flight (MALDI-TOF) mass spectrometry and laser-assisted desorption/ionization – time of flight (LDI-TOF) imaging (MSI). Attention was paid to neutral lipids such as triacylglycerols and other compounds including the male anti-attractant 11-*cis*-vaccenyl acetate for which the expected distribution with high concentration on the tip of the male abdomen was confirmed.

**Novel Aspects:** Lipids were imaged on fruit fly whole body using customized target and data refinement by in-house software.

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# Pressure- and Laser Fluence-Dependence of the Ablation Dynamics of Ionic Liquid Matrices investigated by Fast-Flash Imaging

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Ionic Liquid Matrices (ILM) have emerged in recent years as a promising alternative to conventional solid MALDI-matrices [1]. In comparison, they offer some advantages, most notably concerning the shot-to-shot reproducibility and homogeneity of the signal intensity [2]. This properties are believed to arise from a significantly more uniform ablation process, a better distribution of the analyte in the liquid and the ability to regenerate the sample surface after the ablation. These characteristics also make ILMs perfectly suitable for investigations of the ablation dynamics. Fast-Flash Imaging uses very short light pulses to generate snapshots of short-lived phenomena, such as material ejection following the laser irradiation. Variation of the delay time between irradiation and illumination allows for the investigation of the complete ablation process.

**Method:** The experiments were carried out on a specially designed instrument, which offers the possibility to simultaneously record Fast-Flash Images and mass spectra during a single experiment. The mass spectra were measured on a linear time-of-flight mass spectrometer equipped with a two-stage ion source and a 135 cm long drift region. Ablation and Ionization was achieved by a frequency-tripled Nd:YAG laser (355 nm). The FFI Setup is placed orthogonal to the TOF axis. The illumination source is a solution of Rhodamine 6G in Methanol pumped by a frequency-doubled ND:YAG laser (532 nm). A zoom lens is used for magnification and the image is detected with a CCD camera. A delay generator controls the time-lag between the ablation and the illumination.

**Preliminary Data:** The ablation dynamics were studied using an ILM consisting of α-cyano-4-hydroxycinnamic acid and tributylamine. The general mechanisms however do not seem to change when other ILMs are employed. The laser fluence used in the experiments was determined to correlate to the onset of detectable TOF-MS signals. The fluence does not appear to have a significant effect on the ablation mechanism, higher values do however, as can be expected, lead to the ejection of more material. At $1\times10^{-6}$ mbar, directly after the ablation laser pulse hits the sample, a swelling of the surface is visible. This swelling increases for roughly 50 ns and during this time, fluorescence can be detected from the irradiated region. 50 ns after the ablation pulse, material ejection starts with the formation of beam-like structures originating from the surface. These beams expand into the evacuated space over the sample for about 400 ns, while the density of the plume decreases constantly. After the initial ablation is finished, a cloud-like structure is visible over the sample surface until about 1 µs after the laser pulse, when a second ablation starts to take place. Unlike the beam-like appearance of the initial ablation, this event appears to be composed of clusters and liquid droplets. It is significantly slower then the first ablation, but can be detected for up to 30 µs. These observations are in good agreement with earlier results from post-ionization experiments [3]. To investigate the influence of the pressure on the ablation dynamics, multiple series of Fast-Flash Images were recorded at pressures between atmospheric pressure and $1\times10^{-6}$ mbar. Ablation at values near atmospheric pressure shows a completely different behavior, consisting predominantly of liquid droplets and the second ablation event doesn't occur. At about 100 mbar, the mechanism suddenly changes to the one visible in the high vacuum.

**Novel Aspects:** The ablation dynamics of ILMs are investigated in such detail for the first time.

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# Single cell MALDI imaging

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The knowledge of spatial distributions of biological compounds in single cells is important for understanding cell functions. Commonly used techniques such as optical and staining methods led to important discoveries but lack molecular specificity. MALDI mass spectrometry imaging (MSI) is a widely used analytical technique to visualize spatial distributions of multiple analytes in biological samples. The highest published spatial resolution so far for single cells was 50 µm. In this study we applied MALDI-MSI to analyze single cells on an Orbitrap Exactive MS. A spatial resolution of 7 µm and mass accuracies better than 3 ppm were achieved. Numerous lipids and small metabolites were imaged and identified in a single cell.

**Method:** HeLa cells, an immortalized human cell line derived from cervical cancer, were directly grown on indium tin oxide (ITO) coated glass slides. The cells were fixed with Glutaraldehyde and stained with DIOC6(3). After Matrix coating (DHB, 30 mg/mL in 50:50 acetone/water/0.5% TFA) performed with an in-house build matrix sprayer [1] the cells were analyzed using a home-built atmospheric-pressure scanning microprobe matrix assisted laser desorption/ ionization (AP-SMALDI) imaging source [2] attached to a Fourier transform orbital trapping mass spectrometer (Exactive Orbitrap, Thermo Scientific, Bremen, Germany). Selected ion images were generated using the software package MIRION developed in-house [3].

**Preliminary Data:** An area of 749 × 749 µm on an ITO-slide covered with HeLa cells was rastered with a pixel size of 7µm (107 × 107 pixel). Mass range was m/z 100-1000 and mass resolution was 100000 @ m/z = 200. Several selected ion images with a bin width of ± 0.005 u of different compounds were generated. The selected ion images were generated directly from raw data, i.e. no interpolation or normalization procedure was applied. Selected ion images and optical fluorescence images show excellent correlation of shape and size of the HeLa cells. Even small features of the cells were reproduced. This would not be possible using "standard" pixel sizes of 50 µm or more, which is commonly used in MALDI MSI. High mass accuracy guarantees a confident identification of compounds in the cells. Even in a spectrum of a single pixel a large number of compounds were detected. Several lipid classes such as phosphatidylcholine, sphingomyelin, phosphatidic acid, diglycerides & triglycerides were detected. The mass deviation was in all cases better than 3 ppm (RMS). Also small metabolites such as the DNA bases adenine or guanine as well as Cholesterol were detected. We also demonstrate with the single cell MSI measurements the necessity of high spatial resolution. We combined for the first time high spatial resolution, high mass accuracy and high mass resolution in one MSI measurement for single cells. In combination with the dedicated sample preparation protocol an excellent tool for single cell analysis is now available merging spatial resolution in the low µm range and high mass accuracy, necessary for a confident identification of cell compounds.

**Novel Aspects:** High mass accuracy, high spatial resolution and high mass resolution combined in one MALDI MSI method for single cells analysis.

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# The MALDI-imaging/ MULTI-ARRAY Core Facility: A new Service Platform at the University of Bremen

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The MALDI-imaging/ MULTI-ARRAY Core Facility at the University of Bremen (http://www.maldi.uni-bremen.de) is a newly established institution providing expertise and service in matrix assisted laser desorption/ ionization (MALDI)-imaging mass spectrometry, also called MALDI-imaging, and MULTI-ARRAY® technology combined with computational solutions for data analysis. MALDI-imaging is a powerful tool for the analysis and visualization of (bio)-molecules in their spatial proximity directly from tissue sections, plant samples and bio- or polymer films. By measuring several slices of an object this technique can even be used for the generation of 3D-MALDI-images. Biomarkers can be detected in a molecular discovery process and validated by exploitation of the MULTI-ARRAY® electrochemiluminescence-based platform for high throughput analysis of proteins in serum-, cell and tissue lysates.

**Method:** The MALDI-imaging/ MULTI-ARRAY Core Facility holds the Bruker autoflex speed™ MALDI-TOF mass spectrometer equipped with the smartbeam™-II laser technology providing a repetition rate of 1000 Hz for a maximum performance and guaranteed spatial resolution down to 50 µm. Our service model envisages access to the technology for internal and external users by the straightforward 'sample in – data out' approach. The MALDI-imaging workflow will be executed by our specialists and encompasses sectioning, tissue and requirement-specific sample processing, matrix application using the Bruker ImagePrep™ sample preparation device, measurement, staining and data analysis in a user-tailored fashion. Complimentary data analysis and evaluation can be achieved by specialized and up-to-date developed computational methods provided by long-standing experts in the analysis of MALDI-imaging data.

**Preliminary Data:** Here, we aim to introduce the MALDI-imaging/ MULTI-ARRAY Core Facility and the service we are providing. We present the organization structure and the instrumentation of the Core Facility. The power of the MALDI-Imaging technology will be demonstrated exemplified by own executed tissue samples including 3D-MALDI-imaging pointing out the importance of data analysis methods for improved information extraction from MALDI-imaging datasets using our own computational pipelines.

**Novel Aspects:** Access to the innovative MALDI-imaging technique for external users in combination with up-to-date developed mathematical data analysis methods.

# Simulation of huge three-dimensional imaging mass spectrometry dataset

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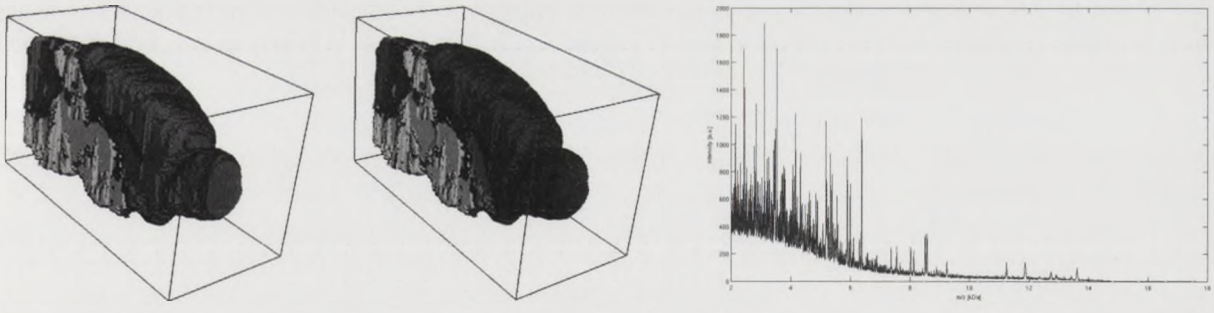
Over the last years matrix-assisted laser desorption/ionization imaging mass spectrometry (MALDI-IMS) has become a useful bioanalytical technique of spatial biomedical analysis. The mass spectra collected over a flat sample reveal the spatial distribution of hundreds of molecular compounds ranging from metabolites to proteins. By slicing a sample into serial sections, measuring each individual section, and then merging all individual 2D MALDI-IMS datasets into one 3D data, one can perform 3D IMS analysis. 3D MALDI-IMS data is huge, reaching a million of spectra and 10-100 GB per dataset. This requires development of special computational methods.

**Method:** With the ever increasing dataset sizes of 3D IMS, computer aided processing becomes more important and new algorithms have to be developed [1]. The evaluation of new methods has to be performed in objective and possibly unbiased manner that requires a gold standard dataset. We propose to use a statistically simulated dataset.A mouse brainsyntheticdataset (127syntheticslices, voxel size 25 µm, 12 annotated anatomic regions, 1.000.000 voxels) is simulated based on the Allen Brain atlas [4]. The spectra simulation is done according to the flight-time-model established by [2]. Certain characteristics such as the baseline and present noise level were modelled according to [3].

**Preliminary Data:** The selected parameters for our model result in 181 peaks, distributed within the m/z range of 2000 to 20,000 Da. This mass range is commonly observed in real-life MALDI spectra. Each simulated spectrum is treated with standard preprocessing algorithms, which include baseline removal and normalization. Automated segmentation of the preprocessed dataset is done with our recently proposed algorithm [1]. The algorithm was specially designed for processing of huge number of spectra. The accuracy score of the obtained segmentation map is measured with statistical comparison measures like sensitivity, specificity, and derivations thereof. Here balanced accuracy i.e. the mean of sensitivity and specificity, seems to be the best measure to reveal a correct segmentation map. This is likely a result of the measure being less affected by different class sizes. Because of the dataset size, which exceeds 100 GB for the unprocessed data alone, it is stored in a HDF5 file container, allowing efficient data access. Data size almost doubles if all intermediate steps are stored, however this allows easy testing of different smoothing parameters that affect the segmentation map.

**Novel Aspects:** We introduced statistical simulatortogenerate gold standard 3D MALDI-IMS data and proposed strategies for statistical evaluation of unsupervised methods.



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# Algorithm for Analyzing Mass Spectrometry Data from Crosslinked Proteins

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Chemical crosslinking of proteins is a non-targeted, exploratory approach to elucidate protein-protein interactions and thus can be used to predict protein function. Amine-reactive crosslinking reagents such as DSS (Disuccinimidyl suberate) or BS3 (Bissulfosuccinimidyl suberate) can be used to covalently link two proteins together. Subsequent mass spectrometry analysis after protein digestion of the crosslinked sample leads to spectra derived from two crosslinked peptides among others. Identification of crosslinked peptides from such spectra is not possible with standard proteomic tools such as OMSSA [1] or Sequest [2]. Different specialized tools are available, but with limitations in regard to database size, running time, usability for high throughput analysis and/or support of different operating systems.

**Method:** The algorithm is implemented in Python. Proteomatic [3] is used for providing a graphical user interface on Windows, Mac OS X and Linux. pymzML [4] is employed for very fast parsing of mzML data and provides handling methods for spectra. The hits are scored with an E-value based on a classic probability score as described in [1]. High mass accuracy MS2 data is desirable to reduce running time via deconvolution of spectra. Deconvolution reduces the number of peaks in a spectrum, because masses are calculated from m/z values. However, it is possible to use standard mass accuracy data without deconvolution. Analysis of high-mass-accuracy mass spectrometry runs with a database containing 574 proteins takes only slightly longer than the measuring time.

**Preliminary Data:** The algorithm was tested with a crosslinked bovine serum albumin (BSA) probe and validated against other crosslinking algorithms. It was applied for evaluation of mass spectrometry data derived from crosslinked samples stemming from complex mixtures such as isolated photosystem I from Cyanidioschyzon merolae or isolated thylakoids from Chlamydomonas reinhardtii. Known interacting chloroplast proteins as well as new unknown interactions were identified. Examples of recognized crosslinked peptides from MS/MS data will be presented.

**Novel Aspects:** The algorithm analyzes MS/MS data from complex mixtures in reasonable time while being free software, user-friendly and operating system independent.

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# Algorithm supported identification of phosphorylated neuropeptides from single insects by means of MALDI TOF mass spectrometry

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In contrast to protein phosphorylation, phosphorylation of neuropeptides in insects is uncommon and only known from a single insect species [1]. In this approach, an algorithm was developed to search MALDI TOF mass spectrometric data of neurohemal tissues for ion signal patterns typical of peptide phosphorylation. The data set included about 1000 mass spectra from more than 100 species of various insect orders. The data confirmed that phosphorylation of insect neuropeptides is very uncommon. However, the data screening enabled the detection of a taxon-specific phosphorylation of CAPA-pyrokinin. This peptide hormone belongs to the few neuropeptides of insects which function is still unknown.

**Method:** In addition to the screening of an already existing data set, further neurohemal organs were dissected and transferred on a MALDI sample plate and rinsed with pure water. A total of 0.5 µl of CHCA matrix solution (Hewlett Packard) was added to each sample. Remaining salts were removed by washing of the dry sample with pure water. MALDI post source decay was performed on an ABI4800 mass spectrometer (Applied Biosystems, Framingham, MA). Processed mass spectra were exported in excel sheets (Microsoft Excel 2002) for subsequent analysis. The search algorithm was written in Visual Basic for Applications (Microsoft Visual Basic 6.0). For probability estimation, neutral losses, mass differences, signal resolution and signal to noise ratio have been taken into account.

**Preliminary Data:** An algorithm for the simple detection of peptide phosphorylations by analysis of specific ion signal patterns from mass spectrometric fingerprint data was applied for the screening of a comprehensive data set of neurohemal tissues from insects. Beside the mass shift of 79.97 Dalton by phosphorylation the neutral loss of phosphoric acid due to post source decay resulting in a mass shift of 98 Dalton with considerably decreased ion signal resolution as well as the occurrence of corresponding sodium and potassium adduct ions were used as reliable markers for automated detection. The analysis of the data set revealed putative phosphopeptides among the CAPA-peptides of the abdominal perisympathetic organs in cockroaches (Blattodea). Tandem mass spectrometry confirmed, that these peptides are phosphorylated forms of the CAPA-pyrokinin (CAPA-PK) and that the phosphorylation site is a serine residue at fifth position. Consensus sequences of various cockroach species show $Ser^{5}$-X-Glu as highly conserved sequence motif, serving as possible recognition sequence for protein kinase. This sequence motif is already described as consensus sequence for Golgi apparatus casein kinases (GCK) [2], which are phosphorylating proteins and peptides destined for extracellular segregation in vertebrates. Species with substitutions of serine 5 and/or glutamic acid 7 had no phosphorylated CAPA-PK. Interestingly, the activity or presence of the respective GCK-like kinase in CAPA-synthesising neurons seems to be taxon-specific, which results in phosphorylation/non-phosphorylation of identical peptide hormone sequences in related species.

**Novel Aspects:** Novel taxon-specific phosphorylation sites of neuropeptides were detected by automated screening of mass spectrometric data for phosphorylation-specific ion signal patterns.

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# AmbiBASE – an open database and analysis software for de novo sequencing and structure analysis

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*De novo* sequencing of unknown peptides and other, more general methods of ab initio MS-based structure analysis are significantly improved in performance by employing restricitive databases that contain information on the boundaries of possible variation and on the interpretation of computational results. Especially natural products can be characterized in structure with much higher confidence, if information on the relevant substance classes is taken into account. In many cases, such substance classes are built up from a defined set of building blocks (amino acids in the case of the peptide substance class) or are modified by a limited set of functional group variations (such as the common modifications in metabolites).

**Method:** Defining a limited set of building blocks and modifiers, and defining lists of possible substance class members is used to rapidly and confidently identify and characterize unknown compounds in natural samples such as insect hemolymph or amphibian skin secretions. The substance database AmbiBASE serves two main tasks: 1) collecting and organizing relevant data on ions, molecules, building blocks, chemical metadata, structural information (MOL- and PDB-files), and literature references, 2) extracting information from the database to allocate it to certain observed ion signals and to provide individual lists of candidates for precursor ions and fragment ions, based on accurate mass. AmbiBASE itself is a MySQL database located on a computer server remote from operating workstations.

**Preliminary Data:** The database currently contains five tables managing data on users, substances, substance classes, adducts/functional groups and literature. AmbiBASE is operated by a user friendly stand-alone software "Ambilys" running on the connected workstations. Ambilys is used for data collection, data organization and data analysis. Data collection capabilities include adding, filtering, deleting, viewing and organizing data. Data analysis functions of Ambilys make use of the data pool to derive suitable sets of possible ion structures and building block combinations. Results can be exported for use in other programs like MS imaging software or spreadsheet calculations. AmbiBASE currently contains data on lipids, mycotoxins, cyclic and linear peptides, amino acids and some other specific substance classes such as phallotoxins and amatoxins. For each substance entry, the name, chemical formula, calculated mono-isotopic exact mass, average (chemical) mass, CAS number and structural data are stored. Data contained in the database can be managed and manipulated by AmbiBASE on the server. Primarily, however, the database is controlled, filled and used through the remote stations running the Ambilys software. A LAN is therefore needed for effectively using the AmbiBASE system. The database can be installed under any common operating system that allows to run a MySQL 4.x or 5.x server. Ambilys was developed under Delphi within a MS Windows operating system.

**Novel Aspects:** A MySQL database supports de novo sequencing and structure analysis of unknown peptides and other compounds, using accurate mass analysis

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# A Metabolomic Study an a Specific Pathway Change in Human Cells During Drug Treatment

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The effect of Rapamycin, an immunosuppressant drug, on the metabolome of HEK 293 (HumanEmbryonic Kidney cell line) was studied. Polar and non polar metabolites were extracted at specific intervals of time and measured using untargeted LC/QTOF MS acquisition. The purine metabolism is known to be targeted indirectly by immunosuppressive drugs such as mycophenolate mofetil. A database was created by extracting the accurate mass of compounds found in the purine metabolism pathway. This database was used to mine data from the untargeted acquisition. A differential expression of these targeted metabolites from this pathway will provide information on how the purine metabolism pathway is affected by Rapamycin treatment.

**Method:** HEK 293 cells are treated with Rapamycin or vehicle only for 16 hrs time points. Metabolites are extracted from drug treated and control cells using a 2:1:2 (Methanol : water : chloroform) mixture and extracted twice. The aqueous layer is collected for LC-MS analysis. The metabolites are separated using a methanol/ water gradient and identified using a QTOF mass spectrometer. Databases of metabolites found in specific pathways are created using a custom software package written to extract and create an accurate mass database from selected KEGG pathways. This database is used for data mining of untargeted LC-MS data.

**Preliminary Data:** The results show that potential metabolites from purine metabolisim pathway start to display statistically up regulation from 45 min onwards post drug treatment. From 2 hrs to 16 hours time point, most metabolites show significant up regulation. Created an accurate mass database of metabolites found in specific pathways. Untargeted metabolomics data acquisition was mined using a database of compounds found in purine metabolism pathway. A time dependent change in intensity is visualized using 'K means clustering. Discovery based, targeted data mining of data acquired from untargeted MS acquisition enabled this metabolomics study to focus on behavior of specific metabolites from specific biological pathway.

**Novel Aspects:** Created an accurate mass database of metabolites found in specific pathways.

# Laser ionization of PAH and phenothiazines in ion mobility spectrometry

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Ion mobility (IM) spectrometry is an established analytical technique for the real time detection of explosives, drugs and industrial chemicals. The underlying principles are the ionization of gaseous substances, the separation of ions in a drift tube, and their detection at a Faraday plate. The resolution of IM spectrometers is limited, but an increase of the analytical selectivity can be achieved by a more selective ionization principle, e.g. REMPI [1]. In this work we have combined IM spectrometry with gas chromatography as a sample introduction method for substances with low vapor pressure. In a second approach, first introduced in mass spectrometry [2], liquid samples are transferred into the gas phase and are subsequently ionized by laser ionization.

**Method:** Two home-built laser IM spectrometers featuring 100 mm drift tubes were used for the investigation of gaseous and liquid samples, respectively. The first IM spectrometer is connected to a gas chromatograph (Agilent, HP 5890 SII). The evaporation source of an APCI unit (Thermo) is used in the second IM spectrometer. Two DPSS lasers (EKSPLA NL204-FiH, NL204/FH), emitting ns pulses (50-300 µJ) at $\lambda = 213$ nm and $\lambda = 266$ nm, are applied for the ionization. The ions were furthermore analyzed and identified by means of another home-made IM spectrometer coupled to an ion trap mass spectrometer (LTQ XL, Thermo).

**Preliminary Data:** GC facilitates a complete preseparation of PAH and phenothiazine mixtures, the introduction of substances with low vapor pressures into the laser IM spectrometer and their reliable quantification. Ion mobilities and corresponding diffusion cross sections (DCS) were determined. All substances, including the isomeric pair perylene/ benzo[a]pyrene, could be distinguished by their DCS. The limits of detection (LOD) were determined for different ionization wavelengths ($\lambda = 213/266$ nm) and pulse energies (50-300 µJ). The LOD of anthracene and promazine are in the low fmol range. The influence of different ionization parameters on the LOD was also determined for the second IM spectrometer, which was used to investigate liquid samples. The LOD are 2-3 orders of magnitude above those of the GC laser IM spectrometer. A special focus of our investigations was on the different ionization mechanisms in the presence and absence of gaseous solvent clusters. In contrast to laser ionization in vacuum (isolated ions), at atmospheric pressure secondary reactions of the primary ions with neutral molecules can be observed. The introduction of solvent molecules increases the number of possible reaction paths. While in the absence of solvent molecules fragment ions, molecular ions and complex ions could be detected, at high solvent densities a significant simplification of the product spectrum was observed. The relative response factors of product ions are strongly concentration dependent, indicating complex secondary reactions.

**Novel Aspects:** The instrumentation of Laser IM spectrometer was extended to the investigations of liquid samples. Furthermore, ionization mechanisms are compared.

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# 7200 Series Q-TOF for GC/MS. A new analytical tool for solving complex analytical problems

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The analytical power of GC/MS with high mass accuracy mass spectrometry is useful in two broadly-defined ways: for the identification of unknown compounds, and to provide increased selectivity for the determination of target compounds, especially in complicated sample matrices. Whether the theoretical gains of the latter application can be achieved in practice depends greatly on the mass accuracy of the mass spectrometer, its mass axis stability over time, and the nature of the matrix interferences (relative retention time, mass, and magnitude). Here we present the design and the function of the new 7200 Series QTOF for GC/MS and how the system can help to solve complex analytical problems.

**Method:** The new Removable Ion Source allows fast swapping of the complete Ion Source, including filaments, in about 30 minutes without venting and the new Internal Reference Mass "locks" mass axis to known background ions, thus acting as a "On the Fly" mass axis correction. Through this a typical mass accuracy < 2 ppm can be achieved.
The fast data rate up to 50 spectra/sec allows deconvolution of closely eluting peaks.

**Preliminary Data:** Examples for the advantageous use of the System in GC-TOF and in GC-QTOF mode are shown for heavy matrix food and environmental samples. The elimination of interference from the matrix at low concentration of the analyte can be illustrated by considering Endrin (10 pg) in leek extract. GC-TOF Data show that with a mass extraction window of ± 0.5 Da there are large matrix interferences at m/z 262,8564 when compared to using a ± 20 ppm mass extraction window. But even with the narrow window of ± 20 ppm the interference is not completely removed. Using MS/MS with high mass accuracy results in clean peak for Endrin at m/z 192.9150. This example shows that only MS/MS (QTOF mode) allows to eliminate the matrix interferences completely and to get a consistent peak shape. Higher resolution, better mass accuracy and faster scan speed always improves analytical results. The use of MS/MS (QTOF mode) allows a further improvement of the interference rejection even for low concentration of the analytes.

**Novel Aspects:** Higher resolution, better mass accuracy and faster scan speed always improves analytical results.

# Intra-laboratory validation of a fast and sensitive UHPLC-MS/MS method with fast polarity switching for the analysis of lipophilic shellfish toxins

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Recently, the mouse bioassay as the long-standing reference method for lipophilic shellfish toxins has been replaced by a LC-MS/MS method by EU Legislation. There have been several reasons for that, among others the lower false positive and negative rates as well as the better precision and accuracy of the chemical assay. This has been shown in the interlaboratory validation study coordinated by the EURLMB, as well as in some other independent interlaboratory validations carried out in the EU, using different LC-MS/MS methodologies. Whereas different HPLC conditions have been used, common for both methods is that the determination of all regulated lipophilic shellfish toxins requires acquisition in positive and negative electrospray ionization (ESI) to gain the best possible sensitivity.

**Method:** However, there is concern that throughput for the LC-MS/MS method is not sufficient for routine use. In this work we show the results of an intra-lab validation for a fast UHPLC-MS/MS method working under acidic conditions. 14 lipophilic marine toxins have been acquired in fast polarity switching and using Dynamic MRM for optimizing the duty cycle of the MS. Whereas azaspiracids, pectenotoxins, 13-desmethyl spirolide C, and gymnodimine have been analyzed in positive mode; yessotoxins have been analyzed in negative mode. The okadaic acid group compounds have been analyzed in both, positive and negative ionization and results for both ionization modes have been compared.

**Preliminary Data:** The validation has been done using the experimental design and samples of the interlaboratory validation study for the EU harmonized SOP coordinated by the EURLMB over a 3-day period. When using Dynamic MRM in fast polarity switching limits of detection (LODs) have been lower and reproducibility and linearity have been better compared to static MRM. The UHPLC separation allows for higher sample throughput in routine use. Compared to the previously used HPLC-MS/MS method LODs have been improved up to a factor of 10 in mussel extract. For the OA group results acquired with negative ionization showed better sensitivity and lower matrix suppression. Matrix effects have been evaluated by comparing standards prepared in solvent with matrix matched calibrations prepared in blank mussel tissue. For accurate quantitation matrix matched calibrations have been used. When analyzing reference mussel materials apparent recoveries have been between 95 and 110% with RSDs below 5% over the 3 day validation procedure. For selected samples new triggered MRM acquisition mode has been applied for additional confirmation of AZA1 and PTX2 showing more than one peak for both, the quantifier and qualifier ions.

**Novel Aspects:** Dynamic MRM in fast polarity switching limits of detection (LODs) have been lower and reproducibility and linearity have been better.

# High Mass Accuracy Provides Enhanced Selectivity for the GC/MS Analysis of Pesticides in Food

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The analytical power of GC/MS with high mass accuracy mass spectrometry is useful in two broadly-defined ways: for the identification of unknown compounds, and to provide increased selectivity for the determination of target compounds, especially in complicated sample matrices. Whether the theoretical gains of the latter application can be achieved in practice depends greatly on the mass accuracy of the mass spectrometer, its mass axis stability over time, and the nature of the matrix interferences (relative retention time, mass, and magnitude.) Here we present some results obtained with typical food samples containing low levels of pesticides.

**Method:** Food samples (okra, frozen blackberry and frozen blueberry) were prepared by the QuEChERS method. The okra was spiked with 300 pesticides at the EU regulatory levels. Samples were analyzed on a GC/QTOFMS system (Agilent 7890 GC: multimode inlet, cold splitless injection, 15M × 0.25 mm × 0.25 u HP-5MS UI analytical column, postcolumn backflush configuration. QTOF mass spectrometer: orthogonal acceleration/single reflectron configuration, 70 eV EI source, 1 M flight tube, 4 GHz sampling rate, dual gain mode, 10 kHz rep rate, 5 Hz data rate, 8 kV ion energy, 10-1700 Dalton mass range, nitrogen collision gas.) Data were analyzed with Agilent MassHunter software.

**Preliminary Data:** Example data is given for several of the pesticides. The elimination of interference from the matrix can be illustarated by considering Flumetralin. Data show that with mass extraction window of ± 0.1 D there are large matrix interferences at m/z 143.0058 and 145.0029 when compared to using a ± 20 ppm mass extraction window. The matrix interferences have been eliminated, and consistent peak shape can be seen for all analyte ions. The higher mass ions, 360.0283 and 404.0320, proved to be more immune to interference, as experience would suggest. For quantitative studies, excellent response linearity is observed in the presence of a 40-fold excess of interferent abundance.

**Novel Aspects:** Selectivity (interference rejection, S/N ratio) greatly improved through the use of accurate mass in GC/QTOFMS for pesticide analysis in food.

# The interactions of metal ions with the benserazide and L-dopa

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One of effect of Parkinson disease (PD) is neuronal loss which leads to the decrease of dopamine (DO) polls. Neurodegeneration process provides the changes in the homeostasis of metal ions: zinc, copper and iron. One of directions of treatment of PD is based on the increase of the dopamine levels in brain by L-DOPA (LD) application. To prevent process of peripheral degradation of LD is administrated with benserazide (BZ). Several investigations suggest that the $Zn^{2+}$ supplementation in PD is warranted. BZ in water solutions is unstable (undergo to two process: oxidation and equilibrium reactions). Presented investigations of interaction of metal ions are important to understand better the possible mechanism of action of the LD and BZ.

**Method:** Experiments were preformed on micrOTOF-Q (BRUKER) instrument with ESI source. Spectra were recorded in the positive ion mode (electrospray voltage 4.5 kV, capillary temperature 180-200°C). The collision energies of MS/MS experiments were 1 and 8 eV. Reagents for analysis were: benserazide hydrochloride (Sigma Aldrich), L-dopa (Sigma Aldrich), $CuCl_2 \times 2H_2O$ (POCH), $Zn(CH_3COO)_2 \times 2H_2O$, (POCH)and $FeCl_2 \times 4H_2O$ (MERCK). Acetic acetate buffer concentration was 0,005M and pH was in the range 4.5-6. Metals were added to all samples in molar relation 0,8 equivalent(eq) to 1eq of BZ concentration of BZ was from 0.5 to $1 \times 10^{-5}$ M. The LD molar relation was 1-4eq to 1eq B. Samples were prepared in argon environment and no visual oxidation process was observed.

**Preliminary Data:** It was shown forming of $M[BZ(H^{-1})]$ complexes where M = $Cu^{2+}$, $Zn^{2+}$ and $Fe^{2+}$. Additionally it was presented that in system contains of the $Cu^{2+}$ and active components of MADOPAR in mass spectrometry measurements conditions the ternary complex contains BZ and LD its forming. It was proved that metal ions: $Zn^{2+}$ and especially $Cu^{2+}$ stabilize the BZ in the prism of equilibrium process of BZ.

**Novel Aspects:** Demonstrated are possible interactions of metals with active components of drugs and impact of metal ion coordination on drug stability.

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# M-pyQuant – High Throughput and Accurate Quantification of Mass Spectrometry Data

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High throughput quantitative analysis of mass spectrometry data requires algorithms that allow fast and exact quantification of molecules. M-pyQuant is such an algorithm based on the Python scripting language, thus can easily be incorporated in other workflows/programs. Isotopic envelopes are calculated based on the chemical formula, thus its matching function can be applied to Lipidomics, Metabolics or Proteomics. Different kinds of stable isotope labeling (e.g. SILAC, 15 N) and variable or fixed posttranslational modifications are supported. The algorithm also allows assessment of incorporation efficiency of the isotopic label which makes analysis of pulse-chase experiments possible.

**Method:** Isotopic envelopes of candidate molecules that origin e.g. from MS/MS identifications or from sequence databases are exactly predicted. The calculation of envelope peak masses and intensities is based on relative isotopic abundances and exact isotopic masses (Berglund & Wieser 2011). In order to quantify peptides their predicted envelopes are matched against MS1 spectra and scaled towards measured peak intensities. The number of matched peaks and the accuracy of the match between measured and predicted envelope are scored. The speed the algorithm is achieved by fast parsing of mzML files (pymzML, Bald & Barth et al. Bioinformatics accepted) and by usage of sophisticated matching functions that are part of M-pyQuant. Parallelization can easily be applied, further increasing the overall matching speed.

**Preliminary Data:** To benchmark the algorithm it was tested on proteomic data. A mixture of unlabeled (light) and 15N metabolically labeled (heavy) samples that had been measured on LTQ Orbitrap (Thermo Scientific) was analyzed. The candidate dataset is based on the complete JGI Chlamydomonas reinhardtii gene model database (v3.1). All protein sequences were in silico tryptically digested considering two missed cleavages. The resulting peptides were filtered for sequence lengths of 7-50 amino acids. Three different charges [2-4] and two isotopic labeling efficiencies (heavy and light) were considered. The algorithm predicted > 7.4 million candidate isotopic envelopes that lie in the measurable m/z range of the mass spectrometer. These candidate envelopes were matched against spectra from one mass spectrometry run and all peptides were quantified without prior knowledge of peptide identities. The algorithm analyzed one mzML file within half a day. For evaluation of this database based approach, peptides were independently identified with the Open Mass Spectrometry Search Algorithm (OMSSA, Geer et al. 2004). Comparison of M-pyQuant and OMSSA results revealed that M-pyQuant is also potentially suited for MS2-free identification. Prediction of sequence specific retention times e.g. as in Krokhin & Spicer (2009) provides additional information that can be used for peptide identification based on MS1 scans. The suitability of M-pyQuant for MS2-free identification has to be further evaluated.

**Novel Aspects:** M-pyQuant is a versatile quantification tool for high throughput mass spectrometry data.

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# Investigation of cartilage and tendon constituents by the use of MALDI-TOF MS

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Fibrillar proteins such as collagens in combination with glycosaminoglycans (GAGs) such as chondroitin sulphate (CS) and hyaluronan (HA) are the main constituents of the extracellular matrix (ECM) of cartilage and tendon tissues [1]. Collagens and GAGs possess important and highly specialized functions in the ECM's physiological organization. The aim of our studies is the simple but reliable detection of characteristic collagen peptides and HA or CS oligosaccharides (obtained by enzymatic digestion or chemical hydrolysis) by MALDI-TOF MS (matrix-assisted laser desorption ionization time-of-flight mass spectrometry). Thus, MALDI-TOF-MS will be used to provide qualitative compositional data of the investigated tissue materials [2] and additionally for the quantitative determination of e.g. the unsaturated disaccharide of CS and characteristic oligopeptides (Gly-Pro-Hyp) derived from collagen [3].

**Method:** In addition to commercially available oligopeptides and CS or HA-derived oligosaccharides, native collagen type I, gelatine, porcine articular cartilage and equine tendon were investigated. All samples were subjected to enzymatic digestion (MALDI MS) as well as HCl (NMR analysis). MALDI spectra were recorded on a Bruker Autoflex device (Bruker Daltonics, Bremen) and NMR spectra on a AMX 300 (Bruker BioSpin, Rheinstetten). To confirm peak assignments, selected mass spectra were also directly recorded from a developed thin-layer chromatography (TLC) plate as essentially described in [4]. Negative ion spectra were recorded in the presence of ionic liquid matrix GCHCA (mixture of 2 tetramethylguandine and alpha-cyano-4-hydroxycinnamic acid), whereas DHB (2,5-dihydroxy benzoic acid) served as positive ion matrix.

**Preliminary Data:** Specific proteases such as trypsin are typically used in "proteomics" to obtain characteristic fragments of a certain protein [5]. However, the insolubility of native, intact collagen represents a significant problem because the majority of common proteases digest only soluble proteins, but do not cleave native collagen. Therefore, the use of bacterial collagenases is suggested as a straightforward approach because this enzyme readily digests native collagen. Although collagenase A digests collagen rather non-specifically, the generated peptide mixture represents a measure of native collagen. Here, we have focused on Gly-Pro-Hyp that is highly characteristic for collagen. One drawback of MALDI-TOF-MS regarding the detection of smaller molecules is the interference of the analyte with matrix ions. This particularly holds as the matrix is present in vast excess over the analyte. From our experience, DHB is the matrix of choice for positive ion detection as DHB provides less intense signals compared to cinnamic acid-derived matrices. In order to evaluate the quantitative relationship between signal intensities and peptide amount, calibration curves were recorded for different tripeptides in the presence of a known amount of Arg-Gly-Asp as internal standard. Due to the high contribution of Gly-Pro-Hyp in the enzymatic collagen digest, the focus was on this tripeptide. It will be shown that quantitative data on the collagen content of cartilage and tendon samples can be obtained by this approach. Additionally, negative ion spectra where recorded by using 9-aminoacridine (9-AA) as matrix. Although peak assignments are much simpler under these conditions, the achievable sensitivity is much lower in comparison to the positive ion spectra. However, negative ion detection is the Method: of choice for the determination of CS degradation products. We will show that the signal-to-noise ratios are reliable concentration measures and the addition of (not yet available) internal standards is not absolutely required.

**Novel Aspects:** This was our first study aiming at the comparative determination of CS and collagen in cartilage and tendon samples.

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# Aplication of fully automated chip-based nanoelectrospray ion trap mass spectrometry for mapping and sequencing of fetal cerebellum gangliosides

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Two native ganglioside mixtures extracted and purified from normal human fetal cerebellum in the 15th and 40th gestational week respectively were subjected to NanoMate HCT MS and CID $MS^2$ analysis under identical experimental conditions. Obtained results indicated that by this method major as well as minor cerebellum-associated ganglioside species could be detected, sequenced and in detail structurally characterized in a high throughput regime, with almost 100% experiment reproducibility at a sensitivity situated in the low picomole range and sample consumption per experiment reduced to femtomole values.

**Method:** The native ganglioside mixtures analyzed in this study were purified from fetal cerebellum, in the 15th gestational week and 40th gestational week and were obtained during routine pedopathological section/autopsy examination. Mass spectrometry was conducted on a High Capacity Ion Trap Ultra (HCT Ultra, PTM discovery) mass spectrometer from Bruker Daltonics, Bremen, Germany. All mass spectra were acquired in the massrange (100-2500) m/z, with a scan speed of 8000 m/z persecond. $MS^2$ was carried out by collision-induced dissociation (CID) in the non-automatic mode of ion selection and fragmentation, using Helium as the collision gas. Fully automated chip-based nanoelectrospray was performed on NanoMate robot incorporating ESI 400 Chip technology (Advion BioSciences, Ithaca, USA) controlled and manipulated by ChipSoft 7.1.1 software.

**Preliminary Data:** Using this experimental setup, MS screening enabled the identification in Cc15 and Cc40 mixtures of 56 and respectively 54 glycoforms with a high degree of heterogeneity in their oligosaccharide sequences and ceramide motifs. By employing CID $MS^2$ molecular ions related GD1 (d18:1/20:0) and GM2 (d18:1/19:0) species were structurally characterized in a high throughput mode. The method provided elevated ionization efficiency, high speed of analysis, high sensitivity and rapidity (10-15 times more sensitive and a few times more rapid compared to classical capillary-based ESI MS protocols), and almost 100% reproductibility (day-to-day, run-to-run) at a sample consumption per experiment situated in the femtomole range. Obtained results also indicated differences in the expression of polysialylated species in the two ganglioside mixtures, which support earlier hypothesis regarding the direct correlation between sialylation degree and brain developmental stage.

**Novel Aspects:** By employing CID MS2 molecular ions related GD1 (d18:1/20:0) and GM2 (d18:1/19:0) species were structurally characterized in high throughput mode.

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# Electron ionization mass spectrometry studies of selected analogous of linezolid

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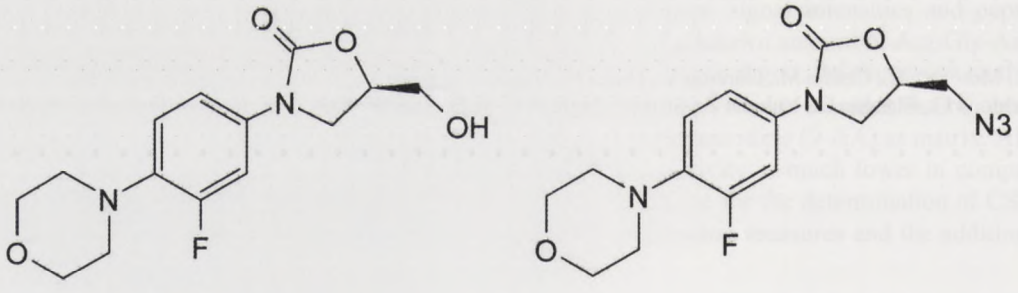
Linezolid is the synthetic chemotherapeutic antibiotic used for the treatment of infections caused by streptococcus and staphylococcus aureus bacteria. This antibiotic possesses the structure of fluorobenzene substituted by two characteristic heterocyclic rings: morpholine and oxazolidinone. Two analogues of this compound with different substituents in oxazolidinone ring (azide or hydroxyl group) were applied as objects of the research.

**Method:** During the characterization of the compounds low resolution electron ionization (EI) spectra were recorded on Finnigan MAT95 double focusing (BE geometry) mass spectrometer. Precursor and product ion spectra in first field-free region (between ion source and magnetic sector) were recorded using appropriate linked scan function: $B^2/E$ = constant and B/E = constant, using the same instrument. Accurate mass measurement of interesting ions were performed at a resolution of 10000 (10% valley definition) by peak matching technique using PFK as a reference.

**Preliminary Data:** Using electron ionization as an ionization technique significant differences in the fragmentation of studied analogues of linezolid were observed. The main process which characterize both compounds is the fragmentation connected with the morpholine ring. Although intensities of peaks corresponding to the appropriate ions revealed predominance of this process for the compound with the hydroxyl group, which is the only process of the fragmentation. Antibiotic with the azide group in the oxazolidinone ring revealed less stability in applied ionization technique. In case of this analogue the observed fragmentation processes are connected with oxazolidinone ring. In spite of the instability of azides and the hardness of the ionization technique the molecular peak was observed in low-resolution EI mass spectra. Also the peak connected with the loss of neutral molecule $N_2$ was presented. However, the peak corresponding to elimination of $N_2$ was not detected in product ion mass spectra of the molecular ion. This observation confirms an assumption that thermal degradation was taking place before ionization. Differences in the abundances and formation the EI fragment ions contributed to make an attempt developing detailed fragmentation pathways. For this purpose the metastable ions spectra were recorded using linked scan technique and accurate mass measurements of interesting ions were carried out.




**Novel Aspects:** Role of substitiuents in fragmentation of linezolid analogous.

# Monitoring drought related protein profiles changes in barley by 2D gel electrophoresis and MALDI-TOF mass spectrometry

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Barley is one of four most important cereals in worldwide production, used for food, feed and malt production. It is considered as a model system due to short life cycle and good morphological, physiological and genetic characterization. Drought is one of the greatest factor limiting plant growth and the productivity of crops. In response to water deficit plants developed various biochemical and physiological mechanisms. Factors, such as drought, cause significant changes in gene expression profile. Products of stress induced genes are classified as directly involved in tissue protection against dehydration and as proteins related with control of gene expression and signal transduction. Identification of this proteins is important for plant breeding programs and can improve a yield under drought conditions.

**Method:** Proteins from leaves and roots for qualitative and quantitative analysis were isolated by phenol extraction. The extracts were dissolved in IEF buffer and submitted for separation by 2D gel electrophoresis. Separated proteins were then stained using Coomassie Brilliant Blue in colloidal version. Obtained gels were analyzed in Image Master 2D Platinum software. Protein spots, which showed changes in expression profile, were excised from gel, digested with trypsin and analyzed by MALDI-TOF or MALDI-TOF/TOF mass spectrometer. The registered mass spectra (Peptide Mass Fingerprint) were compared with these from databases (MSDB, SwissProt, NCBI), using the MASCOT program.

**Preliminary Data:** In our previous studies we created a proteomic maps for root and leaves of barley, consisting of 160 proteins in leaves and 120 proteins identified in roots in samples not subjected to drought (new proteins are added consecutively in ongoing research). In this study changes in protein expression patterns in response to water deficit were monitored in two different cultivars of barley (Maresi and Cam/B1/C1). Plants were grown in greenhouse for three weeks under controlled environmental conditions. After this time, plants were subjected to drought stress. The water level in pots was dropped down to 6% while the control pots were maintained at 12% water level. Leaves and roots samples were harvested after 10 days of drought. The list and the expression profile of drought related proteins are presented on the poster with diagram grouping identified proteins according to their function. We were also able to find differences between two analyzed cultivars in protein expression patterns in the control samples as well as in the stressed ones.

**Novel Aspects:** The future studies will be conducted on a mapping population bred from cultivars analyzed in this study.

# Fast and reliable identification of potential biological warfare agents by the maldi biotyper

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Fast and reliable identification of microorganisms is a requirement not only for clinical routine diagnostics or food control but also for identification of potential biological warfare agents. Cultivation independent detection methods, e.g. real-time PCR and fluorescence *in situ* hybridisation are fast. However, they are limited by their primer or probe sequences, respectively. MALDI-TOF MS fingerprinting combined with a dedicated pattern matching algorithm using a reference spectra library may be an excellent complement of these technologies.

**Method:** For sample preparation biological material was processed according to short inactivation/extraction protocols using trifluoroacetic acid. Mass spectra were acquired in a microflex MALDI-TOF in mass range from 2000 to 20000 Dalton. Reference data sets were created containing species- and subspecies-related information. In addition, a regular database (> 3995 entries) was used for identification.

**Preliminary Data:** The identification of potential biological warfare agents by MALDI-TOF MS could be successfully demonstrated. Potential agents of bioterrorism are often difficult to distinguish from closely related but less virulent species or subspecies. A database consisting of 50 different strains including *Francisella philomiragia, Francisella tularensis ssp. tularensis, Francisella tularensis ssp. holarctica, Francisella tularensis ssp. mediasiatica*, and *Francisella tularensis ssp. novicida* was used for identification of blind-coded samples from the National Institute for Nuclear, Biological and Chemical Protection in Czech Republic. All samples were correctly identified by the MALDI Biotyper to subspecies level. Furthermore, discrimination of *B. anthracis* spores from spores of closely related *B cereus, B. thuringiensis* and *B. mycoides* could be shown. Small, acid-soluble proteins described as biomarkers for Bacillus spores were detected and allowed the differentiation of spores from the *B. cereus* group. Some "white powders" which are potential fakes in a terrorist attack showed no similarities to the spore spectra or other database entries.

**Novel Aspects:** MALDI-TOF MS fingerprinting combined with a dedicated pattern matching algorithm for fast and reliable Identification of potential biological warfare agents.

# Hydrogen Exchange and Hydrogen Transfer Reactions Preceding the Fragmentation of Long-lived Radical Cations of Ethyl Dihydrocinnamate and Related Arylalkanoates

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Nowadays, a vast amount of knowledge has rendered gas-phase ion chemistry of ions a well-rationalized subfield of organic and physical organic chemistry. Site-specific hydrogen rearrangements and hydrogen exchange ("scrambling") processes of different types represent almost ubiquitous features of the fragmentation scenario – be it of small organic [1, 2] or large bioorganic ions [3]. The present contribution is aimed at highlighting new facets of apparently well-established fragmentation reactions, namely, those of the molecular radical cations of ethyl dihydrocinnamate [4, 5] and a number of related arylaliphatic esters.

**Method:** *EI mass and EI-MIKE spectrometry.* All measurements were carried out with a double-focusing instrument, AutoSpec (Fisons, Manchester/UK) with a three-sector, EBE geometry. Fragmentation of the metastable ions in the third field-free region was registered by selecting the precursor ion by the magnetic field and scanning the field of the second electrostatic analyzer. – *Synthesis* (*general*). The deuterium-labelled ethyl dihydrocinnamates were synthesised according to standard procedures starting from appropriately labelled buildung blocks, as described in the literature. All compounds were checked for purity by $^{1}H$ and $^{13}C$ NMR spectroscopy and mass spectrometry. Deuterium contents were determined from both the EI-MS and the $^{1}H$ NMR data.

**Preliminary Data:** An electron ionization study on the fragmentation of metastable molecular radical cations of ethyl 3-phenylpropanoate (ethyl dihydrocinnamate) and related arylalkanoic acid esters was performed by mass-analyzed ion kinetic energy (MIKE) spectrometry. Six deuterium-labelled isotopomers of ethyl dihydrocinnamate were synthesised and studied by MIKE spectrometry. The fragmentation leading to ions $C_7H_7O^+$ (m/z 107) involving migration of the alkoxycarbonyl group [4, 5] was also observed in the 70-eV mass spectra of related alkyl dihydrocinnamates, but it was found to be a high-energy process that does not compete at low energies in metastable molecular ions. Instead, the metastable ions of ethyl dihydrocinnamate undergo competing losses of carbon monoxide, ethanol and the combined loss of these neutral fragments, giving ionized styrene, $C_8H_8^{\cdot+}$ (m/z 104). A highly specific H/D interchange involving the four hydrogen atoms at the benzylic α- and *ortho*-positions was found to precede the losses of ethanol and [ethanol + CO]. This represents another striking case of complete 4H-scrambling [1] that enables the molecular ion to fully equilibrate the interchanging hydrogen atoms prior to fragmentation. A mechanism rationalizing these observations and extending previously suggested mechanisms is proposed involving a series of distonic ions and the intermediacy of an ion/neutral complex. The metastable ions of the related esters exhibit in part similar fragmentation behaviour, but the McLafferty reaction turns out to be more favourable with higher alkyl dihydrocinnamates. For example, *n*-propyl 3-phenylpropanoate and isopropyl 3-phenylpropanoate react through highly distinct fragmentation channels.

**Novel Aspects:** Site-specific complete H-scrambling, distonic ions and I/N-complexes are charactistic features of metastable radical cations of ethyl 3-propylpropanoate and related ω-arylalkanoates

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# ISOQuant - an integrated bioinformatics pipeline for evaluation and reporting of data independent (LC-MSE) label-free quantitative proteomics data

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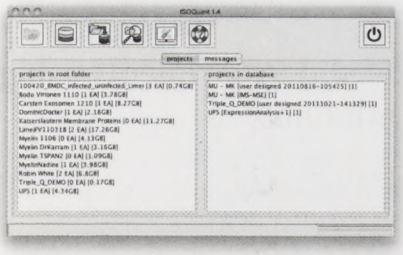
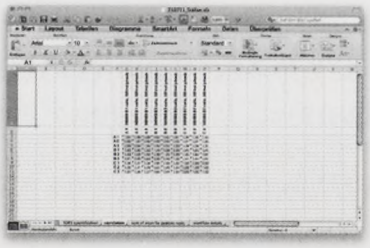
One of the main bottlenecks in the evaluation of label-free quantitative proteomics experiments is the often cumbersome data export for in-depth data evaluation and analysis. Recent developments facilitate the reproducible detection and quantification of up to two thousand proteins within a single 1D LC-HDMSE experiment. However, data-independent, alternate scanning LC-MS peptide fragmentation data can currently only be processed by vendor software, which is limited to analysis on a run-by-run basis. We present the bioinformatics pipeline ISOQuant – an integrated solution for automated in-depth evaluation of label-free LC-MS data, allowing easy data access and export to third party software. ISOQuant facilitates absolute quantification of protein isoforms and label-free relative quantification of peptides and proteins based on annotated accurate mass-retention time clusters.

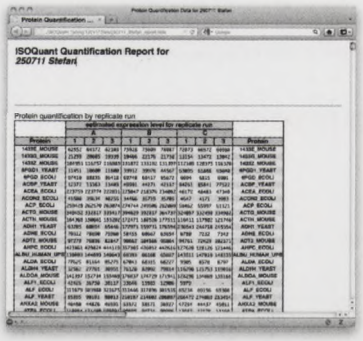
**Method:** ISOQuant consists of in-house developed and adapted third party methods. ProteinLynxGlobalServer™ [1] (PLGS) is used for raw data processing and for peptide and protein identification. Using ISOQuant, PLGS projects are automatically browsed, followed by importing relevant information into a MySQL database and subsequent processing data in multiple stages. Relations between multiple LC-MS runs are built and advanced statistics calculated. Non-linear retention time distortions between LC-MS runs are corrected [2]. Corresponding signals are clustered and subjected to multidimensional intensity normalization. Clusters are annotated by consensus peptides from associated LC-MS runs. Homologue proteins are filtered. Shared peptide intensities are redistributed. Absolute in-sample amounts are calculated [3]. Finally, results of the performed analysis are exported as a set of uniform reports.

**Preliminary Data:** We produced a model data set to evaluate the performance of label-free protein quantification using ISOQuant. Tryptic digests of mouse, yeast and E.coli proteomes were combined in three different ratios and analyzed in five technical replicates. The tryptic peptides (400 ng/injection) were separated by 1D nanoscale UPLC and mass measured with a quadrupole-oa time-of-flight (QTOF) mass spectrometer using data-independent, alternate scanning acquisitions with and without ion mobility separation. Data were processed and analyzed with dedicated algorithms, searching a database containing mouse, yeast and E.coli proteins, identifying over 1500 proteins at a protein false positive rate of <0.5%. The results provided were imported and processed by ISOQuant. Non-linear retention time distortions between LC-MS runs were corrected using a Dynamic-Time-Warping [4] based retention time alignment method, followed by clustering corresponding signals using a Hierarchical-Non-Hierarchical clustering procedure. Signal intensities were subjected to multidimensional normalization correcting systematic errors introduced by variance of technical conditions in different LC-MS runs. To include peptides identified not in all replicates in the quantitation process, peptide identifications were used to annotate previously built signal clusters. Integrating peptide identification information across technical and biological replicates improved average sequence coverage and the reproducibility of absolute protein quantification compared to a standard run-by-run approach using manually exported data. By using filters for replication rate of identification at both peptide and protein level, ISOQuant significantly reduces false positive rates as determined by searching a 5x-randomized database. For label-free quantification, we apply a modified non-linear normalization method in the intensity and retention time dimensions to reduce systematic intensity variations between runs. Applying this normalization approach reduces the variation of measured peptide intensities between technical replicates and improves reliability of label-free quantification. ISOQuant provides easy access to routine application of label-free quantification by significantly reducing evaluation time and by offering standardized data evaluation procedures.

**Novel Aspects:** Novel methods for automated reporting, retention time alignment, signal clustering, multi-dimensional intensity normalization, protein homology filtering, absolute protein isoform quantification.





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# Improved speed and efficiency in small molecule LC-MS(n) experiments using a new automated Panorama Fragmentation

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Isomeric compounds in the mass range 1000 Da are common in complex pharmaceutical, or natural product samples. For the specific structure identification of isomers, MS(n) is necessary making ion trap systems the instrument of choice. However, in ion traps two drawbacks are known: Firstly, the fragmentation efficiency is not constant for the broad precursor ion mass range of interest (100-1000 Da) and especially very small precursor ions show week fragmentation yields. Secondly, the duty cycle for MS(n) experiments on multiple precursor ions could lead to limitations in fast chromatography. Here, we describe major improvements based on the new amaZon speed ion trap which overcome these former limitations. Data isshown on structure elucidation of chlorogenic acids.

**Method:** Extracts from dissolved robusta coffee powder were separated using an UHPLC system (Dionex Ultimate 3000) in a 10 min gradient with Agilent eclipse Plus column (C18, 1.8 µm; 2.1 × 100 mm). Solvents were water/ acetonitrile including 0.1% formic acid, the amaZon speed ion trap system (Bruker) was set up for negative polarity and data dependent AutoMS(3) mode using two precursor ions each per MS/MS stage. The increased speed in LCMS(n) experiments is based on rapid and improved isolation and fragmentation and on multiple new software features. Additionally, a new fast and improved ICC regulation forUHPLC MS(n) has been implemented. Improved efficiency in MS/MS of small molecules is gained the panorama fragmentation mode (PAN).

**Preliminary Data:** With the new panorama fragmentation of the amaZon speed the MS/MS efficiency for small precursor ions (<200 Da) is significantly increased versus previous instruments. A comparison of MS(n) spectra generated with panorama fragmentation versus spectra acquired in standard fragmentation shows unambiguously larger signal to noise values on characteristic fragment ions for the new PAN mode presented here. As a result reproducible fragment ion spectra are generated which can be used for further structure determination and spectra library approaches. The gain in speed in LCMSMS experiments using the new ion trap system is shown by the separation and MS(3) analysis of six different isomers from Caffeoylferuloyl Quinic Acid (CFQA) eluting in a short retention time window. The resulting MS(2) and MS(3) spectra of the isomers are differentiated based on a MS(n) decision tree formerly described in the literature.

**Novel Aspects:** Improved speed and efficiency for MS/MS of small precursor ions in traps.

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# Rapid Analysis of Solid and Liquid Samples by Direct Introduction into a GC-Triple Quadrupole Mass Spectrometer

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A direct introduction of samples can save a lot of time during various analyses. Common direct inlet probes into the ionization chamber are without software control and bear the risk of a source contamination. The ChromatoProbe introduced into a PTV (programmable temperature vaporization) injector can be coupled directly to the ion source with 2 m of uncoated fused silica capillary or via a usual analytical column, with respect to complexity of the sample.

**Method:** Samples range from liquids over slurries to solids. For dirty samples the benefit is the usage of single use microvials, which keep non-volatiles, high boilers and thermally degraded components inside. This decreases runtime per sample and maintenance. Column bake out is usually also not necessary to allow running more samples a day and preserves the column performance.

**Preliminary Data:** Street drugs can be easily investigated as a solid sample or even directly from the introduction of a single hair. Plastics, dyes and drugs may contain residual solvents which can be analyzed with SPME. An alternative is a direct insertion in pieces in a microvial. This sample introduction is less discriminative compared to others. Plant tissues that normally are not considered amenable to GC-MS/MS analysis can be easily investigated with the ChromatoProbe. The injector still keeps all its benefits as split flow of the sample and a stepwise increase of the temperature to mimic a distillation process. All this lowers the risk of contaminating the ion source in comparison to a direct inlet into the ion source. In synthesis control and in combinatory chemistry approaches software features like automatic MS/MS breakdown and the signal stability are a benefit. All changed settings are automatically recorded and can be reviewed during data analysis and allow a convenient workflow.

**Novel Aspects:** Analysis of Solid and Liquid Samples by Direct Introduction by the ChromatoProbe.

# Increasing Protein Identifications on a Q-TOF Mass Spectrometer

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Efficient protein identification by LC-MS/MS techniques depends on both the chromatographic separation of the peptides and optimized MS/MS analysis. Improved separation decreases the number of peptides presented to the mass spectrometer at any given instant and also increases the instantaneous signal for the same amount injected, yielding better detection of lower abundance species. Improved data dependent acquisition more efficiently selects and fragments precursors. This work describes the combined benefit of improving both the chromatographic separation and the data-dependent MS/MS.

**Method:** Experiments were performed on a nanoflow LC QTOF mass spectrometer with custom instrument control software and prototype multilayer polyimide microfluidic chip. The chip format used an enrichment column to trap and desalt the sample prior to transfer to the analytical column. Proteins from E. coli lysate were used for analysis by one-dimensional nanoflow LC-MS/MS. A 1% global peptide FDR was used for database searching of results.

**Preliminary Data:** A new peptidic isotope cluster detection routine was implemented in the data-dependent acquisition algorithm which improves identification of the monoisotopic m/z and centering of the quad isolation window for optimal transmission. Precursor candidates are ranked based on their abundance and precursor "purity," which is a signal-to-noise ratio of precursor ion signal vs. total signal within the isolation window. Precursors that would co-isolate with other peptides, yielding chimeric peptide spectra, were thus filtered based on low purity score. MS/MS accumulation time was modulated to achieve a user-specified target for MS/MS total ion current. Results showed a 44% increase in peptides identified and 42% increase in proteins identified (at a <1% FDR) compared to the standard algorithms. The improved chromaotgraphy resulted in a 28% increase in peptides identified and 18% increase in proteins identified compared to the standard chip. Other designs, fabrication parameters, and operational parameters are being examined to optimize the performance of these new chips.

**Novel Aspects:** Mproved precursor selection combined with improved chromatographic separation results in greatly enhanced protein identification results.

# Accurate quantification of regulated mycotoxins by UHPLC-MS/MS using 13C isotope labelled internal standards

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Between 300 and 400 substances are recognized as mycotoxins – toxic secondary metabolites of fungi, which are often found as contaminants in food. For multi-mycotoxin analysis of food, unified LC-MS based methods can save significant costs and time. However, matrix effects caused by suppression or enhancement of the analyte signal must be addressed.

**Method:** We present a novel method for the quantification of 11 regulated (or soon to be) mycotoxins.

**Preliminary Data:** Acidified shake extraction with subsequent addition of 11 fully 13C-labelled internal standards was followed by UHPLC-MS/MS analysis using an Agilent 6490 QQQ mass spectrometer. Spiking at multiple levels on blank samples of maize allowed recoveries of extraction to be validated as fit for purpose and the sensitivity, accuracy and precision of the method was seen to be commensurate with MRL's associated with all food analysis including that of baby food as outlined by European Commission Regulation No. 1881/2006.

**Novel Aspects:** We present a novel method for the quantification of 11 regulated (or soon to be) mycotoxins.

# Analysis of fluorinated alkyl compounds in biosolid matrix by GC Q-TOF mass spectrometry

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Fluorotelomer alcohols are synthetic compounds used as building blocks for fluorinated polymers and could arise in the environment as intermediate degradation products from those same polymers. Furthermore, fluorotelomer alcohols oxidize to fluorinated carboxylic acids, some of which have been found to be toxic under typical environmental conditions.

**Method:** Sensitive and specific analytical methodology is required to study the fate and transport of fluorotelomers in the environment and gain understanding of human exposure and toxicity.

**Preliminary Data:** Herein, we present a GC Q-TOF method for the analysis of fluorinated alkyl compounds in complex biosolid matrix. Analyte ions were easily extracted from the heavy matrix and comparison to MRM data collected previously suggests detection limits on the order of 5-500 fg on column.

**Novel Aspects:** GC Q-TOF method for the analysis of fluorinated alkyl compounds in complex biosolid matrix.

# Evaluation of SFC-MS Configurations for the Analysis of Lipids, Sterols, and PAHs

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Supercritical fluid chromatography (SFC) is a powerful separation technique, and especially for the separation of apolar compounds, it offers an interesting alternative to normal phase HPLC. Most SFC work has been performed using UV detection, although the coupling to mass spectroscopic detection can largely extend SFC applicability.

**Method:** Initial experiments showed that the hyphenation of SFC with MS results in high sensitivity and good mass spectral quality due to the fact that the major component of the mobile phase is $CO_2$, which evaporates upon entering the source, allowing for analysis at high flow rates. However, the carbon dioxide decompression and expansion after the backpressure regulator (BPR), used in all commercially available instruments, also causes cooling of the transfer line to the MS, which can result in solute deposition, baseline noise and irreproducible results.

**Preliminary Data:** For hyphenation of SFC with MS, different configurations can be used, including effluent splitting before or after the BPR. Effluent splitting obviously reduces sensitivity and alternatives were studied. Best results in term of sensitivity, baseline stability, retention time and peak area repeatability were obtained using a configuration whereby a small (typically 0.2 mL/min) make-up flow is added before the BPR. The total effluent is introduced in the MS (both ESI and APCI were tested). It was also found that heating of a part of the transfer line, just before the ionization source entrance, was needed to obtain optimal peak shape and sensitivity. This SFC-MS configuration was used to analyze several different types of apolar solutes, including lipids, sterols, and polycyclic aromatic hydrocarbons (PAHs). These compounds are notoriously difficult to detect using LC-MS, and comparisons were made between the two techniques. In all cases, similar separation and MS conditions were used.

**Novel Aspects**: SFC-MS configuration was used to analyze several different types of apolar solutes

# Reaction Cell Frontier: Removing oxide polyatomic ion interferences using an innovative reaction cell ICP-MS

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The use of a collision/reaction cell, which is located between the ion extraction lenses and the quadrupole mass filter and pressurized using various inert or reactive gases is now widely accepted in quadrupole ICP-MS as a means to reduce spectroscopic interferences. However, depending on the sample to be analyzed (analytes of interest, matrix species and their concentration), accurate quantification of anaytes by collision/reaction cell ICP-MS is still sometimes problematic due to persistent interferences which cannot be removed by the current configuration.

**Method:** In this study a triple quadrupole ICP MS system. (Agilent 8800 Triple Quadrupole ICP-MS), has been developed based on the Agilent 7700 Series ICP-MS.

**Preliminary Data:** The proposed system has a configuration of quadrupole – collision/reaction cell – quadrupole that follows the off-axis extraction lenses (Omega Lens). Employed in the cell (ORS: Octopole Reaction System) is an octopole ion guide. The first quadrupole, having unit mass resolving power, can select ion species that enter the cell. In contrast to the conventional cell based quadrupole ICP-MS, where all of the ionic species generated in the plasma ion source region enter the cell, the new triple quad configuration can dramatically reduce the spectroscopic interferences as well as background noise. In addition to the improvement of the ion optics arrangement, an improved vacuum system tailored to this configuration has been developed resulting in sensitivity comparable to single quad ICP-MS even though the additional first quadrupole operates as a unit mass resolving filter which does not have perfect ion transmission efficiency.

**Novel Aspects:** New ICP-MS System.

# Spectroscopic studies on chelating ability of 2'-deoxyriboadenosine

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Numerous modifications of DNA have been made in various ways aiming to expand its properties and applications. One of them is the implementation of non-natural base pairs into DNA. Unnatural base pair surrogates can rely on metal ion coordination [1, 2]. The controlled incorporation of metal ions or metal complexes into nucleic acids is a possibility to broaden the scope of their applications. In such a base pair, the natural nucleobases are replaced by ligands with a high affinity towards metal ions.

**Method:** To evaluate the relevant interactions of platinum with histidine modified 2'-deoxyriboadenosine, combined spectroscopic NMR, UV-Vis, ESI-MS andpotentiometricapproaches were used.The chelation sites, stoichiometry of complexes were defined.

**Preliminary Data:** Our interest in understanding the effect produced by the incorporation of imidazole ring in deoxyadenosine molecule on metal coordination was inspired by prior investigation on its role in DNAzymes [3]. The incorporation of imidazole-modified nucleotides can endow DNA with the chemical advantage that histidine provides proteins. In a previous work [26] we reported a binding ability of histamine modified deoxyadenosine towards Cu(II) ions.Now, we present the studies of platinum(II) complexes with a new chelator-type nucleoside,histidine modified 2'-deoxyriboadenosine. Our investigation provide information that the modified deoxyadenosine has capacity to chelate platinum ion. The results pointed to the formation of complexes with stoichiometry 1:1 and 1:2. Obtained complex stoichiometry was confirmed by ESI-MS.

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# Matrix-free laser desorption/ionization mediated by bionanostructures from microalgal cell walls

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Matrix assisted laser desorption/ionization (MALDI) is in many cases the method of choice in bioorganic analytics. Nevertheless, the measurement of small molecules with masses up to 600 m/z is complicated through interfering matrix signals. Consequently, matrix-free LDI approaches moved more and more in the focus. Therefore, nanostructured surfaces can facilitate the LDI of embedded molecules by means of absorbing high amounts of laser energy, resulting in rapid surface heating and ablation of analyte compounds. Here we show that microalgae (e.g. *Thalassiosira pseudonana*, *Prorocentrum minimum*) with their nanopatterned cell walls provide easily accessible alternatives for matrices in LDI-MS. Nanostructure enhanced ionization allows the analysis of a diverse selection of analytes including polymers, sugars, aminoalcohols, and fatty acids without interfering matrix signals.

**Method:** Microalgae shells from *T. pseudonana* and *P. minimum* were purified through oxidation of organic material with sodium hypochlorite (12% Cl), followed by several washing steps with a mixture of acetonitrile and double-distilled water (50:50; v/v) and afterwards stored in double-distilled water. The algae shell suspension was pipetted on a commercial available MALDI target and allowed to dry completely under ambient conditions. Analyte solutions were pipetted on the dry algae shells afterwards.

**Preliminary Data:** We could indicate in our first experiments, that clean algae shells show no signals in the spectra that would interfere with the analytics of small molecules as observed in control spectra generated from pure 2,5-DHB. After optimization of laser energy values and cell wall concentrations the method could be used to investigate a broad range of analytes. It is really robust and high quality mass spectra could be obtained with laser energies from 300 to 350 (max 500, ca 140 µJ) and shell concentrations from 400 µg/ml to 80 mg/ml. Sensitivity and resolution were competitive with that obtained in measurements using the common matrix 2,5-DHB. Besides a series of experiments with polyethyleneglycol 600, we also tested raffinose (trisaccharide, 1 mg/ml) and D-sphingosine (aminoalcohol, 1 mg/ml)) in combination with our bionanostructures. The obtained mass spectra included only substance-specific mass signals, whereas typical interfering masses occurred in spectra generated with 2,5-DHB. We used stearic acid (1mg/ml) in the negative ionization mode and obtained comparable results with as with 1,8-bis-(dimethylamine)naphthalene (DMAN) as matrix. DMAN belongs to the so-called proton sponges and is known to produce mainly molecular ions.

**Novel Aspects:** We thus successfully showed that easily accessible bionanostructures could ionize embedded molecules without interfering signals.

# Comparison of ERLIC-$TiO_2$, HILIC-$TiO_2$ and SCX-$TiO_2$ for global phosphoproteomics approaches

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Reversible phosphoryltions play a critical role in most biological pathways and in signaling studies many advances have been made to identify a maximum number of phosphosites per experiment. Mass-spectrometry (MS)-based phosphoproteomics approaches have been proven to be an ideal analytical method for mapping of phosphosites. However, due to sample complexity fractionation of phosphopeptides prior to MS analysis is a crucial step. In the current study, we compare the chromatographic strategies electrostatic repulsion-hydrophilic interaction chromatography (ERLIC), hydrophilic interaction liquid chromatography (HILIC), and strong cation exchange chromatography (SCX), for their fractionation behavior of phosphopeptides.

**Method:** *HILIC Workflow.* HILIC chromatographic separations were performed on an Akta System (GE Healthcare, Munich,Germany) using a 4.6 mm ID × 25 cm, 5 µm, TSKgel Amide-80 column (Tosoh Biosciences, Stuttgart, Germany). *SCX Workflow.* SCX chromatographic separations were performed on an Akta System using a 1 ml column (Resourse S, GE Healthcare). *ERLIC Workflow.* ERLIC separation was performed on an Akta System using a in house packed Poly-WAX LP column (5 µm particle size, 300 Å pore size; PolyLC, Columbia, MD; Tricorn 5/100; GE Healthcare).

**Preliminary Data:** On the basis of our results, the SCX-$TiO_2$ approach identifies a higher total number of unique phosphopeptides while the ERLIC-$TiO_2$ approach identifies a higher number of multi-phosphorylated peptides. For an in depth analysis, ERLIC-$TiO_2$ can be applied as a complementary method to SCX, as it proved to be especially suited for the fractionation of multi-phosphorylated peptides. Since the SCXFT is enriched in multi-phosphorylated peptides, a reasonable workflow consists of SCX-$TiO_2$ fractionation followed by ERLIC-TiO2 fractionation of the SCX-FT. Our findings also highlight that two rounds of incubation of collected fractions and at least three rounds of FT incubation by $TiO_2$ beads are needed for an in depth analysis of phosphosphorylation dynamics by both SCX and ERLIC.

**Novel Aspects:** We compared the chromatographic strategies ERLIC, HILIC, and SCX, for their fractionation behavior of phosphopeptides.

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# Mass spectrometric de-novo sequencing of the anti cystatin C antibody Cyst-13

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Nowadays scientists are focussed on the use of antibodies in a search of inhibitors for many kinds of amyloidogenic diseases. The great potential of this concept is hidden in the use of monoclonal antibodies (mAb), which recognize specifically one part (epitope) of an antigen. In this regard our work is focused on human cystatin C (hCC), a protein with amyloidogenic properties, and an anti-hCC monoclonal antibody named Cyst-13. Our study proved the suppression of the dimerization of hCC by even low amounts of mAb Cyst-13 [1]. Therefore, the identification of the binding site of Cyst-13 (paratope) may be of high importance for dimerization/oligomerization and fibrillization studies of hCC.

**Method:** The antibody gel bands (heavy and light chains) were cut out from the gel and digested with different endoproteases (e.g. trypsin, Lys-C, Asp-N, Glu-C [2]) [3]. The mixture of proteolytic peptides was analysed by high resolution LTQ-Orbitrap mass spectrometry. For the sequence identification of single peptides, database searches were performed in a first step. For unidentified peptides, a "de-novo" sequencing software tool (PEAKS studio 5.3) was used to determine a suitable peptide sequence directly from its MS/MS data.

**Preliminary Data:** In this work we present the sequence identification of the complete light-chain of the antibody Cyst-13 by a combination of a "Bottom-Up" approach and de-novo data interpretation of mass spectra.

**Novel Aspects:** For the first time, the mass spectrometric de novo sequencing of an anti-hCC antibody is demonstrated.

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# Detection of enzymatically generated dehydrogenated betanidins by LC-MS/MS

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Betanidin (**1**), the basic betacyanin, is the only betalain with the 5,6-dihydroxyl moiety which results in its higher antioxidant activity. In this study, enzymatic generation of dehydrogenated and decarboxylated betanidins with their chromatographic separation and spectrophotometric and mass spectrometric detection (LC-DAD-ESI-MS/MS) was performed. Mechanism of betalains oxidation is of significant interest because these pigments are recently emerging as highly active natural compounds with antioxidative properties and potential benefits to human health [1].

**Method:** The oxidation of betanidin was performed with horseradish peroxidase at the presence of H2O2 and monitored by a spectrophotometric detection at the wavelength range of 350-600 nm. For the LC-ESI-MS/MS analysis of the reaction mixtures, the positive ion electrospray mass spectra were recorded on a ThermoFinnigan LCQ Advantage (electrospray voltage 4.5 kV; capillary 250°C; sheath gas: $N_2$) coupled to ThermoFinnigan LC Surveyor pump utilizing the HPLC gradient Systems 1 and 2. The MS was controlled and total ion chromatograms and mass spectra were recorded using ThermoFinnigan Xcalibur software (San Jose, CA).

**Preliminary Data:** In the initial (up to 10 min) course of the oxidation reaction, the main absorbance band of betanidin **2** diminishes with a shift of $\lambda_{max}$ from 540 nm to 550 nm, and the second band increases at $\lambda_{max}$ 400 nm. This suggests the presence of betanidin *o*-quinone **1**. In further course of the reaction, a hipsochromic shift of λmax of the main absorbance band from 550 nm and a decrease of the second band at $\lambda_{max}$ 400 nm with a batochromic shift towards 410 nm is noticed. A growth in pH results in a stronger hipsochromic shift of $\lambda_{max}$ of the main absorbance band towards 490 nm at pH 7-8. Based on optical and MS/MS data, the structures of 2,17-bidecarboxy-2,3-dehydro-betanidin **3** and 2-decarboxy-2,3-dehydro-betanidin **4** are postulated as the principal betanidin oxidation products formed. The decarboxylation at positions C-2 and C-17 is the most often detected reaction pattern during thermal degradation of betacyanins [2]. One of the oxidation paths is the formation of betanidin *o*-quinone with its rearrangement through the dopachrome or quinone methide derivatives, followed by dehydrogenation and decarboxylation. This results in the formation of **4** as the principal degradation product. Another product formed from **4** on non-oxidative decarboxylation path is **3**. Further oxidation of **3** involves the formation of one of the two quinonoid forms as the oxidized intermediates, e.g. the dopachrome derivative of the indolic system and subsequent rearrangement of the conjugated system of originated structures into the 14,15-dehydrogenated derivatives, leading finally to 2,17-bidecarboxy-2,3-dehydro-neobetanidin **5**, i.e., the compound doubly dehydrogenated at the positions C-2,3 and C-14,15.

**Novel Aspects:** The present study provides evidence for generation of betanidin 5,6-dihydroxyindolic and related structures in acidic and basic media.

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# Jack Bean α-Mannosidase Revisited: Primary Structure and Glycosylation of a Valuable Glycomics Tool

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The enzyme α-mannosidase from Canavalia ensiformis (Jack bean) exhibits a broad substrate specificity, cleaving α 1-2, 1-3 and 1-6 linked mannose residues from both oligosaccharides and glycoproteins. It comprises two pairs of non-covalently linked subunits of molecular weights 66 and 44 kDa, respectively. Even though Jack bean α-mannosidase has been widely used for structure analysis of glycoconjugates for decades neither the amino acid sequence nor the complete glycosylation of the enzyme have been elucidated so far [1]. In the present contribution we communicate the primary structure of both subunits as well as the determination of glycosylation.

**Method:** In the present study a variety of proteases ranging from specific (e.g. trypsin) to rather unspecific (e.g. thermolysin) were used for proteolytic cleavage of α-mannosidase. For *N*-glycopeptide analysis ZIC-HILIC SPE based separation of the resulting glycosylated analyte species was employed [2]. Peptide *de-novo* sequencing and structure elucidation of non-glycosylated and glycosylated peptides were performed by use of nanoESI Q-ToF MS and low-energy CID experiments.

**Preliminary Data:** *De-novo* sequencing of peptides derived from proteolytic cleavages with different proteases gave rise to approximately 90% of the protein sequences of both subunits of α-mannosidase. Intramolecular disulfide bonds could be determined by direct mass spectrometric analysis as described previously [3]. *N*-glycopeptide analysis was performed by use of rather unspecific proteases such as thermolysin that yield short, non glycosylated peptides of low molecular weight and glycopeptides that give rise to signals in the higher m/z range of the nanoESI mass spectra. Furthermore, selective enrichment of *N*-glycosylated species by use of ZIC-HILIC solid-phase extraction furnished samples that almost exclusively comprised glycosylated species. The latter were analyzed both with respect to glycan structure as well as their amino acid sequence. The use of this combined analytical approach provided a deeper insight into the *hitherto* unknown primary structure and glycosylation of α-mannosidase.

**Novel Aspects:** Structural characterization of α-mannosidase from Jack bean by de-novo sequencing and determination of glycosylation are demonstrated for the first time.

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# Phosphorylation-code analysis on cAMP-dependent protein kinase A based on Phos-tag™ SDS-PAGE and MRM mass spectrometry

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Protein phosphorylation is one of the most important posttranslational modifications (PTMs), in particular in the regulation of eukaryotic signal transduction pathways. One goal of current proteomics efforts is to qualitatively and quantitatively describe all phosphorylation sites of a given protein [1]. In this study, Phos-tag™ acrylamide SDS-PAGE (phos-tag) [2, 3] and multi reaction monitoring (MRM) [4] were combined for the analysis of transient phosphorylation patterns of the catalytic subunit of cAMP-dependent protein kinase (PKA-C) overexpressed in *E. coli*. It was attempted to describe the phosphorylation-code of recombinant human and mouse PKA-C in correlation with basic protein kinase function i.e. phosphotransferase activity and inhibitor binding.

**Method:** Co-expression of PKA-C with lambda phosphatase (λPP) with or without additionally co-expression of N-methyl transferase (NMT) in *E. coli* (pRSET-B) was used to produce non phosphorylated PKA-C with or without N-terminal myristoylation. The time course of phosphorylation was analysed with phos-tag in order to separate the different phosphoisoforms. Western blot analysis employing antibodies against the C-terminus of PKA-C (Santa Cruz Biotechnology), against pT197 (Cell Signaling Technology) and against pS338 (Invitrogen™) confirmed different phosphorylation sites. To analyse the exact phosphorylation patterns in each phosphoisoforms, tryptic digests of each band were analysed with MRM on a hybrid quadruple linear ion trap instrument (ABISciex 4000 QTRAP® LC/MS/MS System). MRM transitions were designed with data from data dependent acquisition analysis prior the MRM experiments.

**Preliminary Data:** Phos-tag SDS-PAGE separated the different phosphoisoforms on the basis of their phosphorylation code. Myristoylation on PKA-C showed a more complex phosphorylation pattern on the murine and human isoform 1 respectively. After co-expression with λPP non phosphorylated, inactive PKA-C was detected. Additionally N-terminal modification with the myristic acid did not alter the phosphorylation-code. Auto phosphorylation of the dephosphorylated PKA-C occurred in trans. PKA-C expressed in *E. coli* without λPP and without NMT was hyper-phosphorylated. MRM analysis for all possible serine and threonine phosphorylation sites in the PKA-C produced hundreds of different transitions. For each position we analysed phosphorylated and non-phosphorylated peptides with up to 2 missed cleavages sides, possible deamidation or oxidation as additionally post translational modifications, allowing for up to three different charge states for the Q1 ion and up to three different masses for the Q3 fragment ions. With the transition-list the exact phosphorylation-code of all PKA-C patterns could be analysed. Combined with further functional and structural data we are able to present a new model for the complex phosphorylation pattern of the PKA-C.

**Novel Aspects:** Precise phosphorylation-code analysis of different phosphorylation pattern was established with phos-tag in combination with MRM mass spectrometry.

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# Proteomic Pattern of Interleukin-1/Cycloheximide Cytocidal Effect on HeLa Cells

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Increased release of endogenous tumor necrosis factor (TNF) after interleukin-1 (IL-1)/cycloheximide (CHX) exposure induces cytotoxic activity on HeLa cells. However, this effect is not detectable after selective IL-1 treatment or decreased following CHX [1]. In presented experiments possible changes on proteomic pattern between HeLa cells in response of IL-1/CHX, IL-1 and CHX, were explored. MS-based protein identification was supported by application of difference between predicted and experimental peptides' retention times ($t_R$s) - ($\Delta t_R$) and its utility was examined.

**Method:** Proteomic analysis was performed with the use of LC-ESI-IT-MS/MS. SEQUEST™ algorithm incorporated in BioWorks 3.0 (Thermo Fisher) was used for protein identification. Additionally, it was supported by $\Delta t_R$. This parameter was calculated by applying quantitative structure-retention relationships (QSRR)-based model for peptide tRs prediction in RPLC [2]. It was derived with the use of Dragon (Milano Chemometrics Group) structural descriptors for peptides from 8 known proteins, identified with the highest scores (scoring parameter $X_{corr}$). Predictive ability of the model was verified using peptides from 17 well defined proteins of HeLa cells digests. Evaluated model was finally applied for identification of proteins differentiating all analyzed HeLa proteomes (IL-1/CHX, IL-1, CHX, control).

**Preliminary Data:** Employment of $\Delta t_R$, additionally to MS/MS-derived information, showed that high MS identification correlate with low differences between experimental and predicted peptides' retention times. In contrary, the ΔtR values increased for incorrect determination of the peptides. The study revealed also proteins, which were characteristic exclusively for IL-1/CHX (6), IL-1 (16), CHX (9)-exposed and control (10) HeLa cell cultures. Among them, protein disulphide isomerase (PDIA1_HUMAN: P07237) seems to be one of key proteins responsible for cytocidal effect caused by TNF release after IL1/CHX stimulation of HeLa cells [3].

**Novel Aspects:** Combined application of MS/MS data and $\Delta t_R$ facilitated identification of proteins, typical for HeLa cells in response to IL1/CHX treatment.

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# Automatic MS/MS characterization of N-linked Glycopeptides

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Glycosylation is the most abundant posttranslational protein modification. Involved in many relevant bio-logical processes and pathways, it is crucial to the understanding of many diseases. However, glyco-peptide analysis is still challenging. Due to high glycan heterogeneity and ion suppression effects, abundance of glycopeptides in tryptic digests is low and may require enrichment and separation tech-niques. In addition, interpretation of MS/MS spectra is difficult as classical database search approaches cannot be used when the peptide sequence and the glycan molecular weight are unknown. The proper determination of the peptide mass, i.e., the aglycone, is the key for automated glycan database searches. We developed a software approach evaluating the peptide mass in MALDI-TOF/TOF MS/MS spectra of N-linked glycopeptides.

**Method:** All glycoproteins were reduced, alkylated and digested (trypsin or Glu C) in solution. The digest solutions were separated by nano-LC and the fractions were automatically spotted to a sample holder and sub-sequently analyzed by MALDI-TOF/TOF-MS. All MS/MS spectra were processed as it is routine for proteomics, the peaklists were then used for the detection of glycopeptide spectra based on indicative peak patterns. Database searches were performed using Swissprot and Mascot (Matrix Science) for protein identification and GlycomeDB and GlycoQuest (Bruker) for glycan identification.

**Preliminary Data:** MALDI-TOF/TOF spectra of N-linked glyco-peptides show a characteristic fragmentation pattern. The mass distances within this pattern (17, 83, 120 Da) are used in ProteinScape 3.0 to classify glycopeptide MS/MS spectra and to determine the mass and to ID the aglycone using Mascot. After classification, a list of 57 potential N-linked glycopeptides with their aglycone masses = $(M + H)^+$ was provided. The peptides were matched to different N-glycosylation sites. On the basis of the determined aglycone masses, a glycan database search identified several glycan structures.

**Novel Aspects:** Automated MS/MS with a glycan database for identification of glycan structures.

# Improved Production of RISQ Protein Standards for Absolute Quantification of STAT5 Family Proteins

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Regulatory mechanisms in cellular processes often operate *via* quantitative changes of the proteome. Mass spectrometry based on stable isotope dilution is an excellent tool to study the changes in protein expression. Using stable isotope labeled standards both for peptides and intact protein quantification is a widely recognized and well-established strategy. In terms of absolute quantification only few methods for proteins have been developed so far e.g. the SILAC [1] and FLEXIQuant [2] method. In this study we report an improved method for production of Recombinant Isotope labeled and Selenium Quantified (RISQ) [3] protein standards ready to use as internal standards for absolute quantification of STAT5A (human), STAT5B (human) and STAT5A (mouse).

**Method:** RISQ protein standards were generated by cell-free protein synthesis (RTS 100 *E. coli* HY, RTS 500 ProteoMaster *E. coli* HY, 5-PRIME). Expression vector containing ORF for studied proteins with His-tag at the C-terminus was obtained in LR reaction between donor vectors and pEXP2-destination vector by applying Gateway Cloning technology (Invitrogen). After synthesis the protein standards were purified by $Ni^{2+}$-IMAC and dialysed. Element mass spectrometry (ICP-MS) analyses were performed by double focusing sector field instrument (Element 2, Thermo). The protein standards were digested by trypsin according to the standard protocol. For LC-MS/MS measurements a UPLC-system (Waters) connected to an LTQ-Orbitrap XL instrument (Thermo) was used. Western Blot analyses were done using STAT5A and STAT5 antibody (Santa Cruz Biotechnology).

**Preliminary Data:** C-terminally His-tagged STAT5A (human), STAT5B (human) and STAT5A (mouse) were successfully synthesized as RISQ protein standards which was confirmed by LC-MS/MS analysis. In the reaction medium methionine was replaced by selenomethionine and arginine and lysine for $^{13}C$,$^{15}N$-labeled arginine (Arg + 10) and $^{13}C$-labeled lysine (Lys + 6), respectively. A continuous-exchange cell-free (CECF) synthesis system (RTS 500 ProteoMaster E.coli HY) was compared to batch system (RTS 100 *E. coli* HY) (for STAT5B (human) sequence coverage 40% vs. 13%). Western Blot analysis was applied to verify the efficiency of the applied purification process. This analysis revealed that RISQ standards can be mainly found in flow-through fractions for all types of proteins. Therefore, the purification efficiency has to be improved. One explanation of the fact that RISQ proteins do not efficiently bind to the $Ni^{2+}$ ions inside the column can be high amounts of metals such as $Mg^{2+}$ or $Fe^{3+}$ present in cell-free protein synthesis medium which can bind to His-tag, thus disabling it for binding to the $Ni^{2+}$-column. The presence of metals was confirmed by ICP-MS analysis of flow-through samples. To remove possible metal ions protein samples just after cell-free synthesis were treated with EDTA and after dialysis the samples were subjected to IMAC purification. As an alternative purification method electro-elution technique was employed. Selenomethionine incorporation enables an absolute quantification of the synthesized protein standards by ICP-MS. The absolutely quantified STAT5 family protein standards can be added to cell lysates to determine the abundances of native STAT5 family proteins in the cell by LC-MS/MS.

**Novel Aspects:** Improved cell-free synthesis of stable isotope labeled STAT5 family protein standards for absolute quantification in cell lysates.

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# Interactions of human cystatin C with antibodies as an effective method of fighting Icelandic type hereditary cerebral hemorrhage with amyloidosis

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Amyloidoses are a group of diseases in which the main role is played by abnormal folding of peptides and extracellular proteins. Generation of amyloid deposits by many different peptides or proteins is associated with diseases such as: Alzheimer's Disease (AD), Parkinson's Disease (PD) and many others. Amyloid deposits are also formed by human cystatin C (hCC), and more precisely by its amyloidogenic mutant L68Q [1]. The number of molecules proposed as potent inhibitors of cystatin C dimerization and oligomerization is limited. It was reported that there are studies being conducted, on exogenous factors, which suppress the process of dimerization of both the native form of hCC, and its pathological mutant L68Q [2]. Monospecific antibodies could be one of such potential factors [2].

**Method:** To identify the epitope of the antibodies we applied affinity chromatography techniques, combined with enzymatic digestion of immobilized antibody-hCC complex and analysis of products of enzymatic digestion by mass spectrometry. This method is called epitope excision [3]. It is based on the formation of complexes by antibodies which is attached to the solid support, and human cystatin C (antigen), and then digestion by using several proteolytic enzymes (trypsin, chymotrypsin, Lys-C, Glu C, pronase) and analysis of products by using high-resolution mass spectrometry [3].

**Preliminary Data:** It was proved that catalytic amounts of antibodies present at the conditions which promote formation of dimers, can prevent dimerization of both wild type cystatin C ("wt-hCC") and its highly amyloidogenic mutant L68Q [2]. Twelve different monoclonal antibodies: Cyst10, Cyst12, Cyst13, Cyst16, Cyst18, Cyst19, Cyst23, Cyst24, Cyst27, Cyst28, Cyst29 and HCC3 were investigated in terms of their impact on cystatin C dimerization [4]. In our group we have determined the interaction site of hCC with several anti-hCC antibodies from this group: Cyst13, Cyst16 and Cyst19 [3]. One of the available anti-hCC antibodies, HCC3 interacts with its epitope outside the active center of inhibition, which results in retaining by cystatin C its ability to inhibit proteinase activity in this immune complex [5]. It is very interesting to establish the epitope sequence of this antibody and appropriate studies are being conducted in our laboratory. We are also working on Cyst10, which is the only one which does not suppress dimerization of hCC.

**Novel Aspects:** Determination of epitopes of hCC is essential for planning of potential immunotherapies and designing inhibitors of the dimerization process.

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# Reactions of (Halo)Nitrophenide Ions with Acrylonitrile and Alkyl Acrylates in the Gas Phase. The Case oF [M-2]- Ion Formation

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Activated olefins such as acrylonitrile and acrylates are extensively used in organic synthesis as reactants in condense-phase nucleophilic conjugate addition (Michael reaction) [1-3], nevertheless their intrinsic gas-phase reactivity, in particular with nucleophiles, is still incompletely known. Our previous studies showed that the characteristic feature of the gas-phase ion-molecule reactions of (halo)nitrophenide ions with α,β-unsaturated species like acrylonitrile, methyl acrylate and ethyl acrylate is formation of products having m/z values nominally two mass units lower than the (halo)nitrophenide ion ($M^-$) [4].

**Method:** The mechanistic and structural details associated with $[M-2]^-$ ion formation is the topic of the present work. We report the results of mass spectrometric experiments interpreted by means of computational quantum chemistry. The gas-phase ion-molecular reactions of (halo)nitrophenide ions with α,β-unsaturated compounds were studied employing an API 365 triple quadrupole mass spectrometer (Applied Biosystems), and a Bruker Apex 47, FT-ICR mass spectrometer (Billerica, MA) both equipped with electrospray ionization (ESI) sources.

**Preliminary Data:** Although it is not possible to prove the origin of the $[M-2]^-$ ions directly clear evidence for intermediate β-adduct formation during reaction was found. More specifically, the mechanism involves transformation of the β-adduct comprising an O-atom transfer via a six-membered intermediate, followed by the loss of an HCOR molecule (R = CN, $CO_2Me$, $CO_2Et$). This sequence leads eventually to an $[M-2]^-$ anion with an o-nitrosobenzylic anion structure. Further transformation of the *o*-nitrosobenzylic anion to the more stable *o*-aminobenzaldehyde anion is also considered.

**Novel Aspects:** Nitrophenide ions react with α,β-unsaturated compounds via formation of β-adducts. Unstable β-adducts rearrange to derivatives of o-nitrosobenzylic anion ($[M-2]^-$).

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# Catching Elusive Intermediates: Ion Mobility Mass Spectrometric Studies of Helquats

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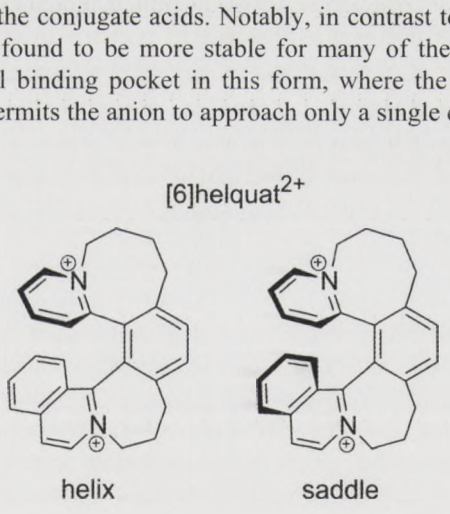
Helquats (HQ) are helical dications, which can be considered as structural hybrids between helicenes and the herbicide diquat. The helix inversion equilibrating the right-handed helix with the left-handed one usually proceeds *via* a saddle-shaped transition state. Reported cases of helicene congeners having saddle-shaped energy minima on their racemization pathways are rare. Yet for one specific [6]helquat, this saddle conformer could even be isolated because it is the kinetically favored product of the synthesis. Further, there are several other HQ derivatives where the presence of a saddle-shaped minimum is predicted by computations, but the saddle form could not be prepared. Our aim was to catch these elusive saddle conformers and to investigate the isomerization of helquats in the gas phase.

**Method:** We studied $HQX^+$ ion pairs (X = counter anion) for several helquats and various anions using ion-mobility mass spectrometry (IM-MS), which enables us the separation not only according to mass but also on the basis of shape. The shapes of the helix and saddle conformers of HQs, especially when they are attached to some counter anion, are rather different; therefore, IM-MS was expected to be a useful tool for their detection. The effects of the anion on the isomerization barrier, on the fragmentation onset, as well as on the stability of the different conformers were also investigated.

**Preliminary Data:** The IM-MS investigation of the isolated helix and saddle forms of the [6]helquat showed that the two conformers can be distinguished and also interconversion between them can be triggered by providing energy. For all other HQs where the saddle form is expected to be a minimum, we observed the isomerization of helix to saddle upon excitation in the mobility spectra. The separation between the saddle and helix conformer depends on both the HQ size and the anion. The obtained ion mobility trends of the different ionic species are in agreement with the hard-sphere cross sections determined from the calculated molecular geometries. With respect to the effect of the counterions, the isomerization barriers are similar for all of them, whereas a linear relationship was found between the fragmentation barriers and the gas phase acidities of the conjugate acids. Notably, in contrast to the free dication and neutral salt, the saddle conformer of the ion pair was found to be more stable for many of the counterions. This observation can be explained by the presence of a central binding pocket in this form, where the anion can interact with both cationic centers, whereas the helix conformer permits the anion to approach only a single quaternized nitrogen atom.




**Novel Aspects:** In the gas phase, anion complexes of certain helicene congeners can be isomerized to more stable saddle-shaped isomers.

# Functional proteomics reveals a regulatory function of the osteoclast-associated receptor in STAT-signaling and cell adhesion of human endothelial cells

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The osteoclast-associated receptor (OSCAR) is a member of the leukocyte receptor complex encoded family of surface receptors. It has recently been identified as an important receptor for osteoclast differentiation, cell activation, maturation of macrophages, monocytes and dendritic cells [1]. Recently, Goettsch et. al could show that in human endothelial cells OSCAR is regulated by oxidized lipoproteins [2]. However the causative role and function of vascular OSCAR in atherosclerosis and other inflammatory conditions is still unknown. Therefore, functional proteomics was used in combination with either a silencing or an overexpression strategy which gave us the ability to analyze the role of OSCAR in human microvascular endothelial cells (HMEC-1) and its possible impact in arterial calcification.

**Method:** To determine cellular functions of OSCAR in human endothelial cells, we either silenced or overexpressed OSCAR in human microvascular endothelial cells (HMEC-1). Protein quantification was performed by nano-UPLC/nano-ESI-MS/MS using the SILAC (stable isotope labeling with amino acids in cell culture) approach. After cell-harvesting cell lysates as well as membrane, cytosolic and nuclear fractions were prepared. After further separation by 1D-SDS-PAGE the entire protein gel lanes were cut into gel slices and digested with trypsin. For identification and quantification of proteins nano-HPLC/nano-ESI-LTQ Orbitrap-MS measurements followed by MaxQuant analyses were conducted. Based on more than 3,300 reproducible quantified proteins involved pathways were extracted by functional annotation clustering of the differentially expressed proteins using the DAVID-program as well as the Ingenuity IPA Software.

**Preliminary Data:** In an in-depth proteomic study using stable isotope labeling with amino acids in cell culture (SILAC) and analyzing different protein fractions (membrane, cytosol, nucleus) more than 3,364 proteins (FDR < 1%) were reproducibly quantified by nano-HPLC/nano-ESI-MS in OSCAR overexpressing or OSCAR deficient endothelial cells. OSCAR overexpression modulated the expression of 145 proteins, which are mainly involved in cell structure and motility, actin/cytoskeleton binding and stress response. Furthermore OSCAR silencing resulted in the regulation of 110 proteins which are mainly involved in cell adhesion, cell communication and actin binding. Besides this, signal transducer and activator of transcription 1 (STAT1) and 3 (STAT3) were regulated in a contrary way upon OSCAR modulation. STAT1 as well as several interferon γ induced proteins show a clear anti-correlation to the OSCAR expression, which could be independently confirmed by immunoblots. As opposed to STAT1 OSCAR overexpression caused a STAT3 activation. Furthermore, we determined a positive modulation of integrin signaling pathway and proteins involved in cell adhesion, which correlates well with an increased adhesion of monocytes to the endothelium after OSCAR overexpression. We studied the function of endothelial- derived OSCAR by using comparative proteomics together with functional cluster analysis of the differentially regulated proteins and revealed a modulation of several functions by either OSCAR silencing or overexpression. Our proteomics analysis pointed out the contribution of OSCAR to STAT signaling pathway and monocyte adhesion. Both of these pathways play important roles in cell activation and inflammation during the generation of an atherosclerosis.

**Novel Aspects:** The osteoclast-associated receptor plays a dual role in the STAT signaling pathway and cell adhesion in human microvascular endothelial cells.

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# Rapid and sensitive detection of pesticides, pharmaceuticals and biomolecules *via* ESI ion mobility spectrometry

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The separation in ion mobility (IM) spectrometry is based on the different drift velocities of ions in an electric field within a bath gas. The drift velocity depends on the electrical field strength and the ion mobility. The latter is an ion-specific property mainly depending on the spatial structure of the ions. Initially, IM-spectrometry was used for the sensitive and fast detection of substances in the gas phase such as chemical warfare agents. The combination of electrospray ionization (ESI) and IM-spectrometry extended the application range to include the analysis of polar substances in liquid samples [1].

**Method:** All results were obtained with a home-made ESI IM spectrometer working at atmospheric pressure. The IM spectrometer consists of a 50 mm desolvation region and a 50 mm drift tube. A Bradbury-Nielsen shutter is used to create a 50-100 µs ion pulse at a frequency of 50 Hz to be analyzed in the drift tube. Purified and dried nitrogen is used as bath gas and methanol-water mixtures are used as standard solvents for all experiments.

**Preliminary Data:** The device facilitates the real-time analysis of liquid samples and is used for the sensitive detection of various pesticides, biomolecules (amino acids, peptides) and pharmaceuticals (neuroleptics), achieving a resolution of $R > 60$ in most cases. Ion mobilities and collision cross sections are reported for 16 different urea-, triazine- and acetanilide-pesticides. The capacity of simultaneously identifying the individual components of simple mixtures is demonstrated. The limits of detection are in the upper nanomolar range for most of the substances investigated. The desolvation and release of ions was a special focus of our investigations. It was found that the dependence of the signal intensity on the temperature in the desolvation region can approximately be described by $\ln I \propto 1/T$. The slope of the respective plots is different for individual substances and allows a characterization of the electrospray process, particularly the efficiency of desolvation and ionization of the molecules. These results complement our previous research on the release of ions in the electrospray plume using spectrally resolved fluorescence images [2].

**Novel Aspects:** The influence of temperature on the desolvation and release of different ions in ESI was investigated at atmospheric pressure.

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# Proteomic profiling of recurrent glioblastoma using data-independent label-free quantification

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Astrocytic gliomas are the most frequent neoplasms of the central nervous system comprising roughly 75% of all neuroepithelial tumors. Grade IV of these tumors, so-called Glioblastoma multiforme, is the most common and most malignant primary brain tumor. Though surgery, chemo- and radiotherapy improve clinical outcome, recurrence is inevitable, and median survival is about one year. Therefore, the elucidation of underlying molecular mechanisms of tumor recurrence, progression and therapy resistance is important. Together with the collaboration partners within the German Glioma Network, we are aiming at systematically characterizing molecular aberrations in pairs of primary and recurrent glioblastomas using a data-independent label-free proteomic approach. The GGN offers the unique opportunity to integrate proteomic data with gene expression results and detailed clinical information.

**Method:** Frozen tissue samples obtained from brain tumor resection were homogenized in GITC followed by ultracentrifugation [1]. Subsequently, protein fractions were precipitated with TCA, separated by short-SDS-PAGE and in-gel digested using trypsin. Peptide concentration after extraction was determined using amino acid analysis (Waters, Manchester, UK). Dried samples were dissolved in 3% aqueous acetonitrile containing 0.1% formic acid (FA) and randomized before undergoing data-independent label-free ion mobility LC-MS/MS ($HDMS^E$) by an extension of the previously described methods [2-4]. Essentially, nanoLC separation was performed using a nanoAcquity LC (Waters) and a quadrupole-IMS-ToF mass spectrometer (Synapt G2 HDMS, Waters). Data were acquired using MassLynx 4.1 (Waters) in $HDMS^E$ positive ion mode. ProteinLynx Global Server (v2.5; Waters) was used for protein identification and label-free quantification.

**Preliminary Data:** In total, twenty paired-brain samples were analysed; ten primary tumors, and ten matched recurrent tumors from the same patient. Using the data-independent label-free quantification approach we could identify approximately 1000 proteins per run resulting in a total number of 2962 non-redundant proteins being identified in more than one run. 392 proteins were determined in all runs exhibiting average sequence coverage of 34.5% spanning from 6.8% to 91.3% sequence coverage. Quantitative analysis revealed 59 differentially regulated proteins with p-value $<0.05$, mean ratio of $>2$ or $<0.5$ and represent in at least two of three technical replicates. Applying the AMRT approach of Silva et al. using yeast ADH as an internal standard we were able to absolutely quantify protein amount over a dynamic range of four orders of magnitude.

**Novel Aspects:** data-independent label-free ion mobility LC-MS/MS based quantification of human tumor tissue.

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# Interaction structure of the complex between neuroprotective factor humanin and Alzheimer's β-amyloid peptide revealed by affinity-mass spectrometry and molecular modelling

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Humanin (HN) is a linear 24 aa peptide recently detected in human AD brain [1]. HN specifically inhibits neuronal cell deathinduced *in vitro* by β-amyloid (Aβ) peptides and by amyloid precursor protein and its gene mutations in familial AD, thereby representing a potential therapeutic lead structure for AD; however, its molecular mechanism of action is not well understood. We report here the identification of the binding epitopes between HN and Aβ(1-40) and characterization of the interaction structure through a molecular modelling study.

**Method:** Wild-type HN and HN-sequence mutations were synthesized by SPPS and the HPLC-purified peptides characterized by MALDI-mass spectrometry (MALDI-MS). The interaction epitopes between HN and Aβ(1-40) were identified by affinity-mass spectrometry using proteolytic epitope-excision and -extraction [2, 3], followed by elution and mass spectrometric characterization of the affinity-bound peptides.

**Preliminary Data:** The affinity-MS analyses revealed HN(5-15) as the epitope sequence of HN, while Aβ(17-28) was identified as the Aβ-interaction epitope. The binding sites were ascertained by ELISA of the complex of HN peptides with immobilized Aβ(1-40) and by ELISA with Aβ(1-40) and Aβ-partial sequences as ligands to immobilized HN. The specificity and affinity of the HN-Aβ interaction were characterized by direct electrospray ionization mass spectrometry of the HN-Aβ(1-40) complex and by bioaffinity analysis using a surface acoustic wave (SAW) biosensor, providing a KD of the complex of of 610 nM. A molecular dynamics simulation of the HN-Aβ(1-40) complex was consistent with the binding specificity and shielding effects of the HN and Aß interaction epitopes.

**Novel Aspects:** These results indicate a specific, strong association of HN and Aβ(1-40) and provide a molecular basis for understanding HN neuroprotection.

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# Methods for Proteomics Characterization of Archeological Organic Residues

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Organic residues of diverse origin are commonly found in archeological artifacts. Conventional analytical methods address their elemental or chemical composition and estimate the relative content of protein, lipid or carbohydrate bulk component. At the same time, the exact protein composition has been neglected because of the common notion that proteins are completely degraded with time. Furthermore, samples compositional complexity with the majority of proteins originating from a variety of wild-bred species with unknown genomes is error-prone and precludes meaningful interpretations. Here we outline a strategy for successful proteomics characterization of organic residual material excavated from an ancient Chinese archeological sites dated to 4th to 1th millennium BC.

**Method:** 5 to 15 mg of dried solid residue material were homogenized by grinding in 2 ml Eppendorf tube. 10% SDS-containing reducing buffer was added and the sample was heated to 60 C with sonication. 50 µl slurry was loaded on 12% polyacrylamide SDS gel. After electrophoretic separation the gel was stained with Coomassie R250 and the gel lane sliced into 4-6 plugs. Proteins were *in-gel* digested with trypsin [1], extracted and analyzed by LC-MS/MS on a LTQ Orbitrap Velos mass spectrometer [2]. Spectra were searched against a comprehensive (NCBI) or customized protein databases by MASCOT software (Matrix Science). When necessary, matched peptide sequences were further evaluated by BLAST and MS BLAST sequence similarity searches [3].

**Preliminary Data:** Archeological evidence suggested the organic material subjected for proteomics analysis could have originated from processed nutritional products. Despite thermal treatment, fermentation and natural aging, we have been able to systematically identify 20-200 proteins by matching 2 to 50 matching peptides, while proteinuous background was relatively minor. Soaking the organic material in high % SDS buffer and direct loading of the full sample on the gel efficiently extracted the protein bulk and did not hamper the subsequent LC-MS/MS such that proteomics analysis was possible on a few mg of excavated samples. The complete analysis of gel lanes enabled the identification of degraded proteins, irrespectively of the actual MW of cleavage fragments. For accurate protein assignment, matched peptides were subjected to sequence similarity searches to identify sequences that could be attributed to either unique organism or a small group of phylogenetically close species. Typical protein background consisted of the components of human skin, sweat, lachrymal and salivary glands (e.g. human serum albumin or amylase), most likely because of material handling, and the mold (mostly *Aspergillus*) proteins. Aging or processing increased protein deamidation and a large number of non-tryptic peptides were detected in tryptic digests. Both factors should be accounted for in database searching settings.

**Novel Aspects:** Method for Proteomics Characterization of Archeological Organic Residues.

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# Comparative proteomic analysis of proteins isolated from Chelidonium majus L. milky sap and Corydalis cava Schweigg. & Koerte tubers (Papaveraceae)

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*Chelidonium majus L.* and *Corydalis cava* Schweigg. & Koerte plants belong to Papaveraceae family. *Ch. majus* milky sap exudes when plant is injured. It is the protoplasm of specialized cells termed laticifers. These cells are located in phloem areas of the vascular system and form an internal, articulated, and fused cell system throughout the whole plant [1]. Milky sap of *Ch. majus* is used for treatment of human papilloma virus infections. *C. cava* develops the sizeable subterraneous tubers, empty inside, scantily covered by fibrous roots and resistant to the attack of various pathogens. In this study we compare the protein composition from *Ch. majus* milky sap isolated from the stems and roots of the plant and *C. cava* tubers, using large-scale tandem mass spectrometry identification (nano-LC-MS/MS).

**Method:** *Ch. majus* stems and roots and *C. cava* tubers were collected in the neighbourhood of Poznan in spring 2010.Protein extracts were separated by SDS-PAGE and gel samples were subjected to LC-MS/MS. After trypsin digestion peptides were extracted from the gel matrix using 1% TFA. Samples were desalted and concentrated and analyzed by liquid chromatography coupled to LTQ Orbitrap XL mass spectrometer (Thermo, USA) in Max-Planck-Institute of Molecular Plant Physiology in Golm. Protein lists of each sample type were combined and protein hits were functionally classified into 11 functional groups.

**Preliminary Data:** In total, 523 proteins were identified: 66 for *Ch. majus* milky sap and 457 for *C. cava* tuber. To achieve quantitative comparison of protein functional categories between analysed samples, Exponentially Modified Protein Abundance Index (emPAI) was employed (Ishihama et al. 2005). The most abundant in proteins are *C. cava* tuber extracts (emPAI sum 108.45), the least *Ch. majus* milky sap (emPAI sum 8.18). The most abundant in stress and defense response proteins are *C. cava* tubers (17.96%), followed by *Ch. majus* milky sap (14.06%). The opposite is for secondary metabolism and storage category – the most abundant is *Ch. majus* milky sap (13.81%), followed by *C. cava* tubers (3.62%). Among defense-related proteins in *Ch. majus* milky sap are proteins with leucine-rich repeats, peroxidase, superoxide-dimutase, lactoylglutathione lyase, monodehydroascorbate reductase. *Ch. majus* milky sap is also rich in secondary metabolism and storage proteins. The most abundant protein from this group is rubber elongation factor (REF),which is an enzyme involved in rubber biosynthesis. *Ch. majus* milky sap, as well as *C. cava* tuber, contains beta-amylase. It is an enzyme which hydrolyzes glucans derived from starch granules to maltose and is present in different plant organs. Similarily as in *Ch. majus* milky sap *C. cava* tubers are highly abundant in superoxide-dismutases, lactoylglutathione lyases but on the other hand unlike *Ch. majus* milky sap they are rich in heat shock (hsp70, 80, 90) and 14-3-3 proteins. They also contain interesting proteins with low abundance: powdery mildew resistance protein PM3b, germin-like protein and late embryogenesis abundant family protein/LEA family protein.

**Novel Aspects:** Comparison of stress- and defense-related protein content of two pharmacologically active plant species from the Papaveraceae family.

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# Proteogenomic analysis of Helicobacter pylori strain 26695

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Since the first sequencing of a DNA-based genome in 1977, genome sequencing developed into a well-automated, high-throughput and inexpensive method. The genome is the carrier of protein sequence information. Usually, proteins are annotated by gene-finding software, which is often prone to incorrect protein assignments like wrong starting sites or stops or missing of short genes. High-resolution mass spectrometry and improved protein and peptide separation techniques empower proteomics to become an essential tool for coding sequence annotation. Existing annotations can be validated by experimental data and novel identification of starting and stop sites, sequencing errors and novel genes or exons can be provided by proteomics. We made an in-depth proteomic study of *Helicobacter pylori* to proof the current coding sequence assignment.

**Method:** *Helicobater pylori* strain 26695 was cultured in BHI medium containing 10% FCS. Cells were harvested by centrifugation at late log-phase and lysed by ultrasonic. Protein separation was either performed by SDS-PAGE or size exclusion chromatography (SEC). Gels were cut into 20 equal pieces and digested with trypsin. SEC was focused on low molecular weight proteins below 30 kDa. SEC fractions were concentrated by C18 spin-columns and digested separately with trypsin, LysC and AspN. MS measurement was carried out on a LTQ Orbitrap XL online coupled to a nanoUPLC. Spectrum files were recalibrated using Maxquant 1.1 and searched against a reverse concatenated database including the NCBI database of and a six-frame translation of *H. pylori* strain 26 695 with Mascot and X!Tandem.

**Preliminary Data:** For peptide identification, Mascot Ion scores had to be greater than both the associated identity scores and 15 or X!Tandem Log(Expect Scores) greater than 2. Protein identifications required at least 2 unique peptides. Protein false discovery rates were below 0.1% for proteins and below 0.5% for peptides. Overall 1107 proteins respectively 70.9% of the *H. pylori* strain 26695 proteome were identified. Peptides which were identified in the six-frame translation but not in the NCBI part of the database were further investigated. The location of these peptides on the genome was carried out by the UCSC genome browser. In this way, we could correct annotations for 5 different proteins. Sequencing errors for two proteins resulted in C-terminal elongated protein sequences. Namely, carbonic anhydrase and hypothetical protein HP0694 were shown to have similar C-terminal sequences to those of *H. pylori* strain J99. Additionally, one previously not annotated protein was identified by three unique peptides highly similar to ferrous iron transport protein A in other strains. The hypothetical protein HP1433 obviously has a wrong start codon assignment, identified by a peptide localized near the N-terminus of the annotated sequence in the same gel fraction. Six different peptides experimentally validated the computationally verified authentic frame shift of the predicted coding region HP0058, reported by Medigue et al. [1]. For validation of our findings, we ordered 12 peptides labeled at the C-terminal amino acid with N15 and C13 isotopes. MS/MS spectra of the ordered peptides were acquired in direct infusion mode at an LTQ-Orbitrap XL mass spectrometer. Peptide identifications by Mascot and X!Tandem as well as the excellent correlation to the experimentally derived tandem mass spectra confirmed our results.

**Novel Aspects:** Proteomic experiments should be included in every DNA sequencing project in order to avoid wrong protein coding sequence assignments.

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# Brood-protective Antimicrobial Proteins and Peptides in Saliva of the Common Earwig (*F. auricularia*)

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Earwigs (*Forficulidae*) are the family of insects wide-spread all over the world [1]. A unique feature of these animals is maternal care. After laying an egg clutch in a nest 5 mm below the ground surface, the female insect stays with it for a considerable time [2]. The female continually shifts the eggs about and cleans them to avoid fungal and microbial growth over the egg surface. It is of interest to find out the mechanism of egg protection and what chemical substances are responsible for antibiotic activity.

**Method:** For the present study salivary glands of the Common Earwig (*F. auricularia*) and egg surfaces were investigated. Our goal was to discover compounds, primarily peptides and proteins, which are secreted by the insect to guard the eggs. Salivary glands were dissected from insect heads and their contents were extracted using MilliQ water. After purification and concentration the extract was analyzed by RP-HPLC coupled to a FTICR mass spectrometer (LTQ-FT, Thermo Fisher Scientific, Bremen). The preparation procedure for the eggs was as follows. Eggs freshly handled by the female were collected and washed with MilliQ water. They were not destructed, since surface substances were of interest only. The obtained solution was purified, concentrated and analyzed with the same approach as above.

**Preliminary Data:** The primary structure of peptides detected in the samples was established by manual de novo sequencing using CID spectra. Amino acid sequences of several peptides detected in salivary glands extracts were elucidated. The major part of them had a mass around 1000 Da and almost all of them contained positively charged basic amino acids. Moreover proteins having a molecular mass of about 10 kDa were detected in the investigated extraction. Their structure was partially established by de novo sequencing, no structurally similar proteins were found in databases. Total amino acid sequencing of these proteins will be scheduled for further investigation.

**Novel Aspects:** Identification and de novo sequencing of bioactive antimicrobial peptides from insects by high resolution mass spectrometry

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# Development of a SRM assay for quantification of urinary proteins

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Several urinary proteins are candidate biomarkers for diverse renal diseases, e.g. microalbuminuria as one early marker of diabetic nephropathy [1]. For the detection and quantification of proteins sensitive, robust, and fast methods are needed to study a larger number of patient samples. LC-MS-targeted proteomics approaches in selected reaction monitoring (SRM) mode proved to be effective for this purpose. SRM-transitions for defined peptides may be obtained from the PeptideAtlas [2]. However, this source does not guarantee that peptides chosen from the PeptideAtlas will yield the necessary signal intensities in SRM. In the present study, we established a SRM assay via an experimental shotgun approach using urinary proteins to define specific transitions for quantification of some urinary candidate biomarkers.

**Method:** *Protein extraction.* Urine samples were centrifuged for sediment removal. Proteins were precipitated with ice-cold acetone. The pellet was resolubilized in ammonium bicarbonate buffer. Total protein concentration was determined using a BCA-assay. *Selection of SRM-transitions.* Urinary proteins were separated via SDS-PAGE, tryptically digested and analysed by HPLC-Chip-Cube ESI-ion trap mass spectrometry. Tryptic peptides were identified by online database search [3]. Transitions were determined by the data from the MS/MS spectra of the ion-trap. Peptides with high Mascot scores were chosen. *Quantification with SRM.* The SRM assay was developed on a HPLC-Chip-Cube ESI-Triple-Quadrupole. The HPLC gradient was adjusted according to the retention behavior of defined peptides. Parameters for ionization and fragmentation were optimized by LC-MS.

**Preliminary Data:** The purpose of this work was to develop a SRM assay for the quantification of urinary proteins based on experimental data obtained from the analysis of tryptic peptides from an extract of urinary proteins. Transitions for defined peptides for SRM may be acquired from existing proteomics databases, e.g. the PeptideAtlas [2]. Peptides chosen from the Peptide Atlas are not necessarily suitable for SRM since the selected peptides might be modified in vivo or will not pass the chromatographic steps prior to the mass spectrometer thus yielding no signals or signals with low intensities. With the developed SRM assay for all targeted urinary proteins quantitative data were yielded in the SRM chromatograms of urine samples thus demonstrating that the strategy described in this study based on experimental data derived from a protein extract for the SRM development is a successful approach and does not necessarily require synthetic peptides for the determination of transitions and for optimizing the mass spectrometric parameters.

**Novel Aspects:** The strategy describes the development of a SRM method by using a protein extract instead of synthetic peptides.

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# Identification of the Disulfide Linkages of Three Antimicrobial Macin Proteins by Proteolytic Cleavage and nanoESI-LTQ-Orbitrap MS/MS Analysis

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Hydramacin-1 (HM-1) [1], theromacin (TM) and neuromacin (NM) are three antimicrobial proteins that belong to the macin protein family. Despite of the high sequential identity including eight conserved cysteine residues, the three molecules showed distinct differences with respect to their biological activity [2]. In order to improve our understanding of the structure-activity relationship, the structures of TM and NM had to be determined. In the present study, a simple and rapid method comprising single or successive enzyme cleavages followed by nanoESI-LTQ-Orbitrap MS/MS analysis was developed to unambiguously assign the disulfide linkages of TM and NM that facilitates the elucidation of their tertiary structures.

**Method:** HM-1 from *Hydra*, TM and NM from *Hirudo medicinalis* were recombinantly expressed in *E. coli*, refolded in a redox system at basic pH and purified with RP-HPLC. Depending on the primary structure, either single enzyme (i.e., trypsin for HM-1 and NM) or successive enzyme treatment (i.e., trypsin and Asp-N for TM) was applied to cleave the purified macin proteins. The resulting peptides were separated by IP-RP-HPLC and analyzed by a LTQ-Orbitrap Velos mass spectrometer.

**Preliminary Data:** The use of sequential enzymatic digestion enabled the production of specific peptide fragments that facilitate the assignment of the disulfide bridges of HM-1, NM, and TM. The results revealed that the eight cysteine residues conserved in all three macins form the same four disulfide bonds: Cys1-Cys6, Cys2-Cys5, Cys3-Cys7, and Cys4-Cys8. TM possesses two additional cysteine residues that form a fifth disulfide bond. The results aided the determination of the TM's tertiary structure by NMR [2].Due to the conserved disulfide pattern of the macins and their high sequence identity, it is very likely that NM shares the same structural features and adopts the same fold as HM-1 and TM. Structurally and functionally, NM appears to be a chimera of TM and HM-1 [2]. The elucidated structure information of the macin proteins might contribute to the rational design of novel potential pharmaceuticals.

**Novel Aspects:** The disulfide linkages of three macin proteins were assigned and their tertiary structures were determined.

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# Further improvements of electrospray mass spectrometry analysis of One-Bead-One-Compound oligopeptide libraries labeled by quaternary ammonium salts

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Split-and-mix technique has become a method of choice in preparation of one-bead-one-compound (OBOC) libraries [1]. Chemical structure of peptide obtained from the resin bead can be identified by ESI-MS methods in a single experiment. The fundamental problem of OBOC peptide library analysis is the small amount of compound obtained from single resin bead. Therefore, an enhancement of detectability is an important goal of MS analysis. The application of quaternary ammonium salts (QAS) increases ionization efficiency and reduces the detection limit, allowing for ESI-MS/MS analysis of trace amounts of compounds [2]. Recently we have investigated the application of such procedure in combinatorial chemistry, in analysis of OBOC peptides libraries. Here we report further improvements of this method.

**Method:** Combinatorial library was synthesized manually using split-and-mix method, on the solid support (TentaGel® HL NH2 resin, 0.56 mmol/g) according to a standard Fmoc synthesis procedure. QAS were prepared on solid support after the peptide synthesis. Iodoacetic acid was coupled to the N-terminal amino acid residue and ε-amino group of lysine in the presence of diisopropylcarbodiimide. Then 1,4-diazabicyclo[2.2.2]octane (DABCO) was added. Enzymatic digestion was performed in 0.01 M ammonium bicarbonate buffer for 24 h. ESI-MS/MS experiments were performed on an FT-ICR (Fourier Transform Ion Cyclotron Resonance) MS Apex-Qe Ultra 7T instrument (Bruker Daltonics, Germany) equipped with standard ESI source.

**Preliminary Data:** Our previous investigations showed synthesis and application of cleavable linker for OBOC combinatorial libraries, composed of lysine with ε-amino group labeled by the *N,N,N*-triethylglycine salt, and methionine, which allows selective cleavage by cyanogen bromide. This method has two major limitations. First, the use of methionine for selective peptide cleavage from the resin by cyanogen bromide eliminates this residue from the library pool. Second, to allow for hits identification by Kaiser test, the library peptide sequences need N-terminal acetylation, however, this makes the MS analysis of the fragment released after enzymatic digestion problematical. Therefore we decided to mark both N- and C-terminus of the peptide by QAS. The introduction of the fixed charge tag on N-terminus allowed for identification of proteolysis products in supernatant. We also developed a new linker cleavable by the Edman degradation method, which contains lysine residue with α-amino group blocked by Boc, whereas the ε-amino group is used for peptide chain assembly. Deprotection of the α-amino group followed by reaction with phenyl isothiocyanate and acidolysis allowed efficient cleavage of a peptide from the resin (Fig. 1). To our best knowledge the Edman degradation has not been previously described in literature as a cleavage method of peptides from a solid support. The ESI-MS/MS analysis of peptides cleaved from a single resin bead using Edman degradation shows signals corresponding mainly to the undigested peptides. The presence of two fixed charge tags located on the opposite sides of peptide results in b and y ion type series (Fig. 2).

**Novel Aspects:** Novel approach to OBOC peptide combinatorial libraries: Edman degradation for peptide cleavage and 1-azonia-4-azabicyclo[2.2.2]octane markers for detectability by ESI-MS/MS.

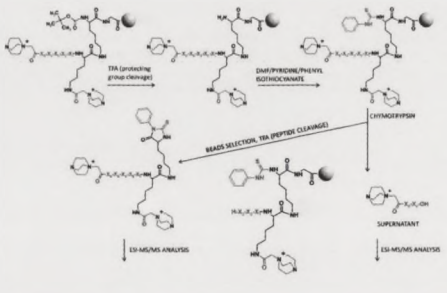


Fig. 1. New strategy for OBOC combinatorial library synthesis and analysis. Gray ball indicates a single resin bead

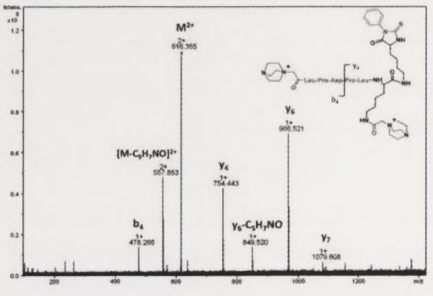


Fig. 2. ESI-MS/MS spectrum of QAS-peptide cleaved from a single resin bead using Edman degradation. The parent ion was 616.355 $[M]^{2+}$

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# Complex formation of the fungicide tebuconazole with zinc and cadmium ions

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Fungicides are bioactive compounds basically foliar applicable. Their application may have side effect onto the chelation or the stabilization of essential/hazardous metals in soil [1, 2]. There exists a risk of enhanced uptake of hazardous metals by the fungicides and also fungicides can stay in crop longer than that corresponding to their biodegradability. This study deals with the formation of complexes of tebuconazole (Teb) and metal salts ($Zn^{2+}$, $Cd^{2+}$) in model solutions using differential pulse anodic stripping voltammetry (DPASV) and in the gas phase using electrospray ionization mass spectrometry (ESI-MS).

**Method:** The measurements were preformed on a Finnigan LCQ Advantage ion-trap mass spectrometer fitted with an electrospray ionization source operated in positive-ion mode. Mixtures of tebuconazole and a metal salt in equimolar concentration ($5 \cdot 10^{-5}$ mol $L^{-1}$) were introduced into the ion source *via* direct sampling. The chosen parameters of ion source were optimal for each mixture. The DPASV measurements used an Eco Tribo Polarograph with the Polar 5 software. The measured solutions consisted of 0.1 mol $L^{-1}$ KCl in deionized water, $MCl_2$ in a concentration of about $10^{-7}$ mol $L^{-1}$ and tebuconazole, which was add to concentrations from $10^{-7}$ mol $L^{-1}$ to $10^{-4}$ mol $L^{-1}$. The accumulation time of metal on mercury drop was 1 min.

**Preliminary Data:** Upon ESI-MS, both metal salts and Teb create three types of complexes: $[MCl(Teb)_n]^+$ ($n$ = 1-3), $[M(Teb)^m]^{2+}$ ($m$ = 2-4), $[M(Teb)_k(Teb\text{-}H)]^+$ ($k$ = 1, 2). The CID spectra of the mass-selected complexes reveal similar dissociation pathways for both metals, while the appearance energies [3] of analog Zn and Cd complexes differ by 20 to 30 kJ $mol^{-1}$. In contrast to the ESI-MS results, the electrochemical behaviors are different for Zn and Cd. Zn and Teb create inert complexes with relatively high stability constants, whereas the complexes of Cd are relatively labile complexes with small stability constants.

**Novel Aspects:** The study helps to understand of different behaviour of tebuconazole in complexes with metals.

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# Mass Spectrometers as a Powerful Tool for Stability Studies and Identification of Degradation Products of Tetracyclines

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People are exposed to negative or even harmful effects caused by pharmaceuticals, their metabolites and degradation products, which occur in the environment. Problems like drug resistance of humans and formation of new strains of bacteria more resistant to available drugs, are becoming more common. Tetracyclines (TCs) are mostly used in human and veterinary medicine in prevention and treatment of bacterial infections. They are very unstable and they start to degrade within a few days. During degradation processes such as dehydration, isomerization, epimerization and proton transfer, many compounds are formed. The aim of the study was determination of TCs' stability and identification of degradation products of tetracycline (TET), doxycycline (DOX) and oxytetracycline (OXY) using LC-MS/MS and LC-QTOF-MS.

**Method:** Tetracyclines methanol solutions were stored in −80, −20, +4 and +25°C in order to investigate their stability. Degradation solution were stored in +25°C in a lighted room for over 200 days. Determination of TCs' stability was performed on LC-MS/MS and identification of degradation products on LC-MS/MS and LC-QTOF-MS in positive and negative ion mode. The separation of analytes was carried on XTerra MS C18 column (30 mm × 2.1 mm; 3.5 μm). Two different types of mobile phases were used. In case 1 the mobile phase was A: 5% ACN in ultrapure water and B: ACN both containing 0.1% (v/v) TFAA (for stability studies) and in the second case – mobile phases were the same as in case 1 without acid (for identification of degradation products).

**Preliminary Data:** The stability of tetracycline antibiotics has been investigated by LC-MS/MS. TCs are strongly polar compounds, being prone to degrade what can be easily observed by the change of the solution color from yellow to brown. The stability studies were lasting for 150 days. The stability results show that the most unstable antibiotic is tetracycline. After storage for 30 days in any conditions TET degrade in almost 50%. However, storing any of the TCs with addition of TFAA provide longer stability. Addition of TEA to the methanol solutions significantly accelerates the degradation process (in Day 0 suspected concentration was a few times smaller). This study showed that tetracycline solutions can be analyzed over a particular period of time without a significant loss of TCs. However, decrease of tetracyclines concentration during stability studies is not a proof of degradation. Only identification of degradation products confirms the statement of degradation (not any other loss of analyte). During degradation tetracyclines are known to subject to such processes as dehydration, isomerization, epimerization and proton transfer. TCs have a common chemical 2-naphthacenecarboxamide structure what make them susceptible for epimerization of dimethylamine group during degradation. After the storage of each tetracycline solution in +25°C in a lighted room for over 200 days degradation products of each tetracycline have been investigated by LC-QTOF-MS. Analysis of fresh and degraded solutions enable identification of known degradation products. In case of TET such compounds as 4-epitetracycline (ETET), anhydrotetracycline (ATET) and 4-epianhydrotetracycline (EATET) have been founds. Analysis of DOX solution confirmed the presence of metacycline (MTET) and demeclocycline (DMC). In case of OXY, 4-epioxytetracycline (EOXY), α and β-apo-OXY were identified. However, a few other unknown compounds have been identified as degradation products and their structures will be presented. The high resolving power of QTOF made it possible to identify different compounds.

**Novel Aspects:** Stability studies and identification of known and unknown degradation products of tetracyclines with usage of MS/MS and QTOF-MS.

# Analysis of human plasma fibrinogen N-homocysteinylation using chemical and mass spectrometric methods

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Homocysteine (Hcy) is sulfur containing amino acid. It is formed from methionine as by-product of the methylation of other amino acids, DNA, RNA, etc. Homocysteine released from cell can be further converted to cysteine by trans-sulphuration or back to methionine by remetylation process [1]. In the absence of cofactors of enzymes catalyzing mentioned transformations – folic acid and vitamins B6 and B12, accumulation of homocysteine in the blood plasma occurs. Homocysteine accumulated under these conditions may be converted into cyclic thiolactone of homocysteine, which due to the high reactivity may react with ε-amino groups of lysine residues in protein chains. Such modifications of proteins have substantial impact on their physico-chemical properties resulting in various pathological conditions – usually hazardous to human health [2].

**Method:** Analysis was aimed both for the location of residues undergoing modification of lysine as well as quantitative determination of plasma fibrinogen modification in individuals with hyperhomocysteinemia. The first stage of the research was optimization methods of sample enrichment. Fibrinogen was modified in vitro by incubation with homocysteine thioloactone in phosphate buffer (pH 7.4). Reaction was carried out for 17 h in 37°C. Protein was further digested with trypsin using standard protocol. Enrichment of modified peptides was conducted based on the reaction of formation of cyclic 1,3-thiazine with aldehyde groups bound to chromatographic resin and the thiol group of homocysteine in the chain attached to lysine residues in fibrinogen peptides. Peptides were analyzed using mass spectrometry methods, MALDI-ToF and LC-MS/MS.

**Preliminary Data:** In presented research we utilized earlier developed in our group approach to localize N-homocysteinylation sites of proteins. To evaluate the homocyteinylation sites of human fibrinogen, standard protein was modified with homocysteine thiolactone *in vitro* and control samples were prepared as well. All analyzes of peptides derived from trypsin digestion were performed on mass spectrometers, both Maldi-ToF and LC-MS/MS systems were used for qualitative analysis and only LC-MS was applied for quantitation of modified peptides. Confirmation of N-homocysteinylation of predicted peptides was possible due to analysis of fragmentation obtained in tandem mass spectrometer (Q-ToF or Ion Trap MS). For our purposes fibrinogen was separated from human plasma samples and digested with trypsin. To fish out only N-homocysteinylated peptides new method using affinity chromatography on aldehyde resin was applied. Persons with high homocysteine level in blood were taken as donors and the correlation between level of fibrinogen homocysteinylation and concentration of homocysteine in blood was done. Confirmation of N-homocysteinylation of predicted peptides was possible due to analysis of fragmentation obtained in tandem mass spectrometer.

**Novel Aspects:** Obtained data allowed to determine the sites of fibrinogen Hcy modification and may be used to develop a diagnostic method.

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# Oligomerisation studies of modified A-beta peptides using mass spectrometry

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Alzheimer's disease (AD) is a progressive neurodegenerative disorder that affects an increasingly proportion of elderly population. The major characteristic of AD is the accumulation of high molecular weight aggregates and plaques in the brain, which comprise the ß-amyloid polypeptide, beta-amyloid (Aß), as a major component. Aß is a 39-43 aa peptide that is formed proteolytically from the large ß–amyloid transmembrane precursor protein (APP) and spontaneously aggregates to Aß–tangles and Aß–fibrils. Recently, the formation of Aß-oligomers has attracted particular interest, since oligomers have been suggested to be a key neurotoxic species

**Method:** For the Aß-oligomers studies by mass spectrometric proteome analysis Aß(1–40) was synthesized using solid phase peptide synthesis, Fmoc chemistry, and the crude peptide purified by HPLC [1]. In addition, two N-terminal prolonged sequences of Aß peptide were prepared; one with the native sequence of the APP proteine, SEVKM-Aß (1-40), and one witht the mutated form SEVNL-Aß(1-40). After synthesis and purification, the peptides were subjected to 5-8 days of incubation at 37°C [2], in 10 mM ammonium acetate buffer, at various pHs, in order to yield oligomers. Further characterization of the aggregates was performed by Tris-Tricin Polyacrylamide Gel Electrophoresis.

**Preliminary Data:** The MALDI-TOF mass spectrometric analysis ascertained the successful synthesis and oligomerization of the peptides. Furthermore, the oxidation product of Aß-Met36 was evidenciated, together with the methionine oxidation of the prolonged Aß peptide.

**Novel Aspects:** N-terminal prolongued Aß peptide oligomerisation.

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# Mass Spectrometric Studies on N-phosphorylation of Ubiquitin

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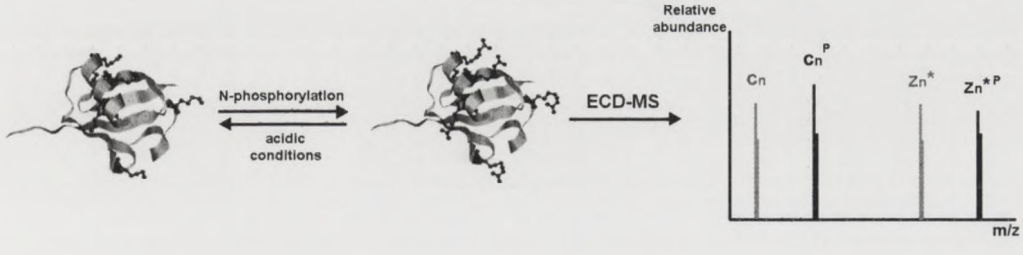
Post-translational modifications of proteins significantly increase the variability of proteome, which makes the analysis of cellular proteins more complicated and requires the use of appropriate techniques to determine the type of occurring modification. Protein *N*-phosphorylation is a post-translational modification of basic amino acid side chains characterized by high instability, which may cause its loss in the analysis using standard techniques and be the reason for lack of knowledge in this area. In recent years the usefulness of mass spectrometry to study this type of modification has been demonstrated (for phosphohistidine [1, 2], and phospholysine [3]). Herein we present the results of our experiments on chemical *N*-phosphorylation of protein – ubiquitin, using ECD-FT-ICR-MS technique to confirm the localization of phosphorylation site.

**Method:** *N*-phosphorylation reaction, both for ubiquitin and for ubiquitinous peptide $UBQ_{59-74}$ YNIQKESTLHLVLRLR, was performed using protocol previously described [3], where monopotassium phosphoramidate was used as a phosphorylating agent. All MS experiments were performed on an Apex-Qe Ultra 7 T instrument (Bruker Daltonics) equipped with a dual ESI source and operated in the positive ion mode. After the calibration with the Tunemix mixture (Bruker Daltonics) the mass accuracy was better than 5 ppm. The obtained mass spectra were analyzed using Biotools software (Bruker Daltonics) to find the fragmentation ions, which contained the phosphoramidate moiety (80 Da).

**Preliminary Data:** The experiment of top-down sequencing of N-phosphorylated protein was performed on ubiquitin, which has eight possible *N*-phosphorylation sites located on the protein surface. The phosphorylation of the protein was performed applying four different weight ratios of protein to phosphorylating agent: 1:5, 1:20, 1:40, 1:100. Only in the case of 1:100 ratio, the significant amount of *N*-phosphorylated product, judged on the basis of the abundance of the peak in ESI-MS spectrum corresponding to the adduct of 80 Da, was observed. To answer the question about the regioselectivity of reaction we performed an ECD-MS experiment on the +10 ion of monophosphorylated ubiquitin $[M^P + 10H]^{10+}$. 100% of the protein sequence was covered, which allowed to localize the phosphorylation site. Surprisingly, only the $His_{68}$ residue was found to be *N*-phosphorylated. No other fragmentation ions containing phospholysine residue (one of the seven possible) have been found. In summary, application of ECD fragmentation method to the top-down sequencing of N-phosphorylated proteins may be useful for analysis of phosphorylation sites without loss of the modification during experiment. We also performed an experiment on the model peptide, $UBQ_{59-74}$ YNIQKESTLHLVLRLR, which is the C-terminal fragment of ubiquitin. In this fragment there are two possible phosphorylation sites – on $Lys_{63}$ and $His_{68}$. We decided to investigate whether a change of the phosphorylating agent concentration and increasing the pH of the solution may influence the degree of phosphorylation of $UBQ_{59-74}$ and thus, if the lysine residue is available for the modification. On the basis of our results it might be concluded that changing the reaction conditions can affect the degree of phosphorylation of the sample, allowing modification of lysine residues in the analyzed model.

**Novel Aspects:** Electron capture dissociation mass spectrometry as a method for analysis of *N*-phosphorylation of proteins.

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# Lithium salt solvation in organoelectrolytes

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Primary lithium-air cells are currently thought to be excellent candidates for high-performance batteries for electric vehicles. Organocarbonates with Li salts are popular electrolytes for $Li/O_2$ system. We aim to investigate solvation of $LiPF_6$, $LiBF_4$ and LiBr with dimethylcarbonate (DMC), ethylenecarbonate (EC) and propylenecarbonate (PC). Solvation alongside with contact ion pairs (CIPs) formation and clustering are important factors for electrolytes' conductivity.

**Method:** Ion mobility spectrometry coupled with mass spectrometry (IMS-MS) is used to obtain information about CIP formation and solvation. Ion formation applies electrospray ionization of dilute solutions of lithium salts in methanol with added organocarbonates. Data were acquired on Waters Synapt G2 HDMS. Chemicals were purchased from Sigma-Aldrich (Prague).

**Preliminary Data:** As a first reference, we have measured electrospray ionization mass spectra LiBr, which show high levels of CIP formation and clustering with the solvent. In contrast, clustering and CIP formation are much less pronounced in the cases of $LiPF_6$ and $LiBF_4$, thus providing mass spectrometric evidence for better performance of these electrolytes in battery applications.

**Novel Aspects:** Occurrence of clustering as a molecular parameter for conductivity. Use of IMS for the study of organoelectrolytes for Li/air batteries.

# Fragmentation of chromophore labelled oligosaccharides using Vis-PD

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Carbohydrates undergo two different types of fragmentation: glycosidic cleavages and cross-ring cleavages. Using low energy vibrational excitation techniques such as Infrared Multiphoton Dissociation (IRMPD) glycosidic cleavage is the predominant fragmentation pathway. Higher energy excitation methods promote additional cross-ring cleavages. In addition to IRMPD various Photodissociation (PD) methods are used for fragmentation of carbohydrates [1, 2]. The PD-experiments reveal diverse fragmentation patterns. Using VUVPD a variety of cross-ring fragments can be identified [2], using UVPD the glycosidic fragments are predominant but additional cross ring cleavages can be observed [1]. In this study, the application of Vis-PD at 457 nm, 488 nm and 514 nm for chromophore labelled oligosaccharides in an FT-ICR mass spectrometer is described.

**Method:** The experiments were performed on an APEX Qe FT-ICR Mass Spectrometer, equipped with a 9.4 T cryomagnet and an Apollo II Combi source, ESI/MALDI version (Bruker Daltonics, Bremen). Electospray Ionisation (ESI) was used in positive and negative-ion mode. Vis-PD experiments were performed with an argon-ion-laser Innova 70c (Coherent, Santa Paula). The fragmentation took place in the ICR Infinity cell. The wavelength 457 nm, 488 nm and 514 nm were applied for PD-experiments. The laser power was adjusted between 0.2 and 2 W and the irradiation time was set between 0.2 and 5 s. The derivatisation was carried out using reductive amination.

**Preliminary Data:** Different labels were attached to Maltopentaose (MP) and the isomers *Lacto*-N-Fucopentaose I and II (LNFP I and LNFP II). Rhodamine 110 and 2-Aminoazotoluene offer adequate derivatisation and fragmentation properties. In the positive ionisation mode the protonated and the sodium cationised oligosaccharides were fragmented by Vis-PD. In negative ion mode the deprotonated species were analysed. In the positive ion mode the fragments of the Maltopentaose-derivate include the reducing end of the sugar. Only glycosidic cleavages (Y type series and Z1 fragment) are monitored. In negative ion mode oligosaccharides show a complementary fragmentational behaviour. Sugar fragments from the non reducing end appear in the spectrum and in addition to glycosidic cleavages (B-, C-, Y- and Z-type) various cross ring cleavages (A and X type) occur. The posESI-Vis-PD spectra show little differences between the isomers LNFP I and II, but in the negative ion mode mayor differences are revealed. The 6-Aminoquinoline derivate of LNPF I was analysed applying Vis-PD as well. It could be demonstrated that the chromophore label influences slightly the fragmentational behaviour of the oligosaccharide. The use of sodium adduct ions as precursors causes major effects on the fragmentation.

**Novel Aspects:** Application of Vis-PD at 457 nm, 488 nm and 514 nm for chromophore labelled oligosaccharides in an FT-ICR mass spectrometer.

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# MeCAT – Analysis of labeled proteins by HighMass-Q-TOF-MS

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The development and application of different strategies for quantitative proteomics is a rapidly growing field in mass spectrometry. Metal Coded Affinity Tagging (MeCAT) that can be used in quantitative proteomics, uses chelate complexes of lanthanides for relative and absolute quantification. Recently, we introduced the new MeCAT-IA reagent, which shows some distinct advantages over the previously used MeCAT-Mal [1]. Here, we present a general characterization of MeCAT-IA-labeled proteins using a high mass quadrupole time-of-flight mass spectrometer (QTOF MS).

**Method:** Model proteins are labeled with the MeCAT-reagent that contains a cysteine-reactive group for quantitative labeling and an elemental tag loaded with a lanthanide ion for quantification. To separate labeled proteins from other sample components HPLC-purification was applied. Analyses were performed on a modified Waters Micromass nano-ESI-TOF MS (MS Vision), equipped with a nano ESI source.

**Preliminary Data:** The analysis of MeCAT labeled proteins on a High Mass Q-TOF instrument allows the detection of labeled proteins via mass spectrometry for the first time. Compared with unlabeled proteins, labeled proteins show the expected mass shifts due to the MeCAT-tag, while the signals from differential labeled proteins could be distinguished.

**Novel Aspects:** We show here an additional possibility to evaluate the MeCAT-labeling of proteins on protein level.

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# MeCAT – Fragmentation behavior of labeled peptides and relative quantification

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The development and application of different strategies for quantitative proteomics is a rapidly growing field in mass sepctrometry. On of the applicable methods is the use of Metal Coded Affinity Tagging (MeCAT) [1-2] that uses chelate complexes of lanthanides for relative and absolute quantification. Recently, we introduced the new MeCAT-IA reagent with iodacetamide reactivity, which shows some distinct advantages over the previously used MeCAT-Mal [3]. Here, we present a general characterization of the fragmentation behavior of MeCAT-IA-labeled peptides using collision induced dissociation (CID), electron impact dissociation (ECD) and infrared multiphoton dissociation (IRMPD).

**Method:** Peptides are labeled with the MeCAT-IA reagent, which contains a cysteine-reactive group for quantitative labeling and an elemental tag harboring a lanthanide ion for quantification. Complex mixtures of MeCAT labeled peptides, like protein digests, are chromatographically separated before analysis by ESI-MS and ESI-MS/MS. For differential labeling in case of relative quantification, MeCAT-IA reagents containing different lanthanide ions were applied and analyzed by HPLC/ESI-MS and ICP-MS.

**Preliminary Data:** Using fragmentation techniques (CID, ECD and IRMPD) unambiguous identification of labelled proteins on peptide level is still possible, as labelled peptides show similar fragmentation patterns as unlabelled peptides. Additionally characteristic fragments of the labelling group can be detected, thus allowing differentiation between labelled and unlabelled peptides, as well as, screening for labelled peptides. Differential labelling is achieved by application of different metals and offers the possibility of differential quantification with HPLC/ESI-MS. This is compared with data from ICP-MS analysis.

**Novel Aspects:** We show here a general characterization of the fragmentation behavior and demonstrate application of MeCAT-IA for relative quantification with HPLC/ESI-MS.

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# Identification of neuropeptides in insect neuro-endocrine system

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In insect, neuropeptides are a large, multifunctional group of substances with plejotropic activity. They regulate almost all physiological processes in the insect body, such as reproduction, development, growth, lipid and carbohydrates homeostasis. In recent years, due to development of new highly sensitive and performance separation techniques, such as high performance liquid chromatography (HPLC) and capillary electrophoresis (CE) combined with mass spectrometry, a huge number of new neuropeptides sequences have been found in the neuro-endocrine system of insects. Here we present the discovery of neuropeptides from two beetle species *Tenebrio molitor* and *Zophobas atratus*. In beetles, which are the biggest insect order, the knowledge about neuropeptides was scanty and limited to only few species.

**Method:** MALDI-TOF MS and MS/MS techniques were used to identify peptides in the methanolic extract of three major neuro-endocrine tissues: brain (B), retrocerebral complex *corpus cardiacum/corpus allatum* (CC/CA) and vetral nerve cord (VNC) of the beetle *Zophobas atratus*. Prior to the MS analysis the extracts were separated by high performed liquid chromatography. The peptides were also identified by MALDI-TOF MS in the ganglia of the ventral nerve cords of two beetles *Zophobas atratus* and *Tenebrio molitor* straight from the VNC dissected tissues without previous extraction. The presence of the peptides in the ventral nerve cord was confirmed by immunocytochemical method.

**Preliminary Data:** Analyses of HPLC fractions from both the CC/CA and B produced numerous mass ions, some of which had identical masses to known beetle peptides. The most abundant mass ions were fragmented for sequence analysis, although inadequate fragmentation of some peptides prevented sequence determination. We identified seven peptides in the *Z. atratus* CC/CA: Zopat-PK-1 (LPHYPRLa), Zopat-MS (pEDVDHVFLRFa), Zopat-MS-2 (pEDVEHVFLRFa), Tenmo-AKH (pELNFSPNWa), Tenmo-PK-2 (SPPFAPRLa), CCAP (PFCNAFTGCa), Zopat-NVP-4 trunc. (GRWGGFA). Two of them Zopat-PK-1 and Zopat-MS-2 are new hormones, for the first time identified in insects. Four peptides were identified in the beetle brain: Zopat-SK-1 (pETSDDYGHLRFa), Zopat-MS (pEDVDHVFLRFa), Zopat-NVP-4 trunc. (GRWGGFA), Zopat-MIP-4 (NWGQFHGGWa) and three in the VNC: proctolin (RYLPT), Zopat-MS-2 (pEDVEHVFLRFa) and Zopat-NVP-4 trunc. (GRWGGFA). Direct tissue profiling of ventral nerve cord ganglia showed presentence of two previously identified in the whole VNC extract peptides proctolin and Zopat-MS-2 in two beetles *Z. atratus* and *T. molitor*. These peptides were also identified by immunocytochemical method.

**Novel Aspects:** Identification of the neuropeptides in the neuro-endocrine system of two beetle species *Zophobas atratus* and *Tenebrio molitor*.

# Human cell-line phosphoproteome mapping using a dual-funnel ETD ion trap

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The essential regulatory mechanism in eukaryotic cell are driven by kinases. They affect the information flow through the signaling processes and consequently exert an influence on cells, tissues and organisms phenotype and functions. Altered regulations of these processes are observed in a variety of diseases, among which are many cancers. Therefore, detecting, characterizing and ultimately quantifying the phosphproteins altered in these pathologies is an important step towards a better understanding of the implicated processes. The low concentration level and ionization efficiencies make it difficult to analyze them directly by mass spectrometry in mixture with other cellular proteins at their natural ratio. We are presenting the results obtained after human cell line IMAC phosphopeptide enrichment followed by an ESI CID/ETD Auto MSn experiment.

**Method:** Cell lysate from Human Raji B cell has been subjected to gel-assisted digestion. Subsequent phosphopeptide purification was performed as previously reported [J. Proteome Res. 2008, 7, 4058-4069], using an IMAC column capped at one end with a 0.5 µm frit disk enclosed in a stainless steel column-end fitting.Tryptic peptides were reconstituted in loading buffer and loaded onto the IMAC column.Automated purification of phosphopeptides has been performed using ACQUITY UPLC™ (Waters, Milford, MA). Eluted peptide samples were vacuum dried, reconstituted in 0.1% (v/v) TFA (40 µL) desalted and concentrated using ZipTipsTM (Millipore, Bedford, CA). LC-MS/MS alternated CID/ETD acquisition has been performed on a dual-funnel amZon ETD ion trap (Bruker)coupled to an Ultimate 3000 nano-LC

**Preliminary Data:** The analyses led to the identification of 95 proteins, 88 of them being identified in each of the three runs. Among these, 93 are phosphoproteins and 87 of them representing 220 phosphorylated peptides have been found in the three replicates. The acquisition speed as well as the high quality of the obtained CID and ETD spectra combined with the use of dedicated bioinfomatics has enabled to map efficiently the phosphorylation sites for most of these peptides. We are now ready to use this technique on a larger scale to proceed to a more systematic mapping of the phosphoproteins present in our sample.

**Novel Aspects:** Human cell-line phosphoproteome mapping by combined ETD and CID in a trap.

# Identification of more than 3000 Proteins in Single LC/MS/MS Runs from a Cell Line Lysate

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A distinct goal in proteomics is still the survey of an entire cell lysate with a maximum number of unambiguously identified proteins. While high resolution instruments are favored for this application, recent improvements in both MS instrumentation as well as in nanoLC separation lead to a much higher number of identified proteins from such a sample in single LC/MS/MS experiments in general. Important criteria for a successful analysis are a high dynamic range, fast MS/MS and good mass accuracy. Results for various mass spectrometer platforms are demonstrated.

**Method:** Human colon adenocarcinoma cell line was trypsin digested and separated by nanoLC (Dionex Ultimate 3000 RSLC nano or Bruker Easy-NanoLC) with Dionex PepMap C18, 150-500 × 0.075 mm analytical column at a flow rate of 300 nL/min with eluent A / B: 0.1% FA in water, 80% ACN 0.1% FA. The nanoLC was coupled online either to the maxis impact QTOF or the amazon speed ion trap was done. For MALDI, fraction collection was done on a Proteineer fc II (Bruker) in 1360 fractions, 10 s each, on a Bruker MTP AnchorChip 1536 TF. The database search was done in NCBI database using Mascot (Matrix Science). Data validation was done by ProteinScape (Bruker).

**Preliminary Data:** Very restrictive criteria were applied in the database search to largely avoid false positive hits. The peptide identity threshold was set to be at least 35, the peptide false discovery rate to FDR < 1%. The list of hits was further screened for multiple or redundant proteins found for the same peptides which were eliminated. More than 12,000 peptides are identified in single runs, resulting in more than 3000 identified proteins, e.g. for the maxis impact, at a FDR < 1%. Merging the data for the maxis and the ultraFlextreme, which is partially complementary due to the different ionization techniques, leads to a 20% increase in identified proteins. All different platforms demonstrate the strength of the individual technologies as well as the advantage of merging data from different ionization modes.

**Novel Aspects:** New instrumentation for identification of 3000 proteins in single LC/MS/MS runs.

# Autoproteolytic Synuclein–αfragments of the intrinsically disordered Parkinson protein might be a key to the elucidation of its oligomerization-aggregation mechanism

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The intrinsically disordered protein alpha-Synuclein (aSyn) is known to be implicated in both idiopathic and inherited forms of Parkinson´s disease (PD). The pathological hallmark of sporadic PD is the formation of intracyoplasmic fibrillar deposits in dopaminergic neurons (Lewy bodies) in the substantia nigra. The 140 amino-acid aSyn consists of three main domains. The hydrophobic NAC domain has a critical role for aggregation, especially the hydrophobic amino acid stretch Val71-Val82. However, how aSyn is involved in the pathogenesis of neurodegenerative disease is not understood at present. Here we report the first biochemical and mass spectrometric structural characterization of autoproteolytic aSyn fragments and its aggregation properties.

**Method:** Recombinant expressions of full-length aSyn and aSyn polypeptides were performed using the *E. coli* expression system BL 21 (DE3) [pLys] strain and the T7 RNA polymerase system. Harvested cells were centrifuged, resuspended in PBS, and heated for 2 min to 100°C; the cell suspension was centrifuged, and the protein was redissolved in PBS. aSyn polypeptides were purified by RP-HPLC and the purity was verified by ESI-MS. Purified aSyn was incubated at 37°C with agitation up to 25 days. Separations were performed by Tris-Tricine PAGE and protein bands were visualized by Coomassie Blue and silver staining. Proteins were extracted by passive elution and characterized by MALDI-MS as well as transferred to a PVDF membrane, and N-terminal sequences determined by Edman sequencing.

**Preliminary Data:** Application of IMS-MS to oligomerization-aggregation mixtures of aSyn *in vitro* recently provided the first identification of specific autoproteolytic truncation and degradation products that were previously observed by gel electrophoresis, but not identified. In particular, a highly aggregating fragment was identified by cleavage between Val71 and Thr72 in the central aggregation domain of aSyn. Aggregation studies of the carboxy-terminal fragment, aSyn (72-140) prepared by both chemical synthesis and recombinant expression, showed a substantially faster fibrillization compared to the intact protein [1]. The *in vitro* oligomerization of aSyn was investigated by incubation at 37°C in sodium phosphate buffer (pH 7.5) for up to 25 days. The formation of oligomers was monitored by Tris-Tricine PAGE. In addition, protein bands with molecular weights lower than that of full-length aSyn were observed, with slowly increasing abundances over two weeks of incubation, thus indicating the formation of truncation and/or degradation products. These bands corresponding to truncation and/or degradation products of aSyn were excised from the gel and the polypeptide extracted by passive elution, and analyzed by MALDI-MS. Comparative *in vitro* studies of the non-aggregating brain protein, beta-Synuclein (bSyn), which lacks the central amyloidogenic domain (72-83), showed neither oligomerization-aggregation nor any autoproteolytic cleavage within 21 days of incubation; identical expression systems and HPLC purification procedures were employed for bSyn and aSyn, thus excluding aSyn degradation by an external protease. Further results provided evidence to exclude a proteolytic cleavage of aSyn by contaminating proteases: Incubation of aSyn with and without addition of a broad spectrum of protease inhibitors yielded identical autoproteolytic degradation.

**Novel Aspects:** Biochemical evaluation of autoproteolytic products is expected to provide a key to elucidate the oligomerization-aggregation mechanism of aSyn underlying its neurotoxicity.

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# Clinical diagnostic of Fabry disease in dry blood spots by fluorometry and multiple reaction monitoring mass spectrometry

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The deficiency of lysosomal enzyme activity is a characteristic feature of lysosomal storage diseases (LSDs), a group of mainly genetic metabolism disorders (e.g., α-Galactosidase, Fabry's Disease; β-Glucocerebrosidase, Gaucher's Disease). The substrates can no longer be processed and are accumulating in the lysosome causing severe disease symptoms leading to multiple organ failure and finally death. For Fabry's Disease (FD) enzyme replacement therapy has become available with high success, thus rendering rapid and efficient diagnosis of key importance. In the present study we have developed and established specific and highly sensitive diagnostics tools using mass spectrometry multiple reaction monitoring (MS-MRM).

**Method:** A fluorometric determination for monitoring of α-galactosidase, β-galactosidase and β-glucuronidase activity was developed as a control assay. A diagnostic study was performed using the dry blood spot (DBS) method with samples from ca. 100 healthy controls and 8 Fabry patients. Tandem mass spectrometry provides not only identification of the product, but also a quantitative determination by introducing a suitable internal standard. A novel assay has been developed by adaptation of the mass spectrometric determination, reducing the time for sample preparation and MS/MS.

**Preliminary Data:** The MS-MRM diagnostics of FD was carried out using a special mixture of substrate and internal standard. Substrate used for enzymatic determination of alpha-galactosidase was (6-benzoylamino-hexyl)-{2-[4-(3,4,5-trihydroxy-6-hydroxymethyl-tetrahydro-pyran-2-xyloxy)-phenyl carbamoyl]-ethyl}-carbamic acid tert-butyl ester (SGLA) ($C_{33}H_{47}N_3O_{10}$; MW 645.7 Da) and the expected product (6-benzoylamino-hexyl)-[2-(4-phenylcarbamoyl)-ethyl]-carbamic acid tert-butyl ester (PGLA)($C_{27}H_{37}N_5O_5$; MW 483.6 Da). The internal standard used was deuterated PGLA($C_{27}H_{32}N_5O_5D_5$; MW 488.6 Da). A typical healthy control sample in the MRM-MS assay gives an average substrate conversion of 6.6 mM /L/ 48 h (2,13 nmols/spot/48 h). A DBS sample collected from a male FD patient yielded an activity < 0,28 nmols/spot/48 h).

**Novel Aspects:** Diagnosis of Fabry`s Disease was resolved using two different techniques based on enzymatic activity of alpha-galactosidase.

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# $CO_2$-Laser Atmospheric Pressure Ionization of Acoustically Levitated Droplets

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Once designed for zero gravity experiments, in recent years levitation devices have experienced a renaissance in microfluidics where they serve as wallless reactors and for contactless sample handling. Until now many optical analytical techniques have been employed to interrogate the contents of levitated droplets, however only one approach towards a coupling to mass spectrometry has been successfully conducted utilizing a combination of corona charging, pulsed high voltage electrodes, MALDI matrix addition to the droplet and desorption with an UV-laser [1].

**Method:** In the presented work we used a single $CO_2$ laser pulse for vaporization and ionization out of previously uncharged droplets following the approach of $CO_2$ MALDI proposed by Hillenkamp 20 years ago [2]. We used water as solvent, glycerol as IR chromophore and amino acids and small peptides as analytes. The droplets were levitated with a home built ultrasonic trap which was set in front of the inlet tube of an atmospheric pressure time-of-flight mass spectrometer (Tofwerk HTOF) in positive ion mode. The $CO_2$ laser (Coherent Diamond C-55A) was gated to ~20 ms by a mechanical shutter. In order to visualize the laser induced vaporization and plume formation, the experiments were accompanied by high repetition rate shadowgraphy.

**Preliminary Data:** The resulting spectra resemble results expected for MALDI conditions and show no fractionation products. This soft ionization is attributed to the low energy coupled to the system by individual photons dictating mere dynamics in the electrical ground state. The observed ions therefore corroborate the lucky survivor mechanism postulated by Karas et al. [3]. This assumption is confirmed due to the fact that only basic analytes such as amino acids and peptides which occur as precharged species produce significant signals. The obtained spectra show also a strong dependency on changes of pH regarding the observed ions and their total and relative intensities. Even though charged droplets are involved no multiple charging of the analyte as would be expected for an Electrospray ionization could be observed. Besides the hydrogenated analyte peaks a strong signal contribution from glycerol and its clusters was observed.

**Novel Aspects:** A simplified ionization scheme for levitated droplets was established. Besides its direct practical aspect it helps understanding classical ionization mechanisms.

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# Digging into MS data: High throughput MS-informatics based on Python

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Mass spectrometry techniques are used in a wide variety of research fields. The amount of data measured can easily exceed several Gigabytes per day and per machine. Conclusions drawn from such large data sets, however, depend on the versatility of the analyzed algorithms and the combination of those into larger workflows or pipelines. As such, the analysis of large amounts of MS data requires a very specialized high throughput informatics support, i.e. MS-informatics.

**Method:** A MS-informatics toolbox based on the Python scripting language: The pyMS-informatics toolbox is presented here. It can be used to easily enhance the quality of MS data through a series of Python modules. For example, through LC-MS alignments in the time domain, one can achieve higher coverage of MS/MS free identification based upon what is known as accurate mass time tags. The result of several hundred RT-alignments is a peptide Library that, for example, can be used to identify biomarkers. Alignments in the intensity domain allow label free quantification.

**Preliminary Data:** Although the tool box is currently being developed in our laboratory, we could already successfully apply our techniques in collaborations (see Höhner et al. 2012 (manuscript in prep.), Barth et al. 2012 (manuscript in prep.)) and publish the interface between the Python scripting language and mzML files, the pymzML module [1].

**Novel Aspects:** high throughput MS-informatics.

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# High Resolution in Mass and Space: AP-MALDI Imaging using Orbitrap-based Instrumentation

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MALDI Mass Spectrometry Imaging (MSI), 17 years after its first announcement [1], has turned into a routine method of highest performance for the molecular histology of biological tissue [2]. The method, providing high resolution and accuracy in mass and space, has been developed for a reliable identification and localization of individual tissue components, and has been applied recently to a number of research areas in pathology, cancer diagnostics, metabolic pathway analysis and plant research. Targeted compound classes included phospholipids, peptides, proteins, drug compounds and metabolites. An atmospheric pressure ion source has been developed for these studies, based on a dedicated microoptical setup that provides highest spatial resolution of 3 to 5 micrometer on tissue in imaging mode on Orbitrap-based instrumentation.

**Method:** A coaxial ion source geometry was employed to irradiate, desorb, ionize and transport biological compounds under atmospheric pressure conditions. Ions are introduced into the mass spectrometer through an extended heated capillary. Transfer efficiency was optimized using both, gas stream dynamics and electrostatic ion focusing. The achievable mass accuracy during tissue imaging operation is typically better than 1 ppm RMS. Imaging speed is higher than 2 pixels per second, depending on mass range and chosen mass resolution. Matrix preparation and on-tissue protein digestion were performed with a new dedicated preparation robot. The presentation will focus on technical developments, including typical examples of applications for demonstrating the instrumental performances.

**Preliminary Data:** The method has been developed as a routine histological technique of high reliability, validity and performance. Combining high spatial resolution (down to 3 µm) with high mass resolution, high mass accuracy, high imaging mass selectivity, $MS^n$ capability and an improved sample preparation technology resulted in an unprecedented quality of imaging data in our experiments. So far phospholipids [2], peptides [3] and drug compounds [4] were imaged with the described setup, from a wide range of biological samples at high spatial resolution. Natural phospholipid diversity was found to be especially useful to represent distinct histological features. Tissue sections were stained (H&E or toluidine) after MS analysis. MS image analysis for all these cases showed excellent agreement with histological structures. Structural features invisible in classical histological staining could be visualized and identified on a molecular basis in our non-targeted MS imaging approach. High spatial resolution protein imaging was established by the development of a dedicated on-tissue digestion spraying device [5]. Results show that imaging mass spectrometry in particular requires highly accurate raw data from FT (orbital trapping or ICR) instruments, whereas low-accuracy data contain a high risk of creating non-reliable, invalid, non-authentic or ambiguous information in imaging experiments of complex biological tissue samples. Fundamental and technical aspects of high performance AP-MALDI imaging, its properties and its prospects will be described, including optical focusing, ion formation and transfer at atmospheric pressure, image acquisition, data analysis and substance identification. The high specificity in mass and space of the method results in an unprecedented information quality and depth which can be advantegeous in a large number of clinical, pharmacological or fundamental applications in the future.

**Novel Aspects:** High performance MALDI imaging at atmospheric pressure using orbitrap-based mass spectrometry.

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# Identification of Carbohydrate-Contacting Peptides in Clinically Relevant Lectins by Proteolytic-Excision Mass Spectrometry

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The emerging physiological significance of carbohydrate (glycan)-protein (lectin) interactions direct increasing attention to the structural analysis of this contact (1). Using human adhesion/growth-regulatory galectins as model, we have developed a robust and reliable approach to identify sequence stretches in contact to the ligand. Combination of proteolytic excision and affinity-mass spectromentry yielded peptides reactive with cognate sugars, as ascertained by biosensor/cell assays.

**Method:** Experimentally, galectins were adsorbed to an affinity resin bearing cognate sugar and proteolytically digested. After washing out unbound galectin fragments, the fragments remaining on the column were eluted, and both fractions analysed by mass spectrometry. The identified CRD-peptides were synthesised by Fmoc-SPPS and their binding properties characterised by affinity-MS. The interactions of lactose with full-length galectins and with identified CRD-peptides were studied by SAW-bioaffinity-measurements and online-bioaffinity-coupling of SAW with ESI-MS. The lactose was immobilised on bioaffinity-sensor-chips in the form of a glycopeptide. SAW measurements were performed with an S-Sens K5 Biosensor instrument in PBS buffer and elution was performed with ACN: 0.1% TFA 2:1 and 10% acetic acid.

**Preliminary Data:** CREDEX-MS of galectins identified at least two carbohydrate-binding peptides for every galectin - in complete agreement with the binding sites of the crystal – or molecular modelling structures. All identified carbohydrate-binding peptides were synthesized and their affinity for lactose demonstrated by affinity-MS. To get more detailed information about the affinity and interaction kinetics of the CRD peptides and full galectins with lactose, SAW-bioaffinity measurements were performed. Some pmol Lactosyl-glycoprobe were immobilized on the SAW chip and dissociation constants of galectin peptides and galectins with lactose determined to be in the µM range. For a real-time study of carbohydrate-lectin interaction a newly developed online-coupling of SAW with ESI-Ion Trap MS was used. With this system the peptides interacting with on chip bound carbohydrate could be studied directly after the elution and it could be proved which peptides interact with the carbohydrate on the chip. This system was tested with synthetic CRD peptides and it was shown that online-coupling of mass spectrometry with the biosensor was successful. The presented results document the validity of the concept to obtain bioactive peptides from lectins (2). These bio-inspired peptides are becoming objects of detailed structural analysis and inspire their tailoring, e.g. by incorporating non-natural amino acids, as medically useful mini-lectins or lectin blockers. Surface acoustic wave measurements were shown as a working tool for determination of dissociation constants as well as in combination with ESI-Ion-Trap-MS for the study of carbohydrate-lectin-interactions.

**Novel Aspects:** Determination of intensity and characterization of peptide/protein-carbohydrate interactions in real time by SAW-bioaffinity analysis and online-coupling of SAW with ESI-IonTrap-MS.

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# *In vitro* Formation, Detection, and Identification of Lysine-derived AGEs

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Glycation (non-enzymatic glycosylation) refers to a chemical reaction of reducing sugars with amino groups of peptides and proteins. Resulting sugar amines (Amadori and Heyns compounds) undergo slow degradation, oxidation and cross-linking reactions yielding advanced glycation endproducts (AGEs), known markers of diabetes, Alzheimer's disease and ageing. Reactive dicarbonyls (e.g. glyoxal and methylglyoxal) generated in processes of monosaccharide autoxidation and lipid peroxidation, are also known to be AGE-precursors [1]. Though AGE formation has been intensively studied by incubation of peptides and proteins with glucose or reactive carbonyls, the contribution of Amadori product degradation to the general AGE pool remains mostly unknown. The absence of reliable sequencing strategies for AGE-peptides is one of the main reasons for this.

**Method:** Therefore, degradation pathways of Amadori peptides were studied and the approaches to identify resulting AGEs were established and validated on the protein level. First, peptides were synthesized on solid phase using Fmoc/tBu strategy with Mtt-protected lysine at the desired glycation site and different acidic and basic residues in $i + 4$-position from the glycated amino acid. The peptides were postsynthetically glycated with α-D-glucose [2] or alkylated with *t*-butyl glyoxal and *t*-butyl pyruvate [3] to obtain carboxymethyllysine (CML)- and carboxyethyllysine (CEL)-peptides, respectively. RP-HPLC purification yielded Amadori and AGE peptides with high yields and purities. Amadori peptides were incubated in triplicates under conditions simulating the cooking process (95°C) for 0, 15, 30, 60, 120 and 240 min using the corresponding unglycated peptides as controls.

**Preliminary Data:** Subsequent RP-HPLC-ESI-QqTOF-MS analysis revealed that 95% of the Amadori peptide degraded within the first 30 min of incubation. Positively charged amino acids in $i + 4$ position from the glycated Lys residue enhanced while negatively charged amino acid delayed the degradation process. The main products were identified by ESI-MS/MS as unmodified and AGE-containing peptides. The latter were represented by the CML and CEL species. Three isomeric oxidation products (most probably regioisomers) were detected at the phenylalanine side chain. Their formation occurred significantly faster in presence of the degrading Amadori moiety than in the control samples. In order to identify CML- and CEL-containing peptides in enzymatic digests, fragmentation patterns of standard synthetic AGE-peptides were analyzed by ESI-QqTOF- and -LTQ-Orbitrap-MS/MS. The side-chain modifications proved to be stable under CID conditions. Thereby the corresponding sites could be unambiguously identified by characteristic mass increments. The same sequencing strategy was applied to human serum albumin (HSA) glycated *in vitro*. Following the removal of reagents by ultrafiltration, disulfide reduction and tryptic digestion, glycated HSA was analyzed by RP-HPLC-ESI-LTQ-Orbitrap-MS in an information-dependent acquisition (IDA) experiment with subsequent database search for carboxymethyl- and carboxyethyl-modified peptides. The results were proved by manual interpretation of the spectra. Thereby, multiple carboxymethylation sites were identified in glycated HSA and the abundance of corresponding peptides could be semi-quantitatively estimated.

**Novel Aspects:** Amadori peptide degradation and AGE formation were studied. MS/MS-based identification of CML and CEL was established on peptide/protein level.

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# Interaction Studies between Guanylylcyclase Peptides and GCAP-2 using (Photo-)Chemical Cross-Linking and ESI-LTQ-Orbitrap Mass Spectrometry

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Two isoforms of retinal guanylylcyclase (ROS-GC 1 and 2) localized in photoreceptor cells are responsible for the synthesis of cGMP, the second messenger in photo-transduction [1]. ROS-GC 1 and 2 are regulated in a $Ca^{2+}$-dependent manner by guanylylcyclase-activating proteins (GCAPs) [2]. GCAPs are hydrophobic N-terminally myristoylated proteins containing four EF-hand motifs, which are responsible for the sensitivity towards $Ca^{2+}$ [3]. Genetic disorders in GCAP genes or parts of the regulatory regions in ROS-GC genes lead to different malfunctions [4]. For the development of novel drugs a detailed molecular knowledge of complex formation between GCAPs and ROS-GC is crucial. Therefore, interaction studies were performed between a ROC-GC1 peptide and GCAP-2 by ESI-LTQ-Orbitrap mass spectrometry.

**Method:** Interactions between a ROS-GC1 peptide (amino acids 965-981 of ROS-GC [5]) and GCAP-2 were assayed using two complementary types of cross-linking chemistry. For determination of larger distances, the homobifunctional amine-reactive, isotope-labeled ($D_0$ and $D_4$) cross-linker bis(sulfosuccinimidyl)glutarate ($BS^2G$) was used. To determine closer interaction areas unnatural photoreactive amino-acids, which are activated by UV-A radiation, were incorporated into ROS-GC peptides. Photo-leucine containing a diazirine-function was used for the replacement of valine and leucine. Intact cross-linked complexes were analyzed by MALDI-TOF-MS. Cross-linking mixtures were separated by one-dimensional gel electrophoresis (SDS-PAGE). For determination of potential cross-linking products gel-bands of interest were excised and digested with trypsin and Glu-C. The peptide mixtures were analyzed by nano-HPLC (Dionex)/nano-electrospray ionization (ESI)-linear ion trap (LTQ)-Orbitrap-MS (LTQ-OrbitrapXL, ThermoFisherScientific).

**Preliminary Data:** The interactions between GCAP-2 and a ROS-GC1 derived peptide – presenting a potential GCAP-2 binding site – were studied by (photo-)chemical cross-linking in combination with high-resolution mass spectrometry. Using the amine-reactive cross-linker $BS^2G$, analysis of gel bands from reaction mixtures with and without $Ca^{2+}$ indicated that a complex between GCAP-2 and GC peptide had been created. In the presence of $Ca2^{+}$ several intramolecular cross-links were identified within N-terminally myristoylated GCAP-2, all of which were in agreement with the published NMR structure (pdb 1JBA) of non-myristoylated GCAP-2. The C-terminal part of GCAP-2, which is not resolved in the NMR structure, seems to be highly flexible as indicated by the numerous intramolecular cross-links within GCAP-2. Analysis of cross-linking samples without $Ca^{2+}$ yielded a limited number of intramolecular cross-linking products of GCAP-2 itself. Several cross-links between the N-terminus of GC peptide and different lysines of GCAP-2 were identified both in the presence and absence of $Ca^{2+}$. These cross-links did not allow us to clearly differentiate between GCAP-2/GC peptide interaction in the absence and presence of $Ca^{2+}$. We conducted further experiments with two GC peptides, in which the photo-reactive amino acid photo-Leu was incorporated either at position Val-3 or Leu-15. After photo-cross-linking, a limited number of cross-links were obtained pointing to a structurally well defined interaction between GCAP-2 and the GC peptide in the presence of $Ca^{2+}$. In the absence of $Ca^{2+}$ the structure of GCAP-2 appeared to be much more flexible as indicated by the large number of diverse photo cross-linking products between GCAP-2 and GC peptide. Based on the distance constraints imposed by the cross-links we were able to create a model of the complex between myristoylated GCAP-2 and the GC peptide that is formed in the presence of $Ca^{2+}$.

**Novel Aspects:** (photo-)chemical cross-linking in combination with high-resolution mass spectrometry for structural protein-analysis

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# Investigation of Calmodulin/Peptide Complexes by Chemical Cross-Linking and LTQ-Orbitrap-MS

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Calmodulin (CaM) is a ubiquitious $Ca^{2+}$ binding protein that binds to numerous target proteins, such as the Munc13 protein family. Munc13 proteins play a major role in synaptic vesicle priming and presynaptic short-term plasticity. The interaction of Munc13 with CaM seems to be the missing link between these processes and changes in residual $Ca^{2+}$ concentrations [1]. Recently, an additional interaction site in Munc13-1 and ubMunc13-2 at position 26 of the identified CaM binding motif (1-5-8) [2] with hydrophobic anchor amino acids at positions 1, 5, 8 and 26 was discovered [3]. The question intriguing is now whether this 1-5-8-26 motif in Munc13 proteins is unique or also found in classical CaM targets, such as skeletal myosin light chain kinase (skMLCK).

**Method:** Chemical cross-linking reactions with the amine-reactive cross-linker bis(sulfosuccinimidyl)glutarate (BS2G) were conducted by adding a 50-fold molar excess of $BS^2G$ to CaM/skMLCK peptide mixtures, which had been incubated in 20 mM HEPES, pH 7.2, at different $Ca^{2+}$ concentrations. For cross-linking experiments with the heterobifunctional cross-linker SBC(N-succinimidyl-p-benzoyl-dihydrocinnamate) [4] a two-step approach was used: First, the amine-reactive site of SBC was reacted with CaM. After quenching and removal of non-reacted SBC, skMLCK peptide was added to labeled CaM. Afterwards, the mixture was irradiated with 4 or 8 J/cm$^2$ UV light (365 nm) to induce the photo-reaction. Analysis of cross-linking products was performed using SDS-PAGE, tryptic *in-gel* digestion, separation of cross-linked peptides by nano-HPLC (Ultimate 3000, Dionex), and nano-ESI-LTQ-Orbitrap-MS/MS (LTQ-OrbitrapXL, ThermoFisher Scientific) analysis.

**Preliminary Data:** Using chemical cross-linking in combination with nano-HPLC/nano-ESI-LTQ-Orbitrap-MS/MS we investigated the complexes of CaM with three skMLCK peptide variants. For these studies, we used skMLCK peptides, in which the CaM binding motif was C-terminally elongated compared to the M13 peptide used previously [5]. Our aim was to elucidate whether there is an additional interaction site in the skMLCK peptide at position 26 of the CaM binding motif as it was observed for Munc13 proteins [3]. Additionally, we exchanged a phenylalanine at position 14 of the CaM binding motif to Ala or Glu in order to investigate the influence of this amino acid on the structure of the CaM/peptide complex. Using the homobifunctional cross-linker BS2G we identified lysines 21, 75, 77, 94, and 148 in CaM to be cross-linked with lysines 3, 7, 8, 20, 21 of the skMLCK peptides. In the cross-linking experiments with SBC, identical lysines of CaM were found to be connected with Met-30, Leu-1, Lys-20/21, Ala-12, and Ala-16 of the skMLCK peptides. All cross-links point consistently to a complex structure that is similar to the NMR structure (pdb entry 2BBM) of CaM with a skMLCK peptide comprising solely the CaM binding motif (M13 peptide) [5]. Our cross-linking data suggest that there is no additional interaction of the skMLCK peptides at position 26 of the CaM binding motif with CaM and that the amino acid at position 14 does not influence the complex structure. This leads to the conclusion that the 1-5-8-26 CaM binding motif of Munc13 is unique and induces an unusual conformation of CaM in the CaM/Munc13 complex.

**Novel Aspects:** The CaM binding behavior of Munc13 differs from that of the classical CaM target skMLCK.

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# Optimization of metal labelling of antibodies by use of ESI-MS

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The unmatched ability of antibodies to bind highly selectively to specific target molecules is more and more applied in various fields of application. For instance, antibodies are applied as pharmaceuticals for treatment of diseases such as instance cancer, autoimmune disorders, heart or inflammatory diseases. Very successful is the application in the enzyme-linked immunosorbent assay (ELISA) for detection of specific proteins, hormones, and many organic substances for instance of environmental origin. For the measurement of the antibody concentration of the formed complexes in the assay, often chromogenic, fluorescent or chemilumescense groups are attached to a primary or secondary antibody.

**Method:** We are investigating metal labelled antibodies, which are detected by inorganic mass spectrometry and in particular with ICP-MS, which is a very sensitive method and the multielement capability of this method can be used for the development of highly multiplexed assays [1]. For such measurements it is important to know the number of metal labels attached to the to the antibody. In this work,we show a new way to determine the labelling degree by use of a modified Waters nano-ESI-TOF-MS (modified by MSVision). This instrument allows very soft ionization, so that intact, non digested antibodies can be measured directly [2]. In the same way the labelled antibody can be analyzed and the labeling degree (number of label atoms) can be determined directly.

**Preliminary Data:** This allows a better understanding of the reactions and binding properties in the labeling process. In our case this is applied to optimize the labeling conditions for p-SCN-Bn-DOTA(Ln) which binds preferentially to free amino groups of lysine [3]. The labeled antibodies are applied in immunoassays based on ICP-MS detection [4].

**Novel Aspects:** With specific digestions, in example papain, the identification of the labeling position in the antibody amino acid sequence is possible [5].

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# Peptide Secondary Structures Studied by Photo-Activated Cross-Linking and ESI-MS/MS

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Photo-activated cross-linking of diazirine-containing amino acid analogues (photo-methionine and -leucine) has been recently introduced to study protein-protein interactions in living cells [1]. The binding of a photo-methionine containing peptide to the active site of trypsin has been reported as well [2]. However, essential parameters, such as stability of the diazirine, reaction time, and preferential targets still need to be elucidated. We used the pure photo-amino acids as well as short labeled peptides to address these basic questions. Peptides were designed with the aim to form stable or instable secondary structures, placing their *N*- and *C*-termini in close proximity or apart from each other. The incorporation of photo-leucine into these peptides allowed determining distances between the peptides' termini by photo-activated cross-linking.

**Method:** Buffered solutions of photo-amino acids and diazirine-labeled peptides (Thermo Fisher Scientific) were subjected to UV-A radiation in a home-built device, proteolytically digested and analyzed by LC/MS and -MS/MS experiments using an analytical HPLC (Agilent 1200 for photo-amino acids) or a nano-HPLC (Ultimate Plus for peptides) system coupled to an LTQ-Orbitrap XL hybrid mass spectrometer (Thermo Fisher Scientific) equipped with a nano-electrospray ionization source (Proxeon, for peptide runs). Precursor ions were typically subjected to CID fragmentation, occasionally to HCD fragmentation and fragment ions were analyzed in the orbitrap analyzer. Potential cross-linked products were identified using the *in-house* software StavroX [3] and validated by manual inspection of mass spectra. Far-UV CD spectra were recorded on a J-810 spectropolarimeter (Jasco).

**Preliminary Data:** Photo-activation of the pure photo-amino acids Photo-methionine and Photo-leucine yielded intramolecular reaction products as well as adducts with solvent molecules. Similar results were obtained with diazirine-labeled peptides. These peptides showed similar far-UV CD spectra as their unlabeled analogues, indicating no significant structural impact of the diazirine group. The peptides were designed to adopt distinct secondary structures, such as reverse turns, as confirmed by far-UV CD spectroscopy as well as structure prediction algorithms. Several intramolecular cross-linked products connecting the peptides' *N*- and *C*-termini were identified, which were in agreement with the proposed peptide structures. Reasonable concurrence was observed between the supposed strength of an intramolecular interaction and the observation of fragment ion mass spectra representing the respective cross-linked products. Likewise, peptides displaying well-defined turns produced singular cross-linked products, whereas fluctuating turns resulted in a more dispersed product pattern. This allowed us to identify an extended range of amino acid residues as reaction partners of the diazirine moieties.

**Novel Aspects:** application of incorporated photoamino acids to study secondary structures in isolated peptides.

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# Study of polymers used in medicine by means of MALDI TOF

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The importance of polymers in medicine is still leading the direction of research. One of important group of polymers are polylactieds (PLLA). Due to the compatibility of this type of polymers with living organisms, they are increasingly used in medicine and related fields, such as controlled drugs operation, the production of sutures or implants.

**Method:** Matrix Assisted Laser Desorption/Ionization – Time of Flight MALDI TOF technique is a method conventionally used in the analysis of polymers, however it is required to find appropriate conditions for the efficient desorption/-ionization process. The quality and reproducibility of the MALDI spectra obtained depends mainly on the measurement conditions and method of sample preparation.

**Preliminary Data:** In this project, we analyzed biodegradable polylactides using common matrices for polymers: 1,8-dihydroxy-9-anthracenone (DT), 2,5-dihydroxybenzoic acid (DHB), 2-(4-hydroxy phenylazo)-benzoic acid (HABA), trans-3-indoleacrylic acid (IAA) and sodium and lithium salts as cationization agents in order to optimize conditions of sample preparation for this type of polymers. PLLA was employed as a model polymer for sample preparation by dried droplet (DD) and solvet free (SF) methods.

# The mass spectra fingerprint of 1-phenyl-pyrazolon[3,4-B]quinoxaline dyes

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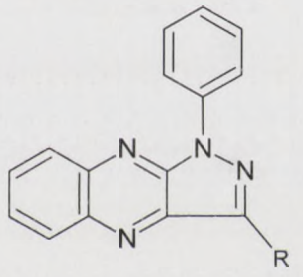
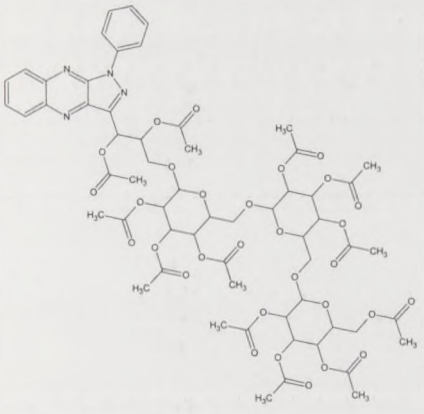
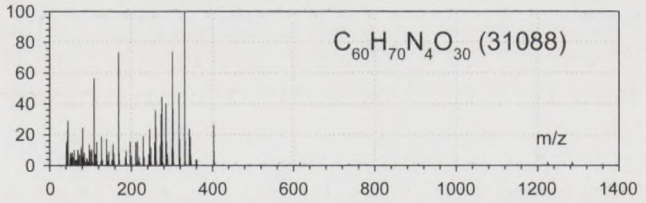
Dyes containing a 1H-pyrazolo[3,4-b]quinoxaline (PQ) moiety have been investigated since 1941. The PQs show applicable properties in antifungal [1], or antibacterial activity [2], separately in the in vitro tuberculosis [3]. The synthesis of 3-methyl-1-phenyl-1H-pyrazolo[3,4-b]quinoxaline (MPPQ) was first described by Ohle in 1941 [4]. Kolemainen et al. [5] analysed the selectivity of the reaction and descri bed the selected PQs as singlet oxygen sensitisers and efficient photoinitiators of free radical polymerisation [6, 7]. C-Nucleoside analogs have a glycosyl group attached to a nitrogen heterocycle at a ring-carbon atom, instead of a ring-nitrogen atom. This carbon-carbon linkage is more stable than the glycosyl carbon-nitrogen bond of true nucleosides, which makes the compounds useful tools for biochemical investigations and for antimitotic and antiviral research. The mass spectra of 1-phenyl-1H-pyrazolo[3,4-b]quinoxalines derivatives (PPQs) were presented in literature as supplementary results. Metwally [2] used MS for the synthetic MPPQ-s identification. Dolejš [8] and Sallam [9, 10] described saccharide derivatives of PPQ, using a similar method of synthesis. The authors of these papers explained the fragmentation partially and applied summary formulas only.



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# Mass spectrometry and ion spectroscopy in organic chemistry

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Mass spectrometry is one of the most important methods in determination of structures of organic compounds. Next to the analytical use it plays more and more important role also in the research of mechanisms of organic reactions. Electrospray ionization allows a direct detection of reaction intermediates from reaction mixtures. Another very fast developing method is action ion spectroscopy, which provides infrared and ultraviolet spectra of mass-selected ions and thus extends a portfolio of the methods for determination of ionic structures [1]. The principle of the method will be explained and several investigations of reaction mechanisms will be presented.

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# Separation and identification of sterol oligomers by HPLC/SEC-MS

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**Abstract:** Phytosterols undergo thermo-oxidative degradation during heating forming high molecular weight oligomers. Size exclusion chromatography was utilized for separation of oligomers prior identification by MS.

**Method:** Phytosterols' standard containing brassikasterol, campesterol, stigmasterol, sitosterol and avenasterol was heated at 180°C for up to 24 h. To test ionization, APCI, APPI and ESI techniques were applied.

**Preliminary Data:** During thermo-oxidative degradation oxidized sterols, volatile compounds, fragmented sterol molecules and oligomers were formed. We established that fragmented sterols, especially oxyphytosterols, react with each other or with sterol molecules forming oligomers, the main product of thermo-oxidative degradation of sterols. Application of high resolution MS further improved elucidation of chemical structure of these compounds. Phytosterol oligomers were observed in frying oils additionally indicating possibility of oligomers formation between sterols and triacyglycerides. Oligomers may have detrimental effect on the nutritional quality of the frying foods.

**Novel Aspects:** As effect of identification we were able to propose molecular structure of the dimers.

# Shotgun Lipidomic Analysis Reveals Considerable Differences in the Plasma Lipid Profile of Female and Male Healthy Subjects

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**Abstract:** Lipidomics is a powerful approach for the comprehensive analysis of metabolic diseases such as e.g. diabetes, obesity, or atherosclerosis. In order to establish the roles of molecular lipid species it is important to define the reference lipidome of healthy individuals. In this study, we analysed blood plasma lipidome of healthy, young men and women to characterize the gender differences. We performed a shotgun lipidomic analysis, an approach which enables high throughput and the broad coverage of lipid species from all major lipid classes.

**Method:** A cohort of 57 healthy individuals (28 male, 29 female) younger than 35 years was selected. The subjects followed a short nutritional protocol and have not received medication, beside contraceptives in women. Internal standards for lipid quantification were added to the plasma samples and lipids were extracted with MTBE as described in [1]. Mass spectrometric analysis was performed on a Q Exactive (Thermo Fisher Scientific) mass spectrometer equipped with a robotic nanoflow ion source TriVersa (Advion Biosciences). Lipid species were identified using LipidXplorer software [2]. The analysis encompassed 15 major lipid classes.

**Preliminary Data:** In this study, a top-down shotgun lipidomic approach was taken to characterize the plasma lipid profile of 28 and 29 young healthy men and women, respectively. For quantification of all identified lipid species internal standards were spiked into the samples prior to lipid extraction by MTBE. Total lipid extracts were directly infused into a Q Exactive mass spectrometer and species identified and quantified using LipidXplorer software. The analysis encompassed > 200 individual lipid species from 15 classes within less than 7 min acquisition time [3] with no carry-over of lipid material between the samples. Shotgun profiling determined the reference values of the content of individual lipids in normal human plasma and revealed striking gender differences that must be accounted for in the future disease specific lipidomic screening.

**Novel Aspects:** Detailed analysis of normal human plasma samples depicting substantial gender differences.

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# Stability study of sweeteners in beverages and identification of potential degradation products using hyphenated techniques

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Sweeteners are commonly used for food production. The majority of sugar substitutes, which are approved for use in food chemistry, are high-intensity sweeteners. Consumer can enjoy a wide range of food products containing sweeteners. Stability of food additives under normal conditions of use and storage is a clue of suitability for any particular food application. Decomposition of sweeteners may result in change of organoleptic properties of foodstuffs. Therefore, the objective of this study was to measure the stability of sweeteners possessing significantly different chemical properties in soft drinks using hyphenated techniques. Moreover, degradation products of sweeteners were identified using LC-QTOF/MS system.

**Method:** In order to evaluate the stability of nine sweeteners (acesulfame-K, alitame, aspartame, cyclamate, dulcin, neohesperidine dihydrochalcone, neotame, sucralose and saccharin) in different storage conditions, cola drink was used as blank for the preparation of fortified samples. The spiked samples were stored at three temperatures: +200°C, +40°C and −200°C for the following time periods i.e. 3 days, 1, 2, 3 and 4 weeks. To determine the effect of storage on the sweeteners stability, analyses of the target compounds were performed using a previously described SPE-HPLC/MS procedure [1]. Identification of degradation products of sweeteners was carried out using LC-QTOF/MS. Chromatographic separation was achieved using C18 column and mobile phases consisting of methanol and water containing 0.1% formic acid.

**Preliminary Data:** The obtained data gave opportunity to evaluate behaviour of sweeteners in different storage conditions. Seven sweeteners i.e. acesulfame-K, alitame, cyclamate, dulcin, neohesperidin dihydrochalcone, saccharin and sucralose were stable regardless of the storage conditions. In case of aspartame and neotame, at temperature +20°C decomposition of compounds was observed. After 4 weeks of storage at room temperature samples contained only about 50% and 20% of the initial concentration of aspartame and neotame, respectively. Aspartame is a dipeptide composed of two amino acids, L-aspartic acid and methyl ester of L-phenylalanie. Depending on environment conditions, there are different pathways for the degradation of aspartame. The reactions include cyclization as well as ester and peptide bond hydrolysis. Diketopiperazine (5-benzyl-3,6-dioxo-2-piperazine acetic acid) was found to be the main decomposition product. It is a result of intermolecular aminolysis. Moreover, L-asparginyl-L-phenylalanine, L-phenylalaninyl-L-aspartic acid were detected. These two isomeric dipeptides are formed when ring of diketopiperazine is opening. L-asparginyl-L-phenylalanine can also be formed by direct hydrolysis of the methyl ester of aspartame. Aspartame can undergo structural rearrangement to form β-aspartame, which was also identified. In case of neotame, four potential products of degradation were identified. The major degradation pathway is ester bond hydrolysis, what results in formation of N-[N-(3,3-dimiethylbutyl)-L-α-asparty]-L-phenylalanine 1-methyl ester. Neotame undergoes a reaction of cyclization, what was confirmed by identification of N-[N-(3,3-dimiethylbutyl)-L-α-aspartymidyl]-L-phenylalanine. Additionally, the isomeric β-neotame and N-fumarylphenylalanie 1-methyl ester were identified. All results within this study were obtained using LC-ESI-MS and LC-QTOF-MS.

**Novel Aspects:** Identification of degradation products of sweeteners using LC-QTOF-MS.

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# SILAC-Based Secretome Analysis of Non-Small Cell Lung Cancer Cell Lines

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Lung cancer is the most common cancer fatality in Europe (335,000 deaths/year)[1] with non-small cell lung cancer (NSCLC) comprising 80-85% of all cases [2]. Survival rates are poor (5-year survival rate lower than 15%) as most patients develop a metastatic disease that is too advanced for curative surgical resection. Even for these patients who undergo surgery, cure is not guaranteed. Tumor-host interaction plays a major role in carcinogenesis, but also in resistance to apoptosis- and necrosis-inducing treatment strategies. This interaction is modified by therapeutic interventions and changes substantially when tumor cells become resistant to a therapeutic agent. Analyzing the secretome of NSCLC cells may provide novel targets to overcome therapeutic resistance of lung cancer.

**Method:** The SILAC (stable isotope labeling with amino acids in cell culture) approach was chosen for secretome analysis of different NSCLC cell lines. For method validation, the *E. coli* MG1655 strain was grown in M9 minimal medium containing $^{12}$C-Arg or $^{13}$C-Arg. After cell lysis and protein concentration determination, $^{12}$C-Arg and $^{13}$C-Arg-labeled cell lysates were mixed in fixed ratios and separated by SDS-PAGE followed by tryptic *in-gel* digestion. The resulting peptide mixtures were separated by nano-HPLC (Ultimate, Dionex) and analyzed by ESI-LTQ-Orbitrap-MS (LTQ-Orbitrap XL, ThermoFisher Scientific). The five most abundant MS signals were subjected to CID-MS/MS in the LTQ. Concentration of conditioned medium was evaluated by ultrafiltration (Sartorius, 3 kDa MWCO) as well as by acetone and TCA (trichloroacetic acid) precipitation.

**Preliminary Data:** To differentially quantify secretomes, a stable isotope label can be introduced by chemical modification or by metabolic labeling [3]. The earliest staget for introducing stable isotope signatures into proteins is by metabolic labeling during cell growth and division [4]. SILAC was chosen as it is a metabolic labeling approach, which is the most accurate MS-based relative quantification method [4]. To validate the SILAC method, *E. coli* strain MG1655 was used. MS analysis of different mixing ratios (100/1-1/100) of $^{12}$C-Arg/$^{13}$C-Arg-labeled cell lysates was performed and different parameters were investigated to minimize the false positive rate. Sorting out the data with heavy/light counts < 2 and heavy/light variability > 10% lead to reproducible results and acceptable thresholds. As these results may not be conferrable to human cell lines, both (heavy and light) states of NSCLC cell lines will be analyzed to yield a reliable control for each experiment. For secretome analysis a method for concentrating the conditioned medium of NSCLC cell lines is required. Therefore, various concentration methods (ultafiltration, precipitation (acetone and TCA)) were evaluated for two NSCLC cell lines (PC9 (erlotinib sensitive) and PC9ER (erlotinib resistant)). Experiments were performed in triplicates. Even though ultrafiltration turned out to be the most suitable concentration method, there is a need to further optimize the conditions for ultrafiltration. Nevertheless, our preliminary data indicate that the secretome of NSCLC cells is mainly composed of low molecular weight proteins and peptides (MW < 30 kDa).

**Novel Aspects:** Analyzing the secretome of NSCLC cells may provide novel targets to overcome therapeutic resistance of lung cancer.

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# Structural characterization of lipoic acid – oligo([R, S]-3-hydroxybutyrate) conjugates with perspective application in cosmetic industry

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Alpha-lipoic acid (thioctic acid) is known as an universal antioxidant, which means that it neutralizes naturally occurring but harmful chemicals known as free radicals. Unlike other antioxidants, which work only in water or fatty tissues, lipoic acid is unusual in that it functions in both water and fat. This gives lipoic acid an unusually broad spectrum of antioxidant action [1]. It is well recognized in the scientific community that delivery systems are highly useful in cosmetics and pharmaceutical disciplines. In recent years an extensive research aimed at the design of novel cosmetic delivery systems has been observed. The attachment of bioactive compounds to polymeric carrier molecules via hydrolysable bonds is one of the promising approaches [2].

**Method:** The knowledge of structure-property relationships is essential for the successful application of polymeric materials, especially in biomedical applications such as tissue engineering, controlled drug delivery and cosmetic modifications. In present work the structure of lipoic acid – oligo([R, S]-3-hydroxybutyrate) (OHB) conjugates obtained via ring opening polymerization of β-butyrolactone initiated with lipoic acid potassium salt was determined by FT-IR, NMR and GPC analyses. The structural characterization of individual macromolecules of obtained conjugates with lipoic acid (including chemical structure of their end groups) was determined with aid of ESI-MS and ESI-MSn techniques.

**Preliminary Data:** Syntheses of oligo([R, S]-3-hydroxybutanoate) bioconjugates with active lipoic acid with required molecular weight, dispersity and chemical structure of the end groups were performed. The structure of the individual bioconjugates chains was determined on the basis of their ESI-MS spectra. The presence of active lipoic acid end group covalently bonded to oligo([R, S]-3-hydroxybutyrate) chains was confirmed by ESI-MS/MS fragmentation experiments performed for the selected conjugate molecules ions and identification of their fragmentation patterns. Moreover, hydrolytic degradation tests of the resulting bioconjugates in laboratory conditions were performed. The release of active lipoic acids into degradation medium was monitored by using ESI-MS technique. The structure of the water soluble degradation products was determined with use of ESI-MS technique. The preliminary comparative cytotoxicity studies on [R, S]-3-hydroxybutyric acid oligomers, pure lipoic acid as well as macromolecules containing lipoic acid covalently bonded to oligo([R, S]-3-hydroxybutyrate were performed at Dr Irena Eris Centre for Science and Research. Cytotoxicity tests showed that the conjugates studied are non-toxic and revealed the potential application of those bioconjugates in cosmetic industry.

**Acknowledgement:** This work was supported by European Regional Development Fund: Contract no. POIG.01.03.01-00-018/08-00 "New generation packaging materials made from plastics subject to the organic recycling" in the frame-work of the Innovative Economy Operational Programme (IE OP).

**Novel Aspects:** Synthesis and characterization on the molecular level of bioactive conjugates for perspective cosmetic applications.

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# Chemical dephosphorylation during $TiO_2$-based phosphopeptide enrichment – Systematic studies by MALDI QIT-Tof MSn and MALDI FT-ICR MS

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There have been many successful efforts to enrich phosphopeptides in complex protein mixtures by the use of IMAC (immobilized metal affinity chromatography) and/or MOAC (metal oxide affinity chromatography) with which mass spectrometric analysis of phosphopeptides has become state-of-the-art in specialized laboratories, mostly applying nanoLC-ESI-MS-based investigations [1-4]. In this study we present a ready to use phosphopeptide enrichment procedure using commercially available $TiO_2$-loaded pipette tips in combination with MALDI MS analyses [5].

**Method:** 5 µl sample were mixed with 2,5 µl 60 mg/ml DHB in 80% acetonitril (ACN)/ 0,1% TFA and 82 mg/ml Citric acid Monohydrate (CIT) in 80% ACN / 0,1% TFA, respectively. NuTips TiO2-Tips (Sunchrom) were equilibrated 5 times with 10 µl of 40% ACN / 0,1% TFA. Samples were aspirated and dispensed 50 times, washed once with 20 mg/ml DHB in 40% ACN/ 0,1% TFA or 28 mg/ml (CIT) in 40% ACN/ 0,1% TFA, once with 10 µl 40% ACN/ 0,1% TFA and eluted with 2,5 µl 25% NH4OH pH>10,5. 1 µl elution solution was prepared on a 600/384 AnchorChip Target with DHB, 2,4,6-trihydroxyacetophenon or Sinapinic acid as matrix. Mass spectra were recorded with 300-500 profiles/ spectrum.

**Preliminary Data:** Nucleophosmin and stathmin are two examples for successful phosphopeptide enrichment of either SDS-PAGE or 2D gel electrophoresis-separated proteins. Using citric acid as additive during sample loading on NuTips $TiO_2$-tips, a similar enrichment success for phosphopeptides can be achieved as compared to applying 2,5-dihydroxy benzoic acid (DHB) for this task. But the DHB-inherited drawbacks are eliminated. In addition, we show that combining DHB and 2,4,6-trihydroxy acetophenone (THAP) as matrix mixture for MALDI-MS measurements retains the sensitivity of DHB for phosphopeptide analysis but adds homogenous crystallization properties of THAP, enabling preparation of evenly distributed matrix surfaces on MALDI-MS anchor targets, a prerequisite for automated MALDI-MS analyses. Finally, high resolution MALDI FT-ICR mass spectrometry after phosphopeptide enrichment suggests that chemical dephosphorylation may occur as a side reaction during basic elution of phosphopeptides bound to MOAC surfaces, suggesting that proteome-wide phosphopeptide analyses ought to be interpreted with caution. By contrast, in-depth analysis of phosphopeptide / non-phosphorylated peptide siblings may be used to estimate stability differences of phosphorylation sites in individual proteins, possibly adding valuable information on biological regulation processes.

**Novel Aspects:** Novel isolation procedure for phosphopeptides and elucidation of dephosphorylation mechanism on $TiO_2$ surface by MALDI-QIT-Tof-$MS^n$ and MALDI-FT-ICR-MS

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# Determination of prohibited drugs in dried blood spots for doping controls by means of a benchtop quadrupole / Orbitrap mass spectrometer

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DBS analysis in doping controls provides numerous benefits, because the sampling is minimally invasive, fast, discreet and robust against manipulation. In the present study a new benchtop mass spectrometer was used combining a quadrupole mass filter, a HCD cell and an Orbitrap analyzer. The combination of an improved heated electrospray probe and stacked ring ion guide (S-lens), yielded in a significantly higher number of ions entering the curved linear ion trap. This enhanced sensitivity is the requirement for a successful DBS analysis concerning drugs of low blood concentration with doping or forensic relevancy. Especially cannabinoids, stimulants and corticosteroids were frequently misused in sport. Their concentration in the circulation during the competition is of special interest accordingly.

**Method:** An assay for 26 model compounds (e.g. $\Delta^9$-tetrahydrocannabinol, tetrahydrocannabinol-9-carboxylic acid, methylhexaneamine, methylphenidate, cocaine, 3,4-methylenedioxyamphetamine, *N*-methyl-3,4-methylenedioxyamphetamine, strychnine, salbutamol, clenbuterol, stanozolol, propranolol, dexamethasone, budesonide) which were detected frequently in positive doping control samples was developed. By means of isotope-labelled internal standards quantification provided reliable results beside qualitative results interpretation. After extraction of DBS with an organic solvent and liquid chromatographic separation of target analytes, mass spectrometry is performed with a high resolution full scan in positive and negative mode. Single product ion mass spectra are acquired using the data-dependent analysis mode (employing an inclusion list) for previously selected precursors of known prohibited compounds with fixed retention time ranges. Besides a sensitive targeted screening, a non-targeted analysis for retrospective data evaluation is possible.

**Preliminary Data:** With the chosen experimental design the determination of various drugs from different classes (stimulants, corticosteroids, anabolic agents, cannabinoids, $\beta_2$-agonists etc.) with one generic sample preparation is enabled which is shown for 26 selected model compounds. Especially the most challenging analytes $\Delta^9$-tetrahydrocannabinol (THC) and its carboxy-metabolite, corticosteroids (e.g. dexamethasone, budesonide), anabolic agents (e.g. clenbuterol, S4 andarine) and stimulants (e.g. cocaine, methylhexaneamine, methylphenidate) are sensitively detected with a limit of detection at 0.25 ng/mL or below. The method was validated for the parameters: specificity, linearity (0-20 ng/mL), precision (< 25%), recovery (mean 60%), limit of detection/quantification, ion suppression, stability and accuracy (85-100%). Six isotope-labelled analogues used as internal standards facilitate a quantitative result interpretation which is of utmost importance especially for in-competition sports drug testing. To our best knowledge this is the first assay to determine THC and its main metabolite THC-COOH from DBS. Considering the fact that THC is one the most frequently detected compounds and an extensively abused drug in general, the relevance of a sensitive assay in this field is obvious. With a LOD of about 0.25 ng/mL (for THC) the presented method yielded comparable sensitivity to formerly developed methods using a volume of 200 to 1000 µL of whole blood. Hitherto DBS analysis in doping controls is not yet implemented despite various benefits that would help reaching a more direct, stable, cost-effective and fast testing protocol. The recent instrumental developments for sensitive mass spectrometers support the introduction of this sampling technology and also the sample preparation is potentially automatable as already reported in various former studies. Although urine specimens still represent the matrix of choice for sensitive long-term detection of generally prohibited drugs, DBS sampling complements the result interpretation particularly for drugs banned in-competition only with an assessable effort.

**Novel Aspects:** The application of this new kind of tandem mass spectrometer supports this interesting sampling technique not only in doping controls.

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# Study of the operational parameter effects on multicomponent mycotoxin analysis by LC-MS/MS

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Mycotoxins are secondary toxic metabolites produced by fungi. They are common food contaminants, and their occurrence in food, beverages and feed has been recognized as a potential threat to the health and well being of humans and other animals. The increase of awareness of mycotoxin harmful effects cases the increase of the demand for analytical methods of high sensitivity and specificity, which will allow their screening in food and feed commodities. Liquid chromatography coupled to tandem mass spectrometry seems to be a perfect fit for the purpose. Systematic study of the operational parameter effects on the analysis of 30 mycotoxin most commonly present in fruits, juices, fruit beverages, beers and wines by LC-MS/MS was carried out.

**Method:** To develop a sensitive and selective multimycotoxin LC-MS/MS method, the effects of operational parameters on both the retention time and the analytical response of selected mycotoxins was studied. The influence of chromatographic and ion source related operational parameters was systematically examined. Experiments were carried out using an ultrahigh performance liquid chromatograph (Agilent UHPLC 1290) coupled to triple quadrupole mass spectrometer (Agilent LC-QQQ 6460).

**Preliminary Data:** The influence of mobile phase solvent and additives on both the retention times and MS signals of mycotoxins was observed. Column chemistry and temperature effected the mycotoxin separation and resolution. Compound-specific optimization of ion source operational parameters appeared to be essential. Co-elutions of compounds were observed. Common MS transitions were identified. The cases of both co-elutions and common transitions were thoroughly analyzed and recognized, to avoid if possible, ion suppressions or enhancement cased by the co-elution and to ensure the method specificity essential for unambiguous mycotoxin identification and their accurate quantification.

**Novel Aspects:** Systematic study of the operational parameter effects on multicomponent mycotoxin analysis by LC-MS/MS was carried out for the first time.

# Studying oxidative and reductive reactions using a potentiostat coupled to MS: Applications in water research and enzymology

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Redox reactions are important chemical reactions deeply involved in chemical production, environmental and biological processes. Latter are like the example of the current effort in removing persistent components prior to the outlet of wastewater treatment plants or biological enzymatic conversion reactions in living systems.

**Method:** ROXY™ potentiostat is an electrochemical apparatus treating molecules oxidative as well as reductive. In the reaction cell several electrodes were applied, e.g. Glassy Carbon, Magic Diamond™, platinum and gold for several relevant molecules using increasing energy in each experiment. The outlet flow was then directly coupled to a time of flight mass spectrometer detecting the products in high accuracy.

**Preliminary Data:** Two research projects will be presented giving details about the experimental conditions and the obtained results, one using pharmaceuticals from waste waters and the other using substrate for the enzyme Chitosanase. The results allow a discussion about the potential as a fast screening technique for this type of molecules and applications.

**Novel Aspects:** Novel molecule oxidation/reduction technique relevant for direct product analysis by mass spectrometry

# MALDI with ligated fullerenes and fullerenes as ligands

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MALDI is frequently used as a reliable means to establish the amount of ligands attained on $C_{60}$. In the first part we present the rare case of a monoadduct, the composition of which had been established by ESI beyond doubt, which shows a reasonably abundant signal for the bis-adduct, when analysed by MALDI. Thereby suggesting a false composition. In the second part we discuss the ion formation behaviour of a zinc porphyrin dimer, carrying six peripheral $C_{60}$ units. Each of the two central zinc porphyrins are decorated with three $C_{60}$ molecules and all moieties are interlinked by a total of seven [4 + 2] cycloadditions: $(C_{60})_3$[ZnPor][ZnPor]$(C_{60})_3$.

**Method:** The ion formation mechanisms occuring in MALDI are investigated using a reflectron time-of-flight mass spectrometer. ESI coupled to a quadrupole ion trap mass spectrometer is used as complementary approach.

**Preliminary Data:** $C_{60}$ Mono-/Bis-Adduct: In cross experiments it is confirmed that the bis-adduct is not present in the sample, but formed in the mass spectrometer. This behaviour is observed with the Prato adduct, which is one of the most important synthons in $C_{60}$ chemistry. The Prato ligand is not accessible as a long-lived species in solution and, therefore, it is normally formed in situ from other components prior to the addition to $C_{60}$. However, it is shown, that the ligand can survive as intact moiety in the special environment that exists in the MALDI source of the mass spectrometer. A formation mechanism for the bis-adduct is proposed whereby the MALDI process of a clean mono-adduct is accompanied by partial loss of the ligand, which subsequently re-attaches to mono-adducts which are still intact. $(C_{60})_3$[ZnPor][ZnPor]$(C_{60})_3$: In direct LDI all fullerene units split off, presumably prior to the actual ionisation event and a single $[ZnPor]^+$ ion results as the major ion accompanied by a lower abundant porphyrine dimer$(ZnPor)^{2+}$. However, electron transfer MALDI with DCTB as the matrix material is thermochemically feasible and gentle enough to produce the intact molecular ion in respectable abundances. Post-source decay of the molecular ion features the loss of several $C_{60}$ units, but not down to the free dimeric porphyrin centre. This intern confirms that in direct LDI dissociations is followed by ionisation, whereas in MALDI ionisation is followed by dissociation.

**Novel Aspects:** Ligand attachment in MALDI, from $C_{60}L$ to $C_{60}L_2$. Differentiation of LDI- and MALDI-contributions to the over-all ionisation process is provided.

# Surrounding the keratin challenge – identification of dermatophyte fungi infection of low abundant fungi proteins in infected skin and nail samples

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The correct diagnosis with accompanying species identification of pathogenic dermatophyte fungi from infected skin and nail samples are of particular importance for correct clinical treatment. However, the characterization of the relative low abundant fungi proteins contained in infected human skin or nail samples is a critical challenge for the analysis by mass spectrometry (MS). Here we present a workflow for the analysis of the seven most common dermatophyte fungi species by LC-MS. The strategy is based on the enrichment of fungal proteins employing ProteoMinerTM hexapeptide extraction technique1, followed by a targeted LC-MS analysis using stringent inclusion and exclusion lists. Thus novel software was developed which has a variety of functions, with the most beneficial for this work being the optimization of inclusion and exclusion lists.

**Method:** Different lysis conditions were tested for protein extraction of samples followed by depletion of high abundant human proteins by using hexapeptide beads (ProteoMinerTM beads[1]). The proteins in the eluate were precipitated, reduced, alkylated and tryptic digested prior to LC-MS/MS analysis. Analysis was performed using a nano-HPLC/nano-ESI-MS/MS (30 min gradient for protein standard, 80 min gradient for complex mixtures) with an Orbitrap LTQ XL ETD (Thermo Scientific) equipped with an chip-based electrospray ionization source (Triversa, Advion). For an improved detection of fungal peptides, inclusion and exclusion lists were prepared using the new program "MS Focus". The performance of the workflow was tested with artificial mixtures of fungi and human nail (1:10) or skin (1:10) samples before being applied to clinical samples.

**Preliminary Data:** The software developed has a variety of functions containing, data recalibration, adaption of retention time shifts, FDR re-evaluation, protein modification statistics, and optimization of inclusion and exclusion lists. The optimization of inclusion exclusion lists includes additional options using protein contaminant sequence files, setting a maximum number of peptides per protein which should be included on inclusion lists, allows intelligent selection of optimal peptides for species detection based on fragmentation behaviour, distribution of peptides over the retention time range, and the usage of a species specific peptide list. These inclusion and exclusion parameters were initially tested in proof of principle experiments using different dilutions of protein standard mixture (containing BSA, cytochrome C, myoglobin, and enolase). Here we were still able to obtain a good protein identification (> % sequence coverage, MASCOT-Score) for enolase even in mixture with 1000:1 excess of three other proteins, if stringent inclusion and exclusion lists were applied. In next more realistic experiments, clinical samples and artificial mixtures (1 mg fungi (trichophyton rubrum): 10 mg nail/skin) were analyzed. Independent of the huge human keratin excess we were able to detect a limited number of fungal peptides. However, in clinical samples the number of fungal peptides detected was so low that species discrimination was not possible due to insufficient number of species-specific peptides. The enrichment with hexapeptide beads and application of stringent inclusion and exclusion lists were then applied. (The inclusion list were constructed from analyses of both pure fungi protein extracts and fungi/human artificial mixtures, whereas the exclusion lists were created on analyses of pure human samples.) Upon employing these techniques we were able to improve the quality of peptide fragmentation spectra as well as the reproducibility of the detection and ultimately were able to improve the number of identified fungal peptides with allowed for discrimination between different dermatophyte genera.

**Novel Aspects:** Optimized detection of low abundant fungal peptides by enrichment using hexapeptide beads and inclusion/exclusion list for LC-MS with "MS-Focus" program.

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# Synthesis and characterization on the molecular level PHA macrodiols

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Poly(hydroxyalkanoates) (PHAs) constitute interesting group of biodegradable and biocompatible polymeric materials, which can be produced from renewable resources. Depending on the bacterial strain and cultivation conditions used for the fermentation process, different kind of PHAs with defined properties can be obtained [1-2]. The thermal an physical properties of PHA depend mainly on the chemical structure of comonomers unit and their sequence distribution [3]. The applications of PHA , particularly in the fields of health, environment, food, agriculture and agro-industries as environmentally friendly materials requires detailed and unambiguous information about their structure. A remarkable instrumental progress in mass spectrometry has made available a number of key methods for the structural characterization of PHA macromolecules [4].

**Method:** The drawback of MS characterization of high molar mass PHA is that, with increasing mass the mass resolution decreases. Furthermore, many MS instruments are limited in the range of mass they can detect. In order to deal with these limitations the PHA biopolymers are usually subjected to partial controlled degradation to obtain PHA oligomers possessing the same composition and sequence distribution as the original one. Several methods of partial degradation have been evaluated for different PHA biopolyester. In this work for controlled depolymerization of selected PHA the reduction reaction with the aid of lithium borohydride was applied. For the characterization of oligomers obtained the NMR spectroscopy and ESI-mass spectrometry was applied.

**Preliminary Data:** The elaborated simple method of controlled depolymerization of PHAs biopolyester leads to the formation (with very good selectivity) of PHA oligomers with the same composition and sequence distribution as the starting biopolyesters and terminated by alcohols end groups. The structural studies of selected commercially available PHA biopolyesters (i.e. PHBV, PHBH, PHO) were performed based on the ESI-MS analysis of their low molar mass oligomers (PHA oligodiols) obtained via reduction with the aid of lithium borohydride. The molecular architecture of individual macromolecules, including the chemical structure of the end groups, was established based on mass-resolved signals in ESI-MS spectra of selected PHBH, PHBV and PHO oligodiols obtained. The composition and sequence distribution were determined based on measurement of the relative intensity of the individual PHA oligodiols molecular ions present at the ESI-mass spectra. The arrangement of comonomer structural units along the PHA chains was verified by tandem ESI-MS/MS experiments performed for the selected molecular ions of PHA oligodiols and investigation their fragmentation pathway. These well-characterized on the molecular level PHA oligodiols will be used for the synthesis of copolymers via condensation process.

**Novel Aspects:** Determination the structure on the molecular level of PHA macrodiols with the aid of ESI-mass spectrometry

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# Synthesis and structural characterization of pesticide-oligo(3-hydroxybutyrate) conjugates

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Currently used forms of pesticides can be harmful to the environment and human who are exposed to those pesticides. Due to weather condition large amount of pesticides never reached their objects and become environmental pollution [1]. Nowadays, there are tends to minimize these negative effects, one of possibilities is using formulations which release active compound in optimal quantities over specific period of time. Polymers, especially biodegradable ones, could be used as carriers in controlled release pesticides formulations [2]. These carriers should be: environmentally friendly and compatible with surrounding environment [3]. All requirements are achieved by oligo(3-hydroxybutyrate) which can be obtained via anionic ring opening polymerization of β-butyrolactone [4].

**Method:** To obtain the pesticide-oligo(3-hydroxybutyrate) conjugates in which active compounds are covalently bonded to polymer carrier, anionic ring opening oligomerization of β-butyrolactone has been used. Reactions were carried out in DMSO under argon atmosphere in room temperature. Potassium slats of pesticide species were used as initiators. The progress of oligomerizations were monitored by the FT-IR spectroscopy based on the intensity of carbonyl group signal of monomer at 1820 $cm^{-1}$ and oligomer at 1735 $cm^{-1}$. When the oligomerizations were completed, oligomers were acidified with cation-exchange resin. After removal of the cation-exchange resin chloroform was added and mixtures were washed with distilled water in order to remove DMSO. Synthesized conjugates were characterized by $^1H$ NMR, GPC and ESI-MS analysis.

**Preliminary Data:** Series of pesticide-oligo(3-hydroxybutyrate) conjugates with different molar mass have been obtained via anionic ring opening oligomerization of β-butyrolactone using the modified pesticide species as the initiators. Molecular weight of the conjugates were controlled by monomer to initiator molar ratio. Molecular mass from GPC analysis was comparable with molecular mass calculated from intensity of end groups in $^1H$ NMR spectrum. The molecular structure of (4-chloro-2-methylphenoxy)acetic acid-oligo(3-hydroxybutyrate) and (2,4-dichlorophenoxy)acetic acid-oligo(3-hydroxybutyrate) conjugates was confirmed by ESI-$MS^n$ analysis.

**Novel Aspects:** Synthesis and structural characterization of (4-Chloro-2-methylphenoxy)acetic acid covalently bonded to oligo(3-hydroxybutyrate).

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# TLC-overlay assay detection of a novel Shiga toxin receptor and its structural characterization by ESI MS

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Shiga toxin (Stx)-producing Escherichia coli (STEC) play an important role as pathogens in humans and pigs. Stx2 and, to a minor extent, Stx1 of STEC strains cause severe kidney and central nervous system complications in humans, whereas Stx2e represents the major virulence factor responsible for the pig edema disease. Cell surface receptors for Stx1 and Stx2 are glycosphingolipids (GSLs) of the globo-series, namely globotriaosylceramide (Gb3Cer) (high-affinity receptor) and globotetraosylceramide (Gb4Cer) (low-affinity receptor). The same receptors have been suggested for Stx2e, only with inverse binding preference. Here we report on a unique recognition of a HexNAc-prolongued Gb4Cer structure, a pentahexosylceramide, by Stx2e which has not been described before. The structure of this novel Stx2e receptor has been elucidated by ESI MS.

**Method:** GSL-Stx-interaction was analyzed in thin-layer chromatography (TLC) overlay assays using Stx-containing bacterial culture supernatants from STEC of various serotypes. TLC-separated GSLs were overlayed with supernatants and bound toxins were detected using anti-Stx1 or anti-Stx2 antibodies, corresponding alkaline phosphatase (AP) conjugated secondary antibodies and indolylphosphate as the substrate. Mass spectrometry was performed on a Q-TOF instrument equipped with a nanospray manipulator. GSL samples were dissolved in MeOH containing 2% formic acid and analyzed in the positive ion mode. Collision-induced dissociation (CID) was performed with argon as collision gas.

**Preliminary Data:** TLC overlay assays performed with a GSL mixture comprising Gb3Cer, Gb4Cer and a pentahexosylceramide and Stx1-, Stx2-, and Stx2e-containing supernatants revealed strong binding intensities of Stx1 to both receptors, Gb3Cer and Gb4Cer whereas Stx2 variants revealed a predominant interaction with Gb3Cer and weak binding to Gb4Cer. Neither Stx1 nor Stx2 recognized at all the pentahexosylceramide structure. Stx2e preparations showed a higher binding activity towards Gb4Cer as compared to Gb3Cer. In contrast to Stx1 and Stx2, Stx2e regardless if derived from human or porcine isolates, exhibited strong binding towards the prolonged globo-series GSL. This uncommon binding specificity has so far not been described for Stx2e species and has also not been observed for any other Stx types. To substantiate the structure of this novel Stx receptor ESI MS and MS/MS experiments were performed. The mass spectrum revealed the presence of a broad variety of higher and lower abundant sodiated species in the m/z region 1440 to 1640. A tentatively assignment to a pentahexosylceramide carrying d18:1 sphingosine and different fatty acyl chains in the ceramide moiety was finally verified by low-energy CID experiments.

**Novel Aspects:** Unique recognition of HexNAc-prolongued Gb4Cer by Stx2e in addition to binding to Gb3Cer and Gb4Cer.

# Towards the Systematic Discovery of Novel Lipid Classes by Shotgun Mass Spectrometry

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Lipids comprise of a wide array of different classes, which in the cells and tissues of living organisms fulfill various structural, metabolic and signaling functions. Despite the wealth of knowledge about molecular constituents of the cells, new types of lipids are still being discovered regularly. However, novel lipids are usually only discovered by chance or by targeted approaches. In order to systematically search for new lipid classes, an unbiased systematic screen would be advisable. We are developing an approach based on shotgun mass spectrometric analysis of total lipid extracts and deciphering the acquired datasets by LipidXplorer software.

**Method:** Crude lipid extracts are prepared from various tissue samples using either the Bligh and Dyer or the Folch extraction method. After drying, extracts are re-dissolved and infused via nanoflow ESI (Advion Triversa) a into a quadrupole-orbitrap hybrid mass spectrometer (QExactive, Thermo Fisher Scientific). Full-MS and MS/MS-spectra with t he precursor ions isolation window of 1 Da are collected over 30-60 minutes using a data-dependent acquisition (DDA) method or a targeted MS/MS (t-$MS^2$) method in conjunction with an inclusion list covering the entire mass range of interest. In this way, either the most abundant peaks in the spectrum or the entire range is subjected to fragmentation. Spectra are then processed using the LipidXplorer software.

**Preliminary Data:** We are currently testing various approaches for the discovery of unknown lipid classes. The pivotal tool in our computational discovery approach is the LipidXplorer software. The full-MS and fragmentation spectra acquired by DDA or by $tMS^2$ contain the most comprehensive structural information about the analytes. With the LipidXplorer software we are able to screen this collection of fragment spectra with scripts for ions of any chemical composition. By using Boolean operators and sum formula arithmetics, we can test combinations of chemical compositions to match fragment peaks and each precursor mass in the full MS spectrum. In the first approach, the data is screened with scripts for known classes of lipids together with scripts containing a broad number of possible heteroatoms. Subtracting the known lipids from the unknown peaks results in a list of candidates of potentially unknown lipids. In the second approach, a large library of scripts is written which consists of plausible variations and modifications of lipid structural elements, (i.e. oxidized fatty acids, different amino acids as head groups etc.). This library is then used to query the collection of fragment spectra for the presence of fragment peaks representing new lipids that are only distantly similar to known lipid classes.

**Novel Aspects:** Computational screening for new lipid classes.

# UHR-Q-TOF Analysis can address common Challenges in Targeted and Untargeted Metabolomics

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Here, we present an ESI-UHR-Q-TOF based analysis of myxobacterial secondary metabolites, which permits to solve several challenges frequently encountered in metabolite profiling studies. Myxobacteria are promising producers of natural products exhibiting potent biological activities, and several myxobacterial metabolites are currently under investigation as potential leads for novel drugs. However, the myxobacteria are also a striking example for the divergence between the genetic capacity for the production of secondary metabolites and the number of compounds that could be characterised to date. Wildtype and mutant strains were analyzed concerning the production patterns of known metabolites and with regard to the discovery of new metabolites.

**Method:** Extracts from Myxococcus xanthus wildtype and mutant strains (4 biological replicates) were separated on a UHPLC RP-C18 column (50 × 2 mm, 1.7 µm particle size). MS analysis was carried out using an ultra-high resolution TOF mass spectrometer. The data was processed using a compound finding algorithm prior to statistical interpretation by principal component analysis (PCA) and t-test. Additionally, a targeted search for known metabolites was carried out using the retention time, mass accuracy and isotopic pattern as identification criteria.

**Preliminary Data:** Since mass accuracy and resolution of TOF instruments are independent of the acquisition rate, they are perfectly suited for a coupling to UHPLC separations. These hyphenations enable a reduction of analysis time in combination with a high chromatographic resolution and therefore permit an increased sample throughput. The UHR-TOF analysis revealed that an acquisition rate of up to 20 Hz did not compromise the achieved mass accuracy or resolution. Acquisition of full scan accurate mass spectra enable the targeted screening for known compounds e.g. from the class of DKxanthenes based on very selective high resolution EIC (hrEIC) traces with small mass windows of 1.0-0.5 mDa. A comparison of several datasets following a "comprehensive feature extraction" combined with a statistical analysis permits an untargeted discovery of novel biomarkers using the same data files as for the targeted analysis. Even a mass accuracy of 0.1 ppm is not sufficient for an unambiguous formula identification for m/z values above 500. A combination of accurate mass data and isotopic pattern information in MS and MS/MS spectra can extend this m/z range for reliable formula suggestions. Examples for novel metabolites from Myxobacteria will be shown.

**Novel Aspects:** Accurate mass data and isotopic pattern information in MS and MS/MS spectra can extend this m/z range for reliable suggestions.

# Using HPLC / DAD / ESI-TOF-MS with fragmentation in the ion source in structural studies of plant secondary metabolites with antioxidant activity

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Considerable interest has developed over the years in fruits and vegetables containing high concentrations of flavonoids, due to their potential biological and health-promoting effects [1-5]. Numerous epidemiological studies indicate that an increase in the consumption of fruits and vegetables is associated with a decrease in the incidence of various diseases like cardiovascular disease, stroke and cancer. The biological effects are in part due to their antioxidant capacity. Among all common fruits and vegetables in the diet, berries, especially those with dark blue or red colors, have the highest antioxidant capacities. The objective of this study was to characterize individual phenolic compounds in some cultivars of chokeberry and elderberry by HPLC-ESI-MS technique.

**Method:** Analyses of appropriate plant extracts derived from some cultivars of chokeberry and elderberry were performed on an Agilent 1200 series HPLC equipped with a diode array detector followed by a time of flight mass spectrometer with an electrospray interface. A 150 × 2,1 mm i.d. ACE C18 column was used for separation. Elution was performed using mobile phase A (5% formic acid aqueous solution) and mobile phase B (methanol). Chromatograms were recorded at 520 nm (anthocyanins) and 280 nm (other phenolic compounds). Column effluent was monitored in positive or negative mode of the MS depending on physico-chemical properties of compounds. Major mass spectrometer parameters were as follows: capillary voltage – 3500 V, scan acquisition – from 50 to 1700 m/z, fragmentor voltage – 150 V.

**Preliminary Data:** The studies revealed, that anthocyanins were the predominant polyphenolic components in chokeberry and elderberry, however the extracts mainly contained cyanidin based pigments. Chokeberry juice contained a mixture of four different cyanidin-glycosides: 3-galactoside, 3-arabinoside, 3-glucoside and 3-xyloside of cyanidin. Cyanidin-3-galactoside and cyanidin-3-arabinoside were found in the highest concentrations. The major anthocyanins in elderberry juice were: cyanidin-3-sambubioside and cyanidin-3-glucoside (eluted together), cyanidin-3-sambubioside-5-glucoside and cyanidin-3-rutinoside with cyanidin-3-sambubioside and cyanidin-3-glucoside predominating. In berries extracts we also found certain amount of derivatives of pelargonidin, malvidin and delphidin. Structural analysis in details also showed rich content of phenolic acids in plant extracts. The appropriate structures were assigned to respective molecules basing on their fragmentation. We identified the following acids in the extracts: benzoic acid, caffeic acid, ferulic acid, chlorogenic acid and neochlorogenic acid. The juices with the active phenolic compounds were used to examine their antioxidant properties, as well as many protective actions in mammalian cells, particularly related to their potential role in colon cancer chemoprevention.

**Novel Aspects:** The fragmentation of compounds, selectively performed in the ion source, could be an alternative to tandem mass spectrometry.

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# Searching protecting groups for 2'-hydroxyl function in RNA synthesis

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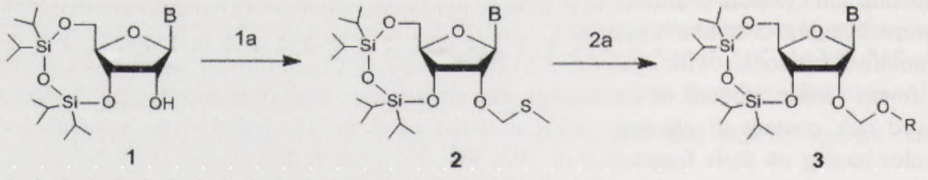
The research aiming to develop at creating an efficient chemical method of RNA synthesis began in the 1950s. Nowadays, the methods of chemical DNA synthesis are developed much better than methods of RNA synthesis. Finding new functions of RNA, and most importantly, discovering the phenomenon of RNA (RNAi) interference, has significantly increased the interest in synthetic RNA. New regulatory functions of RNA, the development of structural research of RNA and numerous reports in the literature presenting practical applications of these oligomers make the demand for synthetic RNA fragments higher. However, chemical RNA synthesis is very problematic and it is a challenge faced by many chemists all over the world.

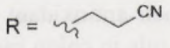
**Method:** Numerous studies have been conducted in many laboratories in order to find new protecting groups for 2'-hydroxyl function, as well as efficient methods of entering them into nucleosides. One of methods of protecting 2'-OH position in ribonucleosides is a method in which a suitably blocked nucleoside (1) component is converted into a more active nucleoside 2'-O-methylthiomethyl derivative (2). Then, the isolated derivative Nr is reacted with appropriate alcohol (to become a part of protecting group) in the presence of N-iodosuccinimide and trifluoromethanesulfonic acid as activators.

**Preliminary Data**: In the last few years, acetal derivatives of formaldehyde have been studied as protecting groups of 2'-OH function in RNA synthesis. Such groups have numerous advantages and the most significant one is a small spacial hindrance. However, entering acetal blocking groups to ribonucleoside requires developing more comfortable and efficient methods than those which have been described in the literature thus far. In my research the MS ESI method is one of the basic methods for identification of obtained products. This method enables me to identify products in studied reactions in a short time without strenuously isolating them. This is of a great help in optimizing the reaction conditions required for introduction of new protecting groups into ribonucleosides.

**Novel Aspects:** Increasing the efficiency of RNA synthesis by developing new protecting groups for the 2'-hydroxyl function to ribnucleosides.




1a ) DMSO, $Ac_2O$, AcOH, 16 h RT, 5 h 45°C
2a ) ROH, NIS, TfOH, - 45°C, DCE, [2]

R = 


B = A, G, U, C

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# Cleaning and separation of Proteins using POROS R2 perfusion HPLC for MALDI MS measurement

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The separation of proteins with RP-HPLC is not so common like the preferentially used method SDS-PAGE. The use of RP-HPLC for the separation of biomolecules such as proteins and peptides is only well established for peptides so far. The main challenges in protein chromatography are sample impurities like detergents and salts as well as insufficient resolution of the system. As a new possibility for cleaning and separating proteins a new method was established using POROS R2 perfusion RP-HPLC. The new method was evaluated using myoglobin and different detergents and then applied to a mitochondrial protein complex, cytochrome bc1 d2Ant. After POROS separation MALDI MS intact mass measurement and trypsin digestion followed by MALDI MS/MS of the separated subunits was performed.

**Method:** The capillary-HPLC system consists of a Eldex MicroProTM Micro/nano HPLC system with 2 ml syringe pumps, a Mistral column oven and a UV detector (SuchChrom, Friedrichsdorf, Germany). The POROS R2 perfusion material (Applied Biosystems, Darmstadt, Germany) is packed in house into fused silica capillaries using a hydraulic pump (SunChrom, Friedrichsdorf, Germany). MALDI MS measurement are carried out on a Voyager STR (AB Sciex, Darmstadt, Germany) in the linear ion mode using sDHB (a 9:1 mixture of 2,5-dihydroxybenzoic acid and 2-hydroxy-5-methoxybenzoic acid) as MALDI matrix and on a AB Sciex 4800 TOF/TOF (AB Sciex, Darmstadt, Germany) using CHCA (α-cyano-4-hydroxycinnamic acid) as MALDI matrix.

**Preliminary Data:** The new LC separation method was tested with myoglobin and various detergents in different concentrations as well as with a mixture of 6 standard proteins for evaluation. In addition the chromatographic parameters like peak capacity, dynamic range and carryover of the system were determined. The peak capacity with ≈ 40 is comparable with other chromatographic approaches. [2]. In the second step the cytochrome bc1 d2Ant complex was investigated as the sample contains Triton-X 100 and SDS. All subunits were measured on the one hand as intact masses as well as their tryptic peptides after digestion. To highlight the advantage of the new method, a comparison with the standard SDS-PAGE system was made. Compared to the LC approach the new method shows a higher number of tryptic peptides and higher sequence coverage compared to the SDS gel. In addition it was possible to detect the strongly hydrophobic Cyt b from the cytochrome bc1 d2Ant complex which was not possible so far with LC based methods.

**Novel Aspects:** Fast and robust method for cleaning and separating proteins from detergent containing samples for subsequent MALDI MS and MS/MS.

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# Comparison of peptide cancer signatures identified by MALDI-MS in serum of patients with head and neck, colorectal and lung cancers

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Mass spectrometry-based analyses of the low-molecular-weight fraction of serum proteome allow identifying proteome profiles/signatures that are potentially useful in classification, detection and diagnostics of cancer [1-3]. Here we compared serum proteome profiles of healthy donors and patients with three types of cancer aiming to identify peptide signatures that were either common for all cancer patients or specific for cancer type.

**Method:** Blood samples were collected before start of the treatment from 35 patients with head and neck cancer, 35 patients with colorectal cancer and 50 patients with non-small cell lung cancer, and from 45 healthy volunteers. Mass spectra of the serum proteome were recorded in the range between 2 and 13 kDa using the MALDI-ToF spectrometry. 131 spectral components (peptide ions) were identified in registered spectra and their abundances in samples from all four groups of donors were used for statistical analyses.

**Preliminary Data:** Similar degrees of overall differences/similarities were observed in all intra-group and inter-group analyses when general features of serum proteome profiles were compared between individual samples. However, classifiers built of selected spectral components allowed differentiation between healthy donors and there groups of cancer patients with 69-74% sensitivity and 82-84% specificity. There were two common peptide species (3766 and 5867 Da) up-regulated in all cancer samples, while other components of classifiers were specific for cancer types. Several spectral components permitted differentiation between lung cancer samples and either head and neck cancer or colorectal cancer samples, while two latter types of samples could not be properly differentiated. Abundances of spectral components that putatively corresponded to fragments of serum amyloid alfa (11 511 and 11 667 Da) were markedly higher in lung cancer samples when compared to samples from three other groups; high abundance of these components corresponded to more advanced cancer. In addition, unsupervised cluster analyses confirmed clear differences of serum peptide signatures characteristic for healthy donors and lung cancer patients. Our data indicates that certain components of serum peptide signatures are common for different cancer classifiers and putatively reflect general response of organism to the disease. However, other components of such signatures are unique and might reflect more specific features related to the type of malignancy and/or degree of its advance.

**Novel Aspects:** Our data indicates that certain components of serum peptide signatures are common for different cancer classifiers.

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# Controlled-pH tissue clean-up protocol for signal enhancement of small molecule drugs analyzed by MALDI-MS imaging

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MALDI mass spectrometry imaging (MALDI-MSI) has recently become an established technique that enables the mapping of molecular species directly from tissue sections. It has been providing information about localization of a wide range of compounds, recently complemented by quantification ability. While MALDI-MSI can offer both high spatial and high mass spectral resolution directly from tissue samples, a major limiting constraint for the application of the technology is that sensitivity can be hampered significantly by endogenous compounds present within the tissue sample. Several protocols have been introduced to enhance the sensitivity of the technique by cleaning-up the tissue mostly for analysis of proteins and peptides. Here, the aim is to develop a clean-up protocol suitable for MALDI-MSI of small molecule drugs.

**Method:** Tissue samples placed on ITO glass slides were desiccated for 15 min prior to clean-up. The predicted solubility properties of the drugs (calculated using ACD Labs software) were used to adjust the pH of the aqueous buffer, i.e. ammonium acetate to the point that the analytes of interest had low solubilities. The tissues were immersed into the washing solution for 10 seconds followed by spinning for 20 seconds. The tissues were introduced into the high vacuum chamber to allow for quick drying. Control and treated tissues were then coated by a uniform layer of the matrix e.g. CHCA or DHB and analyzed by an imaging-enabled MALDI ToF/ToF instrument (UltraFlex II, Bruker Daltonics).

**Preliminary Data:** Several drugs such as cimetidine, imipramine and a new candidate drug in different tissue sections such as brain and lung tissues were tested. The best results were achieved when the pH of the washing solution (100 mM of ammonium acetate) was adjusted to about pH 10 for cimetidine and imipramine and pH 6 for the drug candidate. In these pH values no major delocalization of the drugs were observed, while dramatic delocalization happened when ammonium acetate with the different pH or water were used as washing solutions. The signal to noise ratios for the peaks that corresponded the protonated drugs were significantly increased after treatment. This improvement could be explained by removal of salts and soluble suppressant species from the tissue samples.

**Novel Aspects:** Enhancement of the sensitivity of MALDI-MSI signal of small molecule pharmaceuticals using an improved clean-up protocol.

# Increased sequence coverage of membrane proteins using a multienzymatic strategy for optimized nLC-MALDI-MS/MS

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In comparison with the standard protease trypsin, less specific proteases are able to cleave also in hydrophobic transmembrane regions. The applicability of these proteases to the identification of membrane proteins has been demonstrated. However, the resulting peptides are often hard to analyze using MALDI as ionization technique. The high number of theoretically possible peptides produced by unspecific cleavages is additionally complicating MASCOT MS/MS searches. In this work we present the optimized nLC-MALDI-MS/MS analysis of yeast plasma membrane digests using elastase, chymotrypsin, pepsin and trypsin and additional TMTzero-labeling of peptides. The impact of combining the information of all digests and the improved detection and fragmentation of hydrophobic peptides after labeling was investigated.

**Method:** Yeast plasmamembrane samples were aliquoted and separately digested with chymotrypsin, elastase, pepsin and trypsin. One half of each sample was labeled with TMTzero (Thermo Scientific, Bremen, Germany) according the manufacturer's protocol. Separation of all samples was performed using an Easy nLC system (Proxeon Biosystems, Odense, Denmark) coupled to a SunCollect MALDI spotter (Sunchrom GmbH, Friedrichsdorf, Germany). Spotting was conducted alternatingly on two different vendors' targets. MALDI MS and MS/MS measurements were performed on a 4800 TOF/TOF Analyzer (AB Sciex, Darmstadt, Germany). MS measurements on a MALDI LTQ-Orbitrap XL (Thermo Scientific, Bremen, Germany) additionally gave accurate precursor masses. Both datasets were later on combined. Identification was done using the MASCOT database search engine.

**Preliminary Data:** The high precursor mass accuracy resulting from the combined MALDI TOF/TOF and Orbitrap datasets led to a high number of identified peptides and proteins. Using trypsin as a protease, mostly extracellular and cytoplasmic regions of membrane proteins were identified, whereas the examined less specific enzymes gave peptides from all regions. The additional labeling with TMTzero led to an improved identification of small, hydrophobic and acidic peptides. Combining all identified peptides, the sequence coverages of different membrane proteins could be drastically increased.

**Novel Aspects:** Combination of different enzymes, additional TMTzero labeling and optimized nLC-MALDI-MS/MS measurement leads to increased membrane protein sequence coverage.

# MALDI mass spectral analysis of 10-thioalkyl colchicine derivatives

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Many naturally occurring compounds have both medicinal and poisoning properties but effect on human body depends on dose. One of such compounds is colchicine occurring in plants of Liliaceae family. Colchicine is considered as a classical antimitotic factor [1-2] and therefore is used as a promising chemotherapeutic agent. Its activity is depending on the ability of creating noncovalent complexes with macromolecules such as tubulin in microtubules. It is known that colchicine is able to interfere with the structure of mitotic spindle and hence inhibit mitosis. This is one of the reasons that colchicine and some of its derivatives are useful as anticancer drug components.

**Method:** Till now only colchicine and its interaction with tubulin were examined by MALDI mass spectral techniques [3-4]. This fact has prompted us to examine the series of other biologically active colchicine derivatives. 10-thioalkylcolchicine derivatives were examined by MALDI MS techniques. Three different kind of matrices (2,5-dihydroxybenzoic acid, sinapic acid and dithranol) were used. The measurements, including MS/MS experiments, have been accomplished on Waters Q-TOF Premier instrument, equipped with nitrogen laser MALDI source and MassLynx™ software.

**Preliminary Data:** In this report we examined a series of colchicine derivatives, i.e. 10-thioalkylcolchicines 1-5 (Fig. 1), which have been obtained as model compounds of changed geometry in comparison with colchicine itself. The introduced alkyl chain is likely to change the key-lock binding of the compounds obtained in the living cells. Prior to test their interactions with macromolecules (for example proteins) we used MALDI-TOF and MALDI-Q-TOF mass spectrometry to examine the mass spectral behavior of the compounds 1-5. From the matrices used – DHB, SA and DIT – the last one appeared to give the best response and signal-to-noise ratio. The MS/MS experiment with dithranol as a matrix has been performed and the collision-induced fragmentation of protonated ions $[M+H]^+$ has been analyzed. The data obtained allow to estimate the stability of precursor ions chosen in relationship with the substituent structure

**Novel Aspects:** and seem to be useful for the future applications.

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# Matrix-assisted laser desorption/ionization mass spectral analysis of new sugar receptors, formylphenylboronic hydrazones, and matrix effect in their MALDI mass spectra

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Boronic acid derivatives are well-known as sugar receptors [1] and organic synthetic reagents used to Suzuki-Miyaura cross-coupling reactions [2] as well as Petasis synthesis of α-amino acids [3]. The need for sugar (especially glucose) sensors is continuously growing nowadays due to the rising number of patients with *Diabetes mellitus*, long-term disease, resulting in many organism disorders, like cardiovascular, ophthalmic and mental sufferings. Early detection of raised glucose level in blood and physiological media is a very important and demanding task. Specifically, water-soluble sugar-binding compounds are wanted to construct sugar sensors which can be easily usable.

**Method:** A series of six (1-6) (*ortho-*, *meta-* and *para-*)formylphenylboronic acids hydrazones of Girard T and Girard P reagents (Fig. 1) has been analyzed by means of MALDI mass spectrometry and the influence of matrix choice on the mass spectra has been considered. DHB, dithranol, HABA, CHCA and other matrices have been tested to obtain the best results. The spectra have been obtained on the Waters Q-ToF Premier mass spectrometer.

**Preliminary Data:** In this report we present a series of formylphenylboronic acids derivatives which possess ionic structure and hence are well soluble in water. The quaternary nitrogen atom in such molecules bears a positive charge and this feature enables a deposition of these molecules on ionic layers.

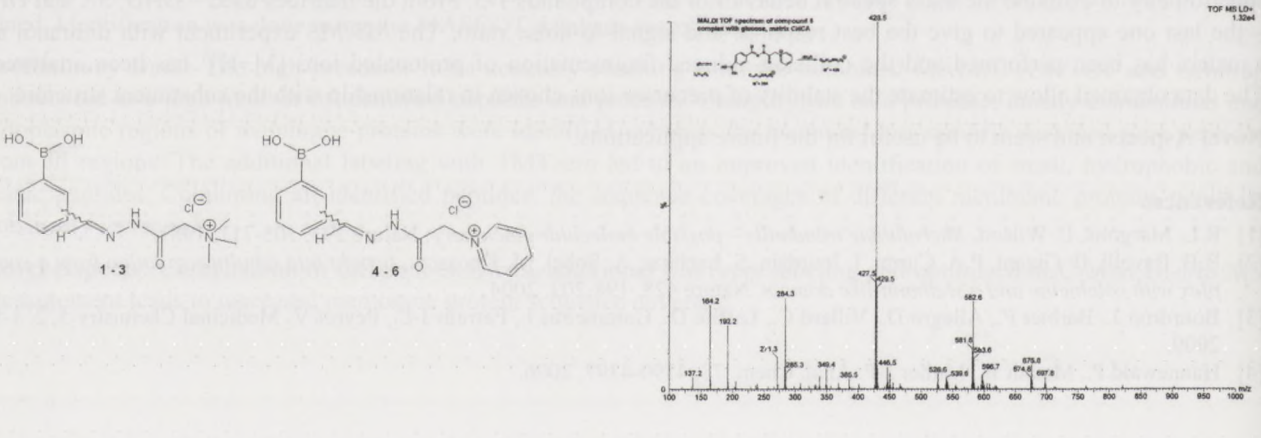


Fig. 1. Structures of compounds 1-6

The interactions of the compounds 1-6 with sucrose has been studied and interesting results obtained will be discussed in more detail.

**Novel Aspects:** The report presents MALDI MS analysis of novel sugar receptors. The optimal matrix is chosen and interactions with sugar shown

Supported by the Ministry of Science and Higher Education (grant number N 204 028636) in the years 2009-2012.

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# Positive ion MALDI-TOF mass spectra are more suitable than negative ion spectra to characterize sulphated glycosaminoglycans

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Glycosaminoglycans (GAG) such as hyaluronan (HA) or chondroitin sulfate (CS) are important constituents of the extracellular matrix (ECM) [1]. Since the discovery of the "sulphation code", particularly (over)sulphated polysaccharides are experiencing considerable interest [2]. Using MALDI-TOF MS, the majority of oligosaccharides can be investigated with only a minimum extent of fragmentation. However, significant but unwanted sulphate loss occurs if the negative ion mass spectra of sulphated carbohydrates are recorded. Using differently sulphated oligosaccharides of the CS type it will be shown that this problem can be reduced by recording the positive ion spectra in the presence of common DHB (2,5-dihydroxybenzoic acid) matrix with only slightly reduced sensitivities in comparison to the negative ion mode.

**Method:** The used HA tetrasaccharide (HA-4) was from Hyalose (Oklahoma City, USA), an oversulphated chondroitin disaccharide (Δdi-trisulphate CS) was purchased from DEXTRA (Reading, UK), while the CS tetrasaccharide (CS-4) was obtained by hyaluronidase digestion of the native CS polysaccharide (from bovine trachea) and subsequent purification by TLC [3]. The tetrasaccharide was scraped from the silica gel and re-eluted with water. Its concentration was determined by using a modified carbazol method. The oligosaccharides were analyzed by negative and positive ion MALDI-TOF MS (Bruker-Daltonics, Autoflex$^{TM}$) using 9-aminoacridine (9-AA) and 2,5-dihydroxybenzoic acid (DHB) as matrices in the negative and positive ion modes, respectively.

**Preliminary Data:** The aim of this study was (a) to investigate to which extent and under which conditions the loss of sulphate [4] from GAG oligosaccharides may be suppressed and (b) to investigate the suitability of DHB and 9-AA as MALDI matrices for the analysis of sulphated oligosaccharides. HA-4, the only GAG without sulphate residues, can easily be identified in both detection modes, whereby the negative ion mode provides slightly higher sensitivity. Regarding the detection of the CS-tetrasaccharide, 9-AA is the matrix of choice in the negative ion mode because all the intensity is collected in a single peak leading to higher sensitivity. However, the intact ion is not detectable but only a fragment subsequent to sulphate loss. In contrast, 9-AA is no suitable matrix for the detection of CS-4 in the positive ion mode. It is surprising that the positive ion mass spectrum (recorded in the presence of DHB) gives the by far best results regarding the detection of the intact CS-4 ion: under these conditions only a small extent of sulphate loss was detectable. This result could be also confirmed by the investigation of even higher sulphated carbohydrates (three sulphate residues) of the CS type that are commercially available. It is concluded that the positive ion MALDI mass spectra in the presence of DHB are the method of choice to detect the intact sulphated carbohydrates with only a moderate extent of sulphate loss. This is particularly important if mixtures of compounds with different sulphation patterns are to be characterized. Thus, the use of liquid crystalline matrices (alone or in combination with CsCl [4]) is not an absolute necessity.

**Novel Aspects:** Detection of intact sulphated CS tetrasaccharides

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# Preserving the chromatographic resolution during the MALDI-spotting process

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Liquid chromatography matrix-assisted laser desorption/ionization (LC-MALDI) has been established in the past as a simple and robust method and has mostly been used to identify proteins in proteomic workflows. When coupling LC and MALDI the first and crucial question is whether the chromatographic resolution is maintained on the way of the chromatographic eluent to the MALDI target and not affected by the conventionally used admixture of matrix solution [1].

**Method:** A tryptic BSA digest was separated on an EASY n-LC (Proxeon) coupled to a MALDI spotting device (SunChrom). In-house packed columns were used with rp18 material. The flow rate was 500 nL/min. The gradient profile was 60 minutes 8-45% B (92% acetonitrile, 0.1% TFA) and 10 minutes up to 90% B. Spotting was carried out in two ways, (A) conventionally using a MicroTee (Upchurch) for admixing matrix solution (CHCA, 3 mg/mL) and spotting every 10 s or 20 s resp., and (B) by direct spotting of the eluent (every 10 s) to the MALDI target – the matrix (CHCA, 1 mg/ml) was spotted in a second step onto each sample spot [2, 3]. All MALDI measurements were carried out using a 4800 TOF/TOF Analyzer (ABSciex).

**Preliminary Data:** The resolution of peptides on the MALDI target could be maintained and even improved, if a spot was produced every 10 s, both for direct and MicroTee spotting. Typical elution time widths of a peptide is between 30 s and 3 min, depending on the particular peptide [2]. Equal or higher signal intensities could be observed for direct spotting. Tailing was reduced by removing the MicroTee. In summary, the direct spotting method (similar to the sandwich method [3]) showed the highest intensities and also the best chromatographic resolution.

**Novel Aspects:** Direct spotting without online matrix admixture yields improved resolution and higher signal intensities and sensitivity.

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# Radiation-related changes in serum proteome profiles detected by MALDI-MS in blood of patients treated with radiotherapy

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Mass spectrometry-based analyses of the low-molecular-weight fraction of serum proteome allow identifying proteome profiles/signatures that are potentially useful in monitoring of response of cancer patients to the treatment. The study was aimed to detect features of human serum proteome that were associated with radiotherapy-related exposure to ionizing radiation.

**Method:** Analyzed group consisted of 46 patients treated with radical radiotherapy because of larynx cancer; patients were irradiated with total doses in a range from 51 to 72 Gy. Three consecutive blood samples were collected from each patient: before the start, 2 weeks after the start, and 1-2 months after the end of radiotherapy. The low-molecular-weight fraction of the serum proteome (2,000-14,000 Da) was analyzed by the MALDI-ToF mass spectrometry.

**Preliminary Data:** Proteome profiles of serum samples collected before the start of radiotherapy and during early stage of the treatment were similar. In marked contrast, mass profiles of serum samples collected several weeks after the end of the treatment revealed clear changes. We found that 41 out of 312 registered peptide ions changed their abundance significantly when serum samples collected after irradiation with a total dose were compared with samples collected at two earlier time points. We also found that abundance of certain serum peptides was associated with total doses of radiation received by patients. The results of this pilot study indicate that features of serum proteome analyzed by mass spectrometry have potential applicability as a retrospective marker of exposure to ionizing radiation.

**Novel Aspects:** Serum proteome analyzed by mass spectrometry have potential applicability as a retrospective marker of exposure to ionizing radiation.

# Increasing sequence coverage in proteomics studies

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Mass spectrometry coupled to reversed phase high performance liquid chromatography has become a common analytical technique in proteomics. Usually, the extracted proteins are digested with a suitable protease and the peptide mixture is separated and analyzed. Commonly, trypsin is the enzyme of choice for proteomics experiments. Digestions with trypsin (or any single enzyme) often result in the identification of large number of proteins, nevertheless with incomplete sequence coverage. If maximum sequence coverage is desired (e.g., studying changes in protein modification or different isoforms), then signals covering ideally all of the protein sequence is needed. Different approaches have been used to improve protein sequence coverage in proteomics. In this study, three different enzymes were used to significantly improve sequence coverage of proteins.

**Method:** Purified mitochondrial membrane proteins from mouse brain were dissolved in 25 mM triethylammonium bicarbonate buffer. Disulfide bridges were reduced in dithiothreitol, alkylated with iodoacetamide and digested with trypsin, chymotrypsin and elastase. Digestion was stopped by freezing at -20°C. The samples were separated on a 15 cm revered phase C-18 column using a nanoflow HPLC. All MS and MS/MS spectra were acquired in positive ion mode using quadrupole-Orbitrap mass spectrometer (Q Exactive, Thermo Fisher Scientific, Bremen, Germany).

**Preliminary Data:** Trypsin, chymotrypsin and elastase are all classified as serine-proteases but their specificity is differs due to the other amino acids present at the base of their respective S1-pocket. While trypsin cleaves exclusively at C-termini to arginine and lysine, chymotrypsin cleaves at C-termini of hydrophobic residues, such as tyrosine, phenylalanine and leucine. Elastase prefers C-termini of residues such as alanine, valine, leucine, isoleucine, serin and threonine. Less specific nature of elastase and chymotrypsin is especially useful when analysing proteins with limited number of R and K in the protein sequence such as membrane proteins. Quadrupole-Orbitrap instrumentation provides rich fragmentation of peptides/proteins. Coupled with the high resolution and high mass accuracy in both MS and MS/MS, such approach enables very reliable identifications, especially for peptides generated using less specific enzymes. The three different enzymes used in the study provided peptides with overlapping sequence enhancing the overall sequence coverage. Depending on the proteins, the increased in sequence coverage varied between 10 and 60%. In total, over 10000 unique peptides were identified. Less than 1% of all the identified peptides were present in all 3 enzymes. About 33% of the identified proteins were detected in triplicate runs of all three enzymes. The detection of particular proteins in all three enzymatic preparations further strengthens quality of the identification results.

**Novel Aspects:** Enhancing sequence coverage in proteomics studies by a combination of three digestion enzymes and high resolution MS and MS/MS analysis.

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# A new Method for the Analysis of 2-AA labeled N-glycans from mAbs by High Resolution RP Chromatography and Mass Spectrometry

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Biopharmaceuticals belong to the most complex pharmaceuticals on the market. The N-glycosylation of these proteins may have effects on the efficacy and safety of the drugs. Therefore, a comprehensive characterization of the glycosylation is necessary [1]. Released N-glycans can be labeled with a fluorescence dye to improve chromatographic separation and to enable UV or fluorescence detection. Using reversed phase chromatography instead of the widely used HILIC enables coupling the LC on-line to a mass spectrometer to identify the eluting N-glycans without ion-suppression through buffer components [3]. 2-AB (2-Aminobenzamid) is the most common used fluorescence reagent [2-4]. We studied the use of 2-AA-label (Anthranilic acid) which potentially offers several advantages like the improved retention and higher resolution in RP-HPLC.

**Method:** N-glycans are released enzymatically from monoclonal antibodies using PNGase-F. Released N-glycans are labeled with 2-AA. Excess label is removed by gel filtration. Purified 2-AA glycans are analyzed using an Agilent 1200 Series HPLC system on-line coupled to an ion-trap mass spectrometer (Bruker AmaZon). The glycans are separated on a Waters ACQUITY UPLC BEH130 C18 column (2.1 mm × 150 mm; 1,7 µm particle size) at a flow rate of 300 µl/min. Mobile phase A consists of 1.0% formic acid in H2O and mobile phase B consists of 1.0% formic acid in 25% ACN in H2O. The mass spectrometer is run in positive ionization mode.

**Preliminary Data:** Previously performed investigations showed the feasibility of RP-HPLC and on-line MS for 2-AB labeled glycans [2]. The use of 2-AA instead of 2-AB together with the more acidic mobile phase showed a similar chromatographic pattern. The glycans eluted in groups, high mannose glycans before non-fucosylated hybrid and biantennary, followed by fucosylated hybrid and biantennary structures. Additionally, the acidic N-glycans were separated according to their neutral group without the need of an ion pairing reagent. The improved binding of the 2-AA glycans to the column allows a steeper gradient resulting in a much shorter run-time and sharper peak profiles. Compared to HILIC chromatography, the "gold standard" for glycan analytics, the new RP-HPLC method offers several advantages: (I) a higher injection volume (e.g. 100 µl) can be injected resulting in higher sensitivity, (II) in contrast to previous investigations the separation of the G1F isomers is achievable, (III) the glycans can be easily identified by MS due to the good MS compatibility of the mobile phase, and (IV) due to the improved retention of the 2-AA labeled glycans, a faster gradient can be used resulting in a shorter run-time. The 2-AA glycans generated predominately double and triple charged $[M + 2H]^{2+}$/$[M + 3H]^{3+}$ ions which can be structurally characterized by $MS^n$ (fragmentation by CID) using ion-trap mass spectrometry in the positive ionization mode.

**Novel Aspects:** Development of a RP-LC/MS method for the analysis of acidic and neutral 2-AA labeled glycans with positive ionization MS.

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# A Novel Platform for Top-Down Proteomics: Chip-Based Nanoelectrospray Combined with Electron Transfer Dissociation Mass Spectrometry

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Recent introduction of electron transfer dissociation (ETD), performed on versatile and ultra-fast trapping instruments, is regarded as an important step with real perspectives in the field [1, 2]. Applied together, ETD and collision-induced dissociation (CID) may significantly increase the sequence coverage and provide added confidence to peptide/protein identification. In the present work, chip-nanoelectrospray (nanoESI) performed on a NanoMate robot was combined for the first time with ETD on a high capacity ion trap (HCT) MS to yield an analytical platform on which top-down peptide/protein analysis by ETD is routinely possible.

**Method:** MS experiments were conducted on a HCT Ultra mass spectrometer (Bruker Daltonics, Bremen, Germany) incorporating an ETD module with fluoranthene as anionic reagent. Fully automated chip-based nanoESI was performed on a NanoMate robot incorporating ESI 400 Chip technology (Advion BioSciences, Ithaca, USA). The robot was coupled to the HCT via an in laboratory made mounting system as described by us before [3, 4].

**Preliminary Data:** Feasibility of the novel approach for peptide identification was first tested on a standard substance P neuropeptide (MW 1347.63 Da). The platform was further applied to a complex synthetic peptide: gonadotropin-releasing hormone (GnRH)-III linked via amide bond to Daunorubicin (Dau)-GFLG, (MW 2257.32 Da). ETD on the $[M + 3H^+]^{3+}$ ion at m/z 753.32 produced two fragmentation processes: a) sequencing of the peptide backbone supported by the c- and z-type of ions as well as by the ions occurring via double cleavage of the peptide bonds; b) N-Cα cleavage of the NH-CO linkage between the peptide and Dau moiety, with the preservation of the labile glycosidic bond. Such a specific fragmentation, accompanied by the formation of $[M + 2H^+]^{2+}$ product ion corresponding to Dau at m/z 257.53 enhanced the further characterization of Dau moiety by a subsequent CID experiment using the alternate ETD/CID mode available on the HCT MS instrument. Using NanoMate-HCT, the first top-down protein analysis by ETD could be demonstrated on a medium-size protein, such as standard horse apomyoglobin (MW 16.95 kDa). In this experiment, of major importance is the molecule multiple protonation under chip-nanoESI conditions which is a fundamental prerequisite of a successful top-down fragmentation by ETD. For the experiment using $[M + 16H^+]^{16+}$ ion detected at m/z 1060.32 as the precursor, the fragmentation by ETD $MS^2$ could be successfully accomplished within only 30 s and with a sample consumption of approximately 12 fmols. ETD mass spectrum summed over the scans acquired for 30 s yielded a number of c- and z-type of product ions corresponding to 80% coverage of apomyglobin sequence. All these results permit a positive estimation regarding the perspectives of the method and its chances to become a tool of routine use in proteomics.

**Novel Aspects:** The novel NanoMate-ETD combination allows reliable identification of peptides and proteins by high throughput top-down experiments of significantly increased sensitivity

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# Advanced LC-MS/MS Tools to Screen for Targeted and Non-targeted Contaminants in Food Samples

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Liquid Chromatography coupled to tandem Mass Spectrometry (LC-MS/MS) is a powerful analytical tool for the analysis of polar, semi-volatile, and thermally labile compounds of a wide molecular weight range, such as pesticides, veterinary drugs, mycotoxins and other food residues. Mass analyzers based on triple quadrupole technology operated in Multiple Reaction Monitoring (MRM) mode deliver highly selective and sensitive quantitative results and are therefore well established for multi-target screening and quantitation of food contaminants. Compound identification is typically performed by monitoring of two MRM transitions and calculating the area ratio of quantifier and qualifier ion. The potential risk of false positive results can be further minimized by the acquisition of full scan MS/MS for each compound detected.

**Method:** However, the use of triple quadrupole based mass analyzers is limited to targeted screening and quantitation. But there is an increasing demand for retrospective and non-targeted data analysis. High resolution and accurate mass instruments are capable of performing targeted and non-targeted screening in a single LC-MS/MS run.Here the AB SCIEX TripleTOF™ 5600 system was used to acquire highly sensitive MS and information dependent MS/MS spectra throughout the LC run. Chromatograms were automatically explored and information on retention time, mass accuracy, isotopic pattern, and MS/MS library searching was used to quantify and identify target analytes with highest confidence. The LC-MS/MS data was further used to retrospectively search for non-targeted and unexpected compounds.

**Preliminary Data:** MS/MS spectra contain the complete molecular fingerprint of a molecule and thus providing an added degree of confidence for compound identification. Here a variety of food samples were analyzed for pesticides, veterinary drugs, mycotoxins and other chemical residues. A fast and generic procedure was used to extract contaminants of various classes. Extracts were subsequently analyzed by LC-MS/MS using a polar embedded C18 phase and different LC-MS/MS techniques. Different analytical setups will be presented and discussed with respect to targeted and non-targeted residue screening.

**Novel Aspects:** Generic QueChERS extraction plus high resolution accurate mass LC-MS/MS.

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# Affinity binding studies of synuclein with anti-synuclein antibodies by online combination of surface acoustic wave biosensor and mass spectrometry

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The surface acoustic waves (SAW) biosensor is a newly developed method able to detect the bioaffinity interaction between two molecular species [1]. Here we describe the combination of SAW biosensor and electrospray ionisation mass spectrometry (ESI-MS), as a highly sensitive technique recently developed in our laboratory which allows the structural identification of affinity-bound ligands together with $K_D$ determination in a wide range of affinities [2].

**Method:** In our work, the anti-synuclein specific antibodies were covalently immobilised on the gold coated surface of the quartz chip, using as a linker a self-assembled monolayer (SAM) of 16-mercaptohexadecanoic acid. The interactions with α-synuclein (αSyn), αSyn (59-80), (1-23), (61-140), (96-140), (1-120), (72-140) peptide antigens were determined. Further, the affinity bound antigens were eluted under acidic conditions (pH 2) and analysed by ESI mass spectrometry using an intermediate sample desalting and concentration step on a guard column. Molecular interaction kinetics between αSyn immobilized on a gold chip and different concentrations of anti-α-synuclein mono/polyclonal antibodies were determined by SAW biosensor.

**Preliminary Data:** The obtained $K_D$ values were in the low nano-molar range. The bioaffinities were comparatively investigated by Dot Blot, ELISA, SAW biosensor and SAW-ESI MS, and the results were found to be in good agreement. Moreover, the SAW biosensor has been employed as an affinity detection method in conjunction with MALDI-MS for epitope determination of αSyn. The SAW-ESI-MS was also applied to the direct analysis of αSyn A30P from mouse brain extracts, indicating this method as a powerful new tool to the molecular characterization of intermediates of protein aggregation.

**Novel Aspects:** This method is a powerful new tool to the molecular characterization of intermediates of protein aggregation.

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# An ion mobility-enabled data independent multi-omics approach to quantitatively characterize urine from children diagnosed with idiopathic nephrotic syndrome

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Synopsis: Idiopathic nephrotic syndrome (INS) is the most prevalent glomerular disease in children. Despite ongoing progress, its pathogenesis is still unknown and the therapy options are confined to gross immune modulation. A variety of methods for diagnostic and treatment purposes are available for the patients; however, the lack of understanding regarding the pathogenic mechanisms underlying INS can lead to poor therapeutic response and adverse side-effects. Here, we describe quantitative proteomic approach to reveal new molecular factors involved in pathogenesis of INS with potential diagnostic and therapeutic significance.

**Method:** Methods: Urine samples were collected from 10 children diagnosed with INS receiving no therapy, and 10 healthy children. Samples were affinity depleted of albumin before digestion with trypsin. Label-free protein expression data were acquired with Synapt G2 using an ion mobility data independent approach, whereby the collision energy was switched between low and elevated energy state during alternate scans and associate precursor and product ions by means of retention and drift time alignment. Data were processed using ProteinLynx Global Server and searched against a human database. Normalized label-free quantitation results were generated using Progenesis LC-MS. In a similar fashion, diluted neat urine samples were analysed using a small molecule profiling approach. The resulting data was analysed using MarkerLynx XS.

**Preliminary Data:** Results: The results of this study showed a significant number of proteins to be over-expressed in the urine from INS patients. More interestingly, the majority of proteins identified as being regulated were secreted glycosylated proteins (e.g. zinc-alpha-2-glycoprotein and cerruloplasmin) in relatively low abundance. Metabolomics analysis showed some statistically significant changes in small molecule content, with homocysteine and uridine elevated, and glutamate, indoline, phenylalanine and glucose reduced in the urine of INS patients. These proteins and small molecules are biomarker candidates that may prove to be useful in the monitoring and treatment of INS.

**Novel Aspects:** These proteins and small molecules are biomarker candidates that may be useful in the monitoring and treatment of INS.

# Analyis of Perfluoroalkylacids and Separation of the Branched Isomers of Perfluorooctanesulfonate

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Perfluoroalkylacids (PFAA) are used in a wide range of applications, because of their strong stability and their inert and non adhering surface properties. But it was observed, that perfluoroalkylcarboxylic acids (PFCA) and perfluoroalkanesulfonic acids (PFSA), in particular perfluorooctanoic acid (PFOA) and perfluorooctanesulfonate (PFOS) are persistent, bioaccumulative and toxic in animal studies. There is a lack of data for the longer and shorter chain PFAA and thus there is need for data on the occurrence of these substances. Especially in the case of PFOS, not only the linear compound but also a considerable amount of branched isomers exist. This may cause problems, because each laboratory has its own method to handle the branched isomers. This paper shows the separation of these compounds using HPLC-MS/MS.

**Method:** Matrix specific preparation methods were developed. Sample solutions were purified and concentrated using solid phase extraction.
*Linear PFAA.* HPLC-System: Agilent 1200 SL; Column: Gemini (3 µm, C18, 110 A, 150 × 2 mm) from Phenomenex; Elution: Gradient elution (Solvent A: Ammoniumacetate 2 mmol/l; Solvent B: Methanol/Acetonitril 60/40).
*Branched isomers of PFOS.* HPLC-System: Agilent 1200 SL; Column: PRONTOSIL 200-3-C30 3.0 (250 × 3 mm) from Bischoff Chromatography; Elution: Gradient elution (Solvent A: Ammoniumacetate 7 mmol/l, pH 8; Solvent B: Methanol). MS/MS-detection was performed with an Agilent 6460 triple quadrupole mass spectrometer equipped with an electrospray interface (ESI) operating in the negative ion mode. MRM settings for linear PFAA are published elsewhere [1]. Quantification was performed using isotope labeled internal standards (Wellington Laboratories).

**Preliminary Data:** Good recoveries for PFCA with six to twelve carbon atoms and PFSA with four, six, seven, eight and ten carbon atoms could be achieved. Because of the surface activity of the PFCA and PFSA, it was important to minimize a potential carry-over from Teflon containing parts of the analytical instrument and/or from highly contaminated samples as far as possible. This is successfully performed by using a trap column between pump and autosampler and an injector-program. By using a mixture of methanol and acetonitril as one solvent component, it was possible to separate all PFAA of interest chromatographically from each other. The limits of quantification were low, despite of the strict criteria of a signal to noise ratio of 3:1 of the second intensive transition at the limit of detection according to SANCO 10684/2009. Not only in the case of PFOS but also in the case of other PFAA, such as perfluorohexanesulfonate and perfluoroheptanesulfonate, a considerable number of branched isomers exist. The focus here is on the branched isomers of PFOS, because only for this PFSA branched isomer standards are commercially available. For determination of Perfluoro-1-methylheptane sulfonate, Perfluoro-4,4-dimethylhexane sulfonate and Perfluoro-5,5-dimethylhexane sulfonate specific transition could be used, but it was necessary to separate the other branched isomers chromatographically from each other, because six substances were determined using the transition m/z 499 to 80. Good separations could be achieved by using a C30 phase and a pH value of 8. The separation of perfluoro-3-methylheptane sulfonate and perfluoro-6-methylheptane sulfonate is very difficult.

**Novel Aspects:** Sensitive analysis of PFAA with different chain length and separation of PFOS-Isomers are possible despite strict quality criteria.

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# Analysis of changes in human endothelial cell and mouse liver proteomes caused by homocysteine thiolactone

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Severe hyperhomocysteinemia caused by genetic or nutritional deficiencies in homocysteine (Hcy) metabolism leads to cardiovascular and neurological pathologies. Hcy is formed from dietary methionine. In addition to its metabolism via transsulfuration and transmethylation pathways, Hcy is metabolized to Hcy-thiolactone (HTL) [1]. HTL is chemically reactive and modifies protein lysine residues in a process called *N*-homocysteinylation, which affects protein structure and function [2], causes cellular toxicity, elicits an autoimmune response, and generates pro-thrombotic *N*-Hcy-fibrinogen [3]. Two enzymes hydrolyze HTL and protect against protein *N*-homocysteinylation: extracellular Hcy-thiolactonase/paraoxonase 1 (PON1) [4] and intracellular Hcy-thiolactonase/bleomycin hydrolase (BLMH) [5]. To identify molecular bases underlying pathology of hyperhomocysteinemia, we studied changes in cellular proteome caused by HTL using HUVECs and *Blmh* and *Pon1* knockout mouse models.

**Method:** HUVECs were treated with HTL and the changes in cellular proteome were examined by 2-dimensional IEF-SDS-PAGE. In addition, we examined how the treatment of HUVECs with *in vitro* prepared *N*-Hcy-protein affects cellular proteome. Proteins were extracted from cells in rehydration solution containing 8 M urea, 2 M tiourea and 2% Chaps. We also induced hyperhomocysteinemia by feeding a high methionine diet (for 8 weeks) in wild type mice and *Blmh* and *Pon1* knockouts, which are deficient in HTL hydrolysis. Mice were killed by CO2, livers collected, frozen at -80°C, and liver proteins extracted using the phenol method. Protein extracts were analyzed by 2-dimensional IEF/SDS-PAGE. Differentially expresses proteins were excised from gels, trypsinized, and identified by MALDI-TOF analysis.

**Preliminary Data:** In HUVECs treated with HTL, the expression of several proteins was decreased (AnexinA6, Anexin5, Anexin3, Plastin3, HSP60), while the expression of other proteins increased (vimentin, UCHL1, TXNDC5 protein). When HUVECs were incubated with *N*-Hcy-protein there was a significant increase of expression of several proteins (A-glucosidase, dnaK-type molecular chaperone, chlorine intracellular channel protein1). In mouse liver we found 5 differentially expressed proteins. Glyoxalase domain-containing protein 4 was found only in *Blmh*-/- mice. The expression of two proteins was decreased by the inactivation of the *Blmh* gene: apolipoproteinA precursor and catechol-O-metylotransferase. Inactivation of *Pon1* increased expression of ferritin light chain 1 and apolipoproteinA precursor. High-Met diet elevated the levels of peroxiredoxin-2 in mouse liver, regardless of the *Blmh* or *Pon1* genotype.

**Novel Aspects:** Proteins whose expression is affected by HTL identify specific biochemical processes whose perturbation might contribute to the pathology of hyperhomocysteinemia.

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# Analysis of FDG (2-deoxy-2-fluoro-D-glucose) fate in the plant cells

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FDG (2-Fluoro-2-deoxy glucose) is glucose analogue which is used in clinical biology as an in vivo glucose radiotracer [1]. It mimics the glucose distribution and after uptake it mainly converts to FDG-6-phosphate which remains in the cell cytoplasm without getting metabolized further to large extent [1]. There have been numerous papers describing the fate of FDG to various metabolic end products like FDG-6-P, FDG-glucononate, FDG-1,6-BiP in the animal cells [2]. However, there has been very little progress in exploring FDG as a radiotracer in plant systems and thus we have been trying to see the applicability of FDG in the plant imaging and also analyzing the FDG transportation and metabolic fate inside the plant system.

**Method:** Plant system: *Arabidopsis thaliana*, 18-FDG (radioactive), 19-FDG(non-radioactive), Orbitrap MS, radio-imaging plates. For imaging, *A. thaliana plants* (rosette stage, 25 day old plants) were fed with 18-FDG (radioactive) through leaves/roots and later imaged with imaging plates to see the distribution of FDG to various other parts. The plant cell suspension were fed with 19-FDG under dark condition and later extracted for soluble sugars with 80% ethanol extraction procedure. The extracts were analyzed for the presence of $^{19}F$ containing compounds by LC/MS/MS (UPLC, Orbitrap).

**Preliminary Data:** Radiotracer Imaging: Imaging plate pictures indicates the transport of the FDG to various plant parts from the FDG fed part. The extent of FDG transportation to other parts is not very high but its distribution clearly showed its tendency to accumulate in young growing parts like apex and roots when fed through leaves [3]. MS analysis: Exact masses for FDG-6-P, FDG-glucononate, sucrose-FDG-6-P conjugate and also UDP-glucose were detected; however, they need to be confirmed using CID spectra.

**Novel Aspects:** Elucidation of FDG transportation and metabolic fate in plant cells.

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# Use of Q-TOF GC-MS to quantify PAH, PCB, and other environmental contaminants in SPMD river water and marine sediments

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Surface water quality remains an area of interest since it is often a source of drinking water and marine life found in the food chain. Analysis of extracts of sediment from river water by GC-MS generates a large number of unknown compounds that need to be identified and subsequently quantified.

**Method:** The complex organic matrix found in sediment samples makes the identification and quantification by low resolution MS difficult.

**Preliminary Data:** In principle, MS can used to elucidate the de novo structure of unknown compounds beginning with the determination of molecular formula of the molecular ion, using accurate mass measurements that have errors less than a few parts per million (ppm). Mass measurements obtained with errors below 2 ppm combined with the isotopic distribution of the mono-isotopic peak can be used to eliminate many of the possible molecular formulae. Quantification requires the exclusion of the interfering matrix ions and thus narrow mass windows for extracted ion chromatographs and extreme mass stability; as well as a large linear range. This poster demonstrates the use of exact mass measurement using a GC-QTOF MS to quantify several classes of pollutants commonly found in sediment extracts of river water.

**Novel Aspects:** This poster demonstrates the use of exact mass measurement using a GC-QTOF MS

# Simultaneous Determination of Metabolic Stability, Metabolite Identification and Profiling Using the Agilent 6550 iFunnel Q-TOF LC/MS System

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Timely and rapid assessment of metabolic stability, metabolite identification and profiling is critical for accelerating lead optimization and enhancing the success rate of drug candidates entering into drug development.

**Method:** Triple quadrupole LC/MS instruments using multiple-reaction-monitoring (MRM) have been the workhorse for quantitative analysis such as metabolic stability and profiling. However, this platform is optimized for high sensitivity target quantitation and not well suited for non-targeted qualitative analysis.

**Preliminary Data:** For these reasons, metabolite identification (qualitative) is often performed in a separate analysis on different types of mass spectrometers. Furthermore, due to the limitation of sensitivity on traditional tandem mass spectrometers, a relatively high substrate concentration (i.e.10-20 µM) is often required in order to identify metabolites with a broad coverage. The ability to obtain quantitation and identification in a single analysis makes metabolic stability, metabolite identification and profiling studies much more efficient. This also has the potential to increase assay productivity and decrease costs in drug discovery and development. Herein, we present an integrated Qual/Quan workflow. The utility of the Agilent 6550 iFunnel Q-TOF LC/MS system for determination of metabolic stability, metabolite identification and profiling in a single experiment is demonstrated in the in vitro buspirone (1 µM) metabolism study in rat liver microsomes.

**Novel Aspects:** New instrumentation.

# Development of an LC-MS/MS method for the simultaneous determination of tocopherols and tocotrienols in human plasma

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Breast cancer is one of the leading causes of mortality in women. Recent studies, both clinical and pre-clinical, have indicated that vitamin E can play a protective role in breast cancer in women. Vitamin E naturally occurs in four forms: alpha-, beta-, gamma- and delta-tocopherols and four corresponding unsaturated analogues, tocotrienols. Tocopherols and tocotrienols are antioxidants and are believed to play a preventive role in diseases associated with oxidative stress including cancer. A majority of conducted studies have been mostly focused on the role of alpha-tocopherol, thought to be the most biologically important form of vitamin E in breast cancer. However, observational studies have failed to consistently support the theory that alpha-tocopherol provides a protection against this disease.

**Method:** The study presents a development of a rapid assay for tocopherols and tocotrienols simultaneous determination in human plasma using reversed phase high performance liquid chromatography coupled with tandem mass spectrometry (RP-HPLC-MS/MS). The mass spectrometry analysis was carried out by the use of Agilent Technologies system (Palo Alto, CA) 1200 LC with atmospheric pressure chemical ionization interface and Agilent Technology 6430 triple quad. The sample preparation was performed with the use of liquid-liquid extraction technique.

**Preliminary Data:** The separation of four tocopherols and three tocotrienols was successfully achieved with Cosmosil 2.5 πNAP column using RP-HPLC technique. LC and MS/MS parameters were optimized for the future analysis of vitamin E constituents in human plasma. The chromatographic run was carried out isocratically with mobile phase composed of methanol : water (90:10; v/v) over 13.5 min at a flow rate 0.5 ml/min.

**Novel Aspects:** The novelty of the proposed method lays in a simultaneous determination of seven tecopherols and tocotrienols resolved with the use of RP-HPLC.

# Application of Modern Mass Spectrometric Methods in Neuroscience Research: Mapping and Sequencing of Human Brain Gangliosides

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Structural elucidation of individual ganglioside components in mixtures extracted from human brain represents a fundamental requirement for the determination of their composition in defined brain regions and for correlating the specificity of these structures with the specialized function of each area of the central nervous system in health and disease. An important part of the brain's learning and memory system is the caudate nucleus (NC), located within the basal ganglia of the brains of many animal species. Various disorders as: depression, Huntington disease, attention deficit disorder (ADD), schizophrenia, obsessive compulsive disorder (OCD) and PAP syndrome are strongly associated with this part of the brain.

**Method:** In the present work a high-throughput screening of complex gangliosides based on fully automated chip nanoelectrospray (nanoESI) mass spectrometry (MS) was performed. Sequencing of the isolated ganglioside species was accomplished by tandem MS using collision-induced dissociation (CID) at low energies. The native mixture of gangliosides used in this study was extracted from a 42 years-old adult caudate nucleus according to the method of Svennerholm and Fredman [1], as modified by Vukelić et al. [2]. In order to obtain a high-throughput analysis system that integrates automatic chip-based ESI infusion, ultra-fast ion detection and multistage sequencing at superior sensitivity, we used a NanoMate robot for sample infusion [3, 4], coupled to a high capacity ion trap mass spectrometer (HCT MS).

**Preliminary Data:** NanoESI chip $MS^1$ screening in negative ion mode enabled the identification of over 50 glycoforms exhibiting a high degree of heterogeneity in the ceramide motifs and alterations such as fucosylation, *O*-acetylation and lactonization. By this system, a more realistic representation of ganglioside heterogeneity, when compared against the thin-layer chromatography (TLC) pattern, or previously acquired MS data was achieved. The feasibility of chip-based nanoESI HCT multistage CID $MS^n$ for polysialylated ganglioside fragmentation was tested on two interesting brain-associated structures of GD1 and GM1 containing long-chain fatty acids in their ceramide moiety. MS2 obtained by accumulating scans at variable ion excitation amplitudes, gave rise to the first fragmentation patterns from which sialic acid (NeuAc) attachment sites diagnostic for GD1b and GM1a structural isomers could be unequivocally determined without the need for sample derivatization or supplementary investigation by other analytical or biochemical methods. Besides the identification of the sialylation sites, $MS^2$ fragmentation provided a complete characterization of the oligosaccharide sequence and documented the (d18:1/32:1) and (d18:1/34:2) ceramide constitutions for GD1 and GM1, respectively.

**Novel Aspects:** Obtained $MS^2$ data indicated the presence in the NC sample of unusual mono- and disialylated ganglioside species containing long-chain fatty acids.

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# MS-based lipidome and proteome analysis on oxidation products of 1-palmitoyl-2-linoleyl-phosphatidylcholine and their reactions with cysteine, histidine and lysine residues

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Lipids, containing unsaturated fatty acyl residues, are susceptible to oxidation by reactive oxygen species, especially during oxidative stress. The resulting carbonyl-containing lipid peroxidation products can readily react with nucleophilic amino acid residues, changing the structures and functional activities of proteins. This can lead to oxidative stress related disorders. Mass spectrometry (MS) was used to investigate protein adducts with lipid-derived aldehydes, such as hydroxy/oxo-nonenal, malondialdehyde, and acrolein. Nevertheless, reactive lipid-containing carbonyl compounds are poorly investigated. MS analysis of carbonyl compounds usually relies on chemical derivatization to enhance the ionization, while fragmentation of the derivatized compounds in tandem MS should provide structural information and specific fragment ions or neutral losses.

**Method:** Here we present an MS-based study of linoleic acid peroxidation products (LaPP) obtained by incubating linoleic acid and 1-palmitoyl-2-linoleoyl-sn-glycerophosphatidylcholine (PLPC) overnight with or without $H_2O_2$ in the presence or absence of CuCl. The carbonyl-containing products obtained by oxidation of PLPC were derivatized with 2,4-dinitrophenylhydrazine (DNPH), and extracted with to the Bligh and Dyer protocol. Underivatized (hydroxylated carboxylic acids) and derivatized (carbonylated species) LaPP were analyzed by ESI-LTQ-Orbitrap-MS. Under the same oxidative conditions, three synthetic peptides (Ac-PAAPAAPAPAEXTPV-OH; X = Cys, His or Lys) were incubated with linoleic acid and PLPC. As strong oxidative conditions promoted significant peptide oxidation, PLPC was first oxidized under mild conditions (air or Cu(I)), then incubated with model peptides, and finally analyzed by nano-RPC-ESI-Orbitrap-MS.

**Preliminary Data:** The tandem mass spectra of PC-containing LaPP displayed fragment ions characteristic for phosphatidylcholines, derived from neutral losses of trimethylamine, phosphorycholine, esterified plamitic acid and linoleic acid derived peroxidation products. The DNPH-derivatized lipid-containing carbonyls were identified by the mass shift of 180 m/z units compared to the underivatized compounds. With this shotgun lipidomics strategy we were able to identify in total 55 different LaPP of which 26 contained reactive carbonyl groups. The strongest oxidation conditions ($H_2O_2$ and Cu(I), i.e. a Fenton-like reagent) yielded 51 LaPP, whereas air oxidation produced only 12 LaPP. Independent of the oxidation conditions, around half of all LaPP were short-chain (oxidative cleavage) and the others long-chain peroxidation products (oxygen addition). Stronger oxidative conditions caused significant oxidation of the amino acid side chains, as well as oxidative modifications of proline and threonine residues, and peptide backbone cleavage. Furthermore, LC-MS of the model peptides incubated with linoleic acid or PLPC under optimal oxidative conditions identified ten LaPP-derived peptide modifications at lysine, nine at cysteine and only three on histidine residue. Three high molecular weight LaPP, still esterified to the PC backbone, were detected on Lys-containing peptides. Three new LaPP-derived mass shifts (86, 88 and 102 m/z units) were obtained at cysteine residues.

**Novel Aspects:** Combined MS-based lipidomics and proteomics approach providing access to amino acid residues modified by LaPPs.

# Determination of volatile organic compounds as markers for early detection of lung cancer by SPME-GC-TOF/MS

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Volatile organic compounds (VOCs) in exhaled breath provide valuable information about state of health human. Analysis of breath has numerous advantages in comparison with traditional diagnostic methods. It is a non-invasive technique, painless and agreeable for patients. Particularly important from medical diagnostic is the possibility cancers of early detection without necessary using invasive methods. Analysis of breath composition may assistance identify potential markers which in the future will facilitate diagnosis lung cancer. To determination VOCs present in human breath samples are using solid phase microextraction (SPME) and gas chromatography-time of flight-mass spectrometry (GC-TOF/MS) for the extraction, separation and analysis of these samples. GC-TOF/MS technique to rapid determination compounds present in human air, at the level of parts per billion (ppb) is applied.

**Method:** The GC-TOF/MS analysis was performed on gas chromatograph 7890 A (Agilent, Waldbronn, Germany) coupled with spectrometer TruTOF (Leco, St. Joseph, MI, USA) equipped with CP-Porabond-Q (Varian Inc., Middelburg, The Netherlands) 25 m × 0.25 m × 3 μm column. Oven temperature program was as follows: initial 40°C held for 2 min, then ramped at 10°C/min to 140°C and the ramped at 5°C/min to 270°C and held for 5 min. The temperature of the split-splitless injector was 200°C. The acquisition of chromatographic data was performed by means of Chroma TOF software (Leco). A manual SPME holder and carboxen/polydimethylsiloxane (CAR/PDMS) (75 μm) coated fiber (Supelco, Bellefonte, USA) were used for the SPME method.

**Preliminary Data:** The SPME/GC-TOF/MS application to detection compounds in human breath samples were discussed. The technique was applied to determination composition breath the 23 patients with lung cancer and 31 volunteers. The total number of compounds identified in sample of breath equal 55. On the basis of the analysis using GC-TOF/MS compound which enables an indication group of persons with lung cancer was isopropyl alcohol. The statistical analysis allowed to extract the compounds with concentrations level significantly different between groups of healthy persons and patients with cancer diagnosis. Combination of non-parametric test with supervised and unsupervised classification methods enabled to predict five compounds propane, carbon disulfide, 2-propenal, ethylbenzene and isopropyl alcohol which separated two research groups of patients and healthy controls.

**Novel Aspects:** SPME-GC–TOF/MS was used to the determination of VOCs in breath from patients with lung cancer and healthy persons.

# Robust LC-MS/MS method for the determination of simvastatin in human plasma

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Simvastatin, 2,2-dimethylbutanoic acid (1S,3R,7S,8S,8aR)-1,2,3,7,8,8a-hexahydro-3,7-dimethyl-8-[2-[(2R,4R)-tetrahydro-4-hydroxy-6-oxo-2H-pyran-2-yl]ethyl]-1-naphthalenyl ester, is a highly effective cholesterol-lowering agent, which is widely used in the treatment of hypercholesterolemia.

**Method:** A liquid chromatography – electrospray ionization-mass spectrometry (LC-ESI(+)-MS/MS) method has been developed and validated for the determination of simvastatin in human plasma. The mass spectrometry system consisted of an Alliance 2695 series liquid chromatograph with a gradient pump, coupled to a Quattro micro API triple quadrupole mass spectrometer (Waters). A stable isotope-labeled internal standard (simvastatin-d6) was used in the study. After liquid-liquid extraction samples were analyzed on C18 column. Gradient elution was applied with mixtures of ammonium acetate solution and acetonitrile.

**Preliminary Data:** All validation parameters met acceptance criteria requested by EMA [1] and FDA [2]. Calibration curve was linear in the range of 0.1-20.0 ng/mL. To ensure desired sensitivity of simvastatin determination, the instrument response (peak height) was sum of two transmissions: m/z 419.34 > 199.10 and m/z 419.34 > 225.10. The matrix effects calculated according to EMA and Matuszewski et al. (2003) [3] were not significant. According to EMA guideline the possibility of back-conversion of a metabolite (simvastatin acid) into parent analyte during extraction procedure and in the MS source was assessed and was found not to be significant. The method was applied in the bioequivalence study of simvastatin formulation in humans. Among 117 incurred samples reanalysis, acceptance criteria were met for 114 (97.4%) of the repeats, which confirmed that the bioanalytical method provides reliable results. Over 3000 analyses on one HPLC column indicate robustness of the developed method.

**Novel Aspects:** Development and validation of a robust liquid chromatography/tandem mass spectrometry method for the determination of simvastatin in human plasma.

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# Analysis of urinary nucleosides as potential cancer markers by LC-TOF-MS AND LC-MS/MS

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Nowadays, the term "universal tumor marker" does not exist in clinical practice. Many of commonly used biomarkers are not sufficiently sensitive and specific. Hence, there is a need to find the appropriate ones that could diagnose cancer in its early stage and therefore increase chances for recovery. According to the literature, it has been noticed that RNA's metabolites, namely nucleosides seem to play a significant role in cancer diagnosis [1]. During RNA turnover, nucleosides are excreted intact into the urine so their levels are higher in cancer patients in comparison to the healthy ones. In the present work we focused on determination of nucleosides from urine samples using LC-TOF-MS as well as LC-MS/MS.

**Method:** Urine samples (n = 80) derived from both cancer patients and healthy volunteers were qualitatively and quantitatively analyzed using liquid chromatography-time-of-flight mass spectrometry (6200 LC-TOF-MS, Agilent Technologies) and liquid chromatography triple quadrupole mass spectrometry (6430 LC-MS/MS, Agilent Technologies), respectively. In both cases analysis on urine samples was performed after sample pretreatment procedure including solid-phase extraction with phenylboronic acid gel column (Varian PBA, Agilent Technologies). The nucleosides were separated using gradient elution consisted of 0.05% formic acid in water (A) and 0.05% formic acid in methanol (B) with pH 2.7 on Zorbax Extend C-18 column (2.1 × 50 mm, 1.8 μm) at 8°C. The obtained data sets were analyzed using univariate and multivariate chemometric techniques (Mass Profiler Professional Software, Agilent Technologies).

**Preliminary Data:** Liquid chromatography time-of-flight mass spectrometry enables the analysis of either nucleosides (targeted analysis) or nucleosides and other metabolites (untargeted analysis) that could be statistically significant in cancerogenesis. Statistical methods like U-Mann Whitney unpaired test as well as one-way ANOVA, used for targeted data set, extracted four statistically significant nucleosides in cancer diagnosis, namely 3-methyluridine, 1-methyladenosine, inosine and guanosine. The statistical approach used for untargeted data set extracted 193 statistically significant entities, however tandem mass spectrometry is needed for structure confirmation of the most relevant metabolites in cancerogenesis. Moreover, LC-MS/MS brings the information about exact differences in nucleosides' concentrations between cancer patients and healthy volunteers.

**Novel Aspects:** This study concerns analysis of urinary nucleosides in a holistic way using both qualitative and quantitative techniques.

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# Liquid chromatography – mass spectrometry profiling of triterpene glycosides in Eryngium sp.

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Eryngium is the largest genus in the Apiaceae family. It comprises of approximately 250 species, many of which have a long tradition of use in folk medicine. As revealed by taxonomic and molecular biology studies, relationships between Eryngium species are very complex [1]. Thus, smaller, subgeneric taxons such as sections and subsections were early recognized within the genus [2, 3]. According to this classification, the three species known to occur in Poland, *Eryngium campestre*, *Eryngium maritimum* and *Eryngium planum*, belong to three different sections [3]. The goal of our study was to investigate whether there are any qualitative or quantitative differences in triterpene glycosides contents between these species.

**Method:** Lyophilized plant material was extracted with 80% MeOH. After concentration under reduced pressure, samples were purified using SPE on Waters Oasis HLB cartridge columns. One µl aliquots of the samples were then analyzed on a LC-MS system (Thermo Advantage Max ion trap with Surveyor HPLC for profiles and fragmentation studies, Waters TQD with Acquity UPLC for quantitative analyses).

**Preliminary Data:** Concurring with previous phytochemical studies on the members of *Eryngium*, primary triterpene glycosides detected in our work were derivatives of acylated R1 and A1 barrigenols [4, 5]. For the three investigated species, we produced saponin profiles of rosette leaves and roots. In aerial parts of both *E. maritimum* and *E. planum* we observed few signals tentatively belonging to unknown triterpene glycosides. Based on their MS/MS fragmentation patterns, they could be classified as derivatives of acylated R1 barrigenol. The same primary compounds, producing [M-H]-ions at m/z 1099 and1083, were present in extracts from both species. However, *E. planum* extracts seemed to contain several saponins that were absent in *E. maritimum*. We were unable to detect any compounds corresponding to known or unknown saponins in rosette leaves of *E. campestre*. Methanolic extracts from roots of studied species contained different set of saponins, although main sapogenols seemed to be identical. Again, some similarity was observed between profiles of *E. maritimum* and *E. planum*, where compounds with deprotonated ions at m/z 909 and 967 were observed as primary saponins. Isolation, purification and subsequent NMR analysis allowed establishing their structures as, respectively, 3-O-β-D-glucopyranosyl-(1→2)-β-D-glucuronopyranosyl-22-O-angeloyl-A1-barrigenol and 3-O-β-D-glucopyranosyl-(1→2)-β-D-glucuronopyranosyl-21-O-acetyl, 22-O-angeloyl-R1-barrigenol. In the negative ion electrospray mode, MS/MS fragmentation patterns of these two compounds displayed rather unusual loses of 270 mass units as base peaks on their spectra. We believe these ions were generated via a $^{1,4}X$ cross ring cleavage of glucuronic acid moiety. As with the saponins of rosette leaves, once again *E. planum* seemed to have larger variety of compounds than *E. maritimum*. *E. campestre* contained completely different array of saponins in roots [4]. The differences, in comparison to the other two species, involved not only characteristics and sequence of the C-3 oligosacharides, but also the variety of acids acylating C-21 and C-22 hydroxyl groups of aglycone.

**Novel Aspects:** Identification of novel acetylated triterpene glycosides in Eryngium. Unusual fragmentation pattern of triterpene glucuronides. Metabolic profiles of saponins in Erygnium.

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# Monitoring changes in barley (Hordeum vulgare) leaf metabolome under drought stress

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Barley (*Hordeum vulgare*) is an important cereal grown for food, animal feed and as a material for making malt used in production of alcohol. In a ranking of the cereal crops in the world, barley is in fourth place after wheat, rice and maize. Drought is a major environmental factor affecting plant growth and development, contributing to yield loss. Therefore it is interesting to understand the mechanism underlying the abiotic stress tolerance. Nine cultivars originating from Europe, North America and Syria were grown in a greenhouse under controlled watering conditions.

**Method:** Drought was applied at different growth stages in three different variants: (1) 10-day long drought introduced at the 3 leaf seedling stage; (2) drought for the same period of time introduced at the onset of flowering; (3) plants submitted to both periods of drought. Plants were collected in three time points after beginning of corresponding drought period (on day 2, 6 and 10). Qualitative and quantitative analysis of primary and secondary metabolites were performed using GC/MS and UPLC/DAD/UV methods, respectively. The resulting sets of chromatograms were processed in order to align them and remove baseline shifts and data were submitted to mathematical analysis in search of peaks significantly different between the studied barley cultivars and watering conditions.

**Preliminary Data:** We were able to identify different classes of compounds in leaf extract samples: amino acids, organic acids, sugars, sugar alcohols, fatty acids and sterols identified in result of GC/MS and phenolic compounds analyzed using LC/MS and UPLC/UV/DAD methods. Among the identified metabolites the most abundant were sugars, especially sucrose with high overall content in samples. Plants exposed to water deficit revealed changes in metabolite profiles in comparison with control plants, particularly in abundances of some amino acids, sugars and flavonoid glycoconjugates. From UPLC/UV data supported with LC/MS analyses we were able to identified series of phenolic compounds. Application of statistical methods permitted to correlate metabolic profiles registered with GC/MS and UPLC/UV data.

**Novel Aspects:** The aim of the future studies will be the analysis of metabolite changes in mapping population derived from two parents.

# Identify Unknown Compounds in GC-MS Based Metabolic Profiling: GC-APCI-TOF Analysis of a Corynebacterium Glutamicum ΔprpD2 Mutant Strain

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GC-MS based Metabolomics studies are well established and typically employ electron impact (EI) ionisation. Unfortunately, many possible biomarkers remain "unknown" until now due to the lack of reference spectra for the majority of biologically relevant compounds. GC-TOF with more gentle atmospheric pressure ionisation (APCI) can preserve the molecular ion information and delivers accurate mass and isotopic pattern information. These data enable sum formula generation for known and unknown target compounds. We used a GC-APCI-MS based metabolic profiling approach to analyse metabolite extracts of a prpD2 mutant Corynebactrium glutamicum strain. The benefits of high resolution MS data in combination with GC separations to facilitate structure elucidation will be presented.

**Method:** C. glutamicum prpD2 strains were grown on glucose, with a propionate pulse in the exponential growth phase. Two technical replicates of each culture were harvested by centrifugation before and 1 h after the propionate pulse. Derivatized methanolic extracts were analyzed by GC-MS using a HP-5MS column interfaced to a Q-TOF-MS by an APCI source operated in positive mode. Data were acquired from 85-750 m/z at 4 spectra per second. Extracted feature intensities were normalized to the intensity of the internal standard ribitol before analyzing the data by Principal Component Analysis (PCA). Sum formula for selected target compounds were determined utilizing both accurate mass and isotopic pattern information in MS and MS/MS spectra.

**Preliminary Data:** PCA analysis of the GC-APCI-TOF data following feature extraction and normalization revealed a clear separation according to the carbon sources used for cultivating mutant C. glutamicum prpD2 cells. Utilizing exact mass and isotopic pattern information 18 possible sum formulae were calculated for a compound mainly responsible for this observed sample grouping. By combining accurate mass and isotopic pattern information of MS and MS/MS spectra sum formulae suggestions for this compound could be reduced to a single sum formula. This precursor sum formula, C19H43O7Si4, as well as calculated sum formulae for fragments and neutral losses were in accordance to trimethylsilylated 2-methycitrate. The structural hypothesis could be confirmed by comparison to the reference standard. An accumulation of 2-methylcitrate in C. glutamicum cells metabolizing propionate could be expected in cells lacking 2-methylcitrate dehydratase activity, catalyzing the conversion of 2-methylcitrate to 2-methyl-cis-aconitate in the methylcitric acid pathway.

**Novel Aspects:** Benefits of high resolution MS data in combination with GC separations to facilitate structure elucidation

# Diversity of phenolic compounds in barley (Hordeum vulgare)

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Barley is one of the most widespread and widely adapted crops grown under contrasting environmental conditions. Adaptations to new environments, different agricultural practices and selection have further added to the complex diversity in phenotypes. Multiplicity of cultivars and forms of barley encouraged us to extensively study differences in polyphenols profiles in this species. These compounds have a variety of functions in plants. A crucial role is associated with the response to unfriendly environmental conditions and their influence on the feeding quality of plant. Liquid chromatography with tandem mass spectrometry supported with nuclear magnetic resonance provide opportunities to identification and structural analysis the metabolites profiles in plants.

**Method:** Evaluation of poliphenols based on 9 cultivars originating from Europe, North America and Syria and six SSD lines derived from two cultivars, Maresi and Cam B1 as well as the parental lines in different growth conditions. HPLC/PDA/ESI/MSn was used for the analysis of methanolic extracts from leaves of barley. In parallel, purification of phenolics from above 1 kg of barley leaves was conducted. The procedure included: (1) isolation of several fractions using preparative flash column filled with polyamide followed by (2) multiple purifications of the fractions on preparative RP C18 HPLC column. In addition, sets of 13C and 1H NMR spectra have been recorded for 9 compounds obtained in sufficient amount and purity.

**Preliminary Data:** Several papers characterize secondary metabolites in barley, however, the use of flash chromatography/preparative HPLC purification of compounds from a large amount of plant material in allowed us to identify numerous phenolics not described before in this plant species. Coupling of HPLC with ESI-MSn enabled the structural studies of two hundreds and fifty of compounds mentioned above. The main groups of metabolites are O-glycosides or/and C-glycosides of flavones: apigenin, luteolin, tricin and chrysoeriol. In addition, flavanone naringenin as well as flavonol quercetin and its derivatives were observed. However, other phenolics such as hydroxycinnamic acids and their glycoconjugates as well as hydroxycinnamoyl polyamines: hordatine A, hordatine B and their glycoconjugates were also found. The sugars can be acylated with hydroxycinnamic acids (p-coumaric, caffeic, ferulic, sinapic acid) and in a low proportion with malonic acid. Patterns of the glycosylation and sugar acylation were changeable in different grow condition or developmental stages.

**Novel Aspects:** Our study brought an overview of complexity of poliphenols in barley and extend the knowledge about ecogeographically differenced cultivar.

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# Deuterated oligodeoxynucleotides containing 5-fluoro-4-thiouridine as a tool of elucidating the photocrosslink reaction mechanism

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Photocrosslinking has been shown to be an extremely useful and versatile technique for structural studies in relation to nucleic acids and protein interactions Appropriate photoactive unit in macromolecule chain upon irradiation can form covalent link with other molecules positioned in its vicinity. This phenomenon is based on the ability of the reactive sites to absorb the energy of the applied light and use it to form the bond. Among the different candidates for the photoprobe moiety, 4-thio-5-halogenopyrimidines appear to be especially suitable We have observed [1] that these beneficial effects culminate in the case of 4-thio-5-fluoro-uracil (FSU) nucleoside

**Method:** The FSU nucleoside (in 2'-O-methylated form) was synthesized and incorporated into oligo deoxy nucleosides *via* solid supported synthesis [2]. The oligomers were irradiated in the form of the duplex with complementary oligodeoxynucleotide chain using argon-ion laser emitting the light at 351 nm. The obtained photoadducts were separated by HPLC and subjected to enzymatic digestion. The undigested photocrosslink core was isolated and its Maldi-TOF spectrum helped to establish its nucleoside composition. The process was repeated with selectively deuterium-labeled (at thymine $CH_3$) oligonucleotide. The MS spectra of labeled and unlabelled products were compared.

**Preliminary Data:** The oligonucleotides duplexes which were irradiated could form the crosslink with the opposite thymidine residue at the 5' side:

5' C G A T A C G A **X** A 3'
3' T G C T A T G C **T** A T T 5'
or at 3'side :
5' C G A T A C G A **X** A 3'
3' T G C T A T G C T A **T** T 5'

In both cases the crosslink core is composed from the FSU unit plus one adenosine and one thymidine (it was confirmed by Maldi-TOF analysis of the digestion product). The most straightforward way to decide which is the way of photocoupling was to use isotope-labelled thymidine positioned in one of the sites. We have synthesized appropriate $d_3$-deoxythymidine and incorporated it into selected positions of both strands. After irradiation the enzymatic digest was purified and subjected to the MS analysis, revealing the direction of photoreaction. Perspectives. The utility of the photoprobe, which was already very interesting, is greatly enhanced once the details of the reaction mechanism were revealed. Analogous reaction was observed with 5-chloro-4-thiouridine and presently it is under study.

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# ESI-MS/MS Applied to Elucidation of Stoichiometry and Binding Site of the Peptide-Metal Complexes

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Somatostatin (SST) is a peptide hormone that regulates the endocrine system and affects neurotransmission and cell proliferation via interaction with G-protein-coupled somatostatin receptors and inhibition of the release of numerous secondary hormones. Radiolabeled somatostatin derivatives, such as $^{90}$Y-[DOTA])$^{0}$-Tyr$^{3}$-octreotide and $^{177}$Lu-DOTA$^{0}$-Tyr$^{3}$-octreotate, are successfully being used for tumor imaging and experimental peptide receptor-mediated radiotherapy in patients with somatostatin receptor-positive tumors [1]. We have synthesized a series of SST/octreotide analogues in which a role of the metal chelator is played by a combination of His and Asp residues located outside of the macrocycle formed by the disulfide bridge between the Cys$^{3}$ and Cys$^{8}$ residues:

Ac-His-His-(Cys-Phe-Trp-Lys-Thr-Cys)-His-His-$NH_2$
Ac-His-Asp-(Cys-Phe-Trp-Lys-Thr-Cys)-His-Asp-$NH_2$
Ac-Asp-His-(Cys-Phe-Trp-Lys-Thr-Cys)-Asp-His-$NH_2$
Ac-Asp-Asp-(Cys-Phe-Trp-Lys-Thr-Cys)-Asp-Asp-$NH_2$.

We have studied the interactions of these peptides with Cu(II) and Zn(II) ions using ESI-MS/MS.

**Method:** Peptides were synthesized according to the standard Fmoc procedure on a Rink resin. Cys side chains were protected by Acm groups. After cleavage from the resin, disulfide bridge was formed by simultaneous removal of the Acm groups and oxidation by $I_2$ in $CH_3COOH$. Peptides were purified by preparative HPLC. $10^{-4}$ M peptide, as well as $CuNO_3$ and $ZnCl_2$ solutions were prepared by dissolving them in $10^{-2}$ M $NH_4OAc/H_2O$ : $CH_3CN$ (1:1) mixture. Peptide solutions were mixed with the metal ions solutions (1:1) and incubated for 24 hours at 5°C (pH ~ 7). MS/MS experiments were performed in the positive ion mode on apex-ultra or micrOTOF-Q spectrometer (Bruker) with ESI source. Precursor ions were fragmented applying argon as a collision gas.

**Preliminary Data:** ESI-MS was used to follow the progress of the removal of Acm groups and the oxidation of Cys residues by I2. Extending of the reaction time increased the yield of the cyclization but it also resulted in a significant oxidation of the Trp residues. ESI-MS and MS/MS experiments clearly show that all of the synthesized octreotide analogues bind both Cu(II) and Zn(II) ions forming mainly 1:1 peptide-metal ion complexes. In some cases we observed binding of two metal ions by a single peptide molecule, however dissociation of the additional metal ion from the complex required very low energy which suggests its weak binding to the peptide ligand. CID experiments resulted in rich fragmentation spectra. Analysis of the MS/MS patterns of the 1:1 peptide-metal ion complexes enabled us to draw some conclusions about a possible interaction sites of the peptides with metal ions.

**Novel Aspects:** ESI-MS/MS measurements of the peptide-metal complexes allow to elucidate the most probable peptide region to bind the metal ion.

### References

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# New decarboxylated and dehydrogenated betacyanins analyzed by LC-MS/MS

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The most restrictive factor in the widespread application of natural pigments as food colorants is usually their thermolability [1]. Betalains is a group of thermolabile water soluble, nitrogenous pigments found in botanical species belonging to families of the order Caryophyllales. From these red-violet or yellow-orange pigments betanin and its C-15 isoform derived from red beet root (*Beta vulgaris* L.) are extensively used as food colorants in low temperature products. Betalains are known to be very sensitive to several factors, including high and low pH, higher temperature or water activity. Some studies have already elaborated the conditions under which these pigments (mostly betanin) retain their attractive color and even discovered some of their degradation products [1].

**Method:** For the LC-ESI-MS/MSanalysis of the heated extracts, the positive ion electrospray mass spectra were recorded on a ThermoFinnigan LCQ Advantage (electrospray voltage 4.5 kV; capillary 250°C; sheath gas: $N_2$) coupled to ThermoFinnigan LC Surveyor pump. The MS was controlled and total ion chromatograms and mass spectra were recorded using ThermoFinnigan Xcalibur software (San Jose, CA). Helium was used to improve trapping efficiency and as the collision gas for CID experiments. The relative collision energies for MS/MS analyses were set at 30% (according to relative energy scale).

**Preliminary Data:** Because the identification of betacyanin dehydrogenation and decarboxylation products is crucial in determination of betacyanin degradation mechanism in Hylocereus polyrhizus fruit and *Beta vulgaris* L. root preparations, the results of dehydrogenation experiments performed in betacyanin ethanolic and aqueous solutions are reported. As expected, the appearance of neophyllocactin and neohylocerenin was confirmed by their pseudomolecular masses at m/z 635 and 693, respectively, in addition to the well-known neobetanin m/z 549. All these compounds exhibited lower polarity than their parent betacyanins, the absorption maxima around $\lambda_{max}$ 490 nm characteristic for neobetanin and only one chromatographic peak resulting from the loss of the chiral center at C-15. The subsequent fragmentation ions at m/z 387 from the loss of a glucose moiety and 343 from the next loss of $CO_2$ confirmed the existence of these compounds. Structures of new dehydrogenated betalains derived from phyllocactin and hylocerenin were also suggested. Prolonged heating of the *H. polyrhizus* extracts resulted in very complex decomposition mixtures, which were subsequently analyzed by LC-DAD and LC-MS/MS. A group of compounds possessing absorption maxima at $\lambda_{max}$ 507-509 nm appeared in higher quantities after prolonged heating and was identified as bidecarboxylated betacyanins and 2,15,17-tridecarboxy-betacyanins.

**Novel Aspects:** Structures of new dehydrogenated betalains are analyzed.

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# Evaluation of the Performance Improvements Needed in an ESI-QTOF-MS System for Qualitative and Quantitative Multi-Target Pesticide Screening in Food

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Fast and comprehensive full scan accurate mass screening becomes a valid alternative in food control in particular if hundreds of pesticides have to be proved in a reasonable time frame. Additionally to the high number of targets the technique takes advantage of unknown evaluation and retrospective analysis. However, practical studies of the performance of current high mass accuracy mass spectrometers reveal certain limitations. Due to matrix background, unambiguous identification and correct quantitation is not always achievable at required trace levels. Besides of additional MS/MS-based result confirmation workflows, the general improvement of the instrument performance itself is of interest. This study evaluates the performance enhancement achieved using a redesigned QTOF which is improved with regard to requirements for food safety analysis.

**Method:** For this study, a representative subset of 50 out of >1000 pesticides was chosen, with regard to their relevance for routine monitoring. Seven solvent and matrix based dilution series (0.01 pg/µL – 1 ng/µL, QuEChERS-extracts: cucumber, strawberry, wheat flour, leek, orange, ginger) of the pesticide-mix are analyzed by an UHPLC-QTOF system. Data acquisition is performed in alternating full scan resp. bbCID mode using different acquisition rates up to 20 Hz. Automatic data evaluation is performed using dedicated target screening software. The basic instrument performance criteria mass accuracy, resolution, detection limits, linearity, dynamic range and reproducibility of the system are evaluated for the solvent based pesticide samples. Additionally, statistics for the pesticide identification rates and false positive/negative results for all samples are generated.

**Preliminary Data:** The novel instrumental setup provides enhanced performance especially with regard to mass accuracy (better than 1ppm), detection limits (usually fg/µL range) and accessible mass band width. The latter allows for screening methods, that can cover a significantly wider scope of analytes in one method (m/z range <100 to >1000). Especially the performance for low (m/z <200) and very low m/z signals (m/z <100) is substantially improved, which is important for confirmation experiments in broad band CID (bbCID) mode, since often particularly low mass fragments are significant for the unambiguous identification of compounds (like for distinguishing the coeluting compounds aldicarb and butocarboxim where the detection of the low mass fragments m/z 89 and 75 is essential). For most compounds calibration curve characteristics as a linear range up to ~4 orders of magnitude with an R >0.999 are observed. Peak area reproducibility is in the range of a few percent (medium concentration levels), sometimes below 1%. The mass accuracy stability of the system enables the use of selective high resolution extracted ion chromatograms (hrEIC) with trace widths below 1mDa. We found that mass accuracy performance and isotope pattern quality are widely independent of the acquisition rate. Statistical evaluations will be shown for acquisition rates up to 20 Hz. Performance criteria for screening protocols given in the EU decision 2002/657/EC require a mass resolution of 20,000 for the assignment of identification points for LC-hrMS data. This requirement is met for the complete m/z range that was evaluated. Due to the enhanced system performance identification rates increase, whereas false positive and false negative results caused by interfering matrix compounds are considerably reduced (detailed statistical data will be shown).

**Novel Aspects:** Redesigned QTOF which is improved with regard to requirements for food safety analysis.

# Development and validation of bioanalytical LC/MS/MS assay for quantifying of two pyridine derivatives with potential endothelial activity in mice serum

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Quaternary pyridinium salts such as 1,4-dimethylpyridinium (1,4-DMP) and 1-methylpyridinium (1-MP) chloride have recently become a subject of great interest because of theirs potential endothelial and vasoprotective activity [1]. This activity may be the result of structure similarity to 1-methylnicothinamide (MNA), that antithrombotic, anti-inflammatory and vasoprotective action is a consequence of its stimulation of endothelium to produce prostacyclin ($PGI_2$) in the $COX_2$ dependent mechanism. The aim of this work was to develop and validate the analytical method for simultaneous determination of 1,4-DMP and 1-MP in mice serum using LC/MS/MS technique for the purpose of pharmacokinetic studies.

**Method:** The chromatographic separation of investigated compounds was achieved on the Aquasil C18 (4.6 × 150 mm, 5 µm, Thermo Scientific) analytical column. Mobile phase consisting of acetonitrile-water (40:60, v/v) with addition of formic acid (0.1%, v/v) was delivered isocratically by the Dionex UltiMate 3000 HPLC system with the constant flow of 0.8 mL/min. Triple quadrupole mass analyzer TSQ Quantum Ultra with heated electrospray ionization (H-ESI) interface was employed as a detector. The stable isotope-labeled analogues of the quantified compounds were used as an internal standards (1,4-DMP-$d_3$ and 1-MP-$d_3$).

**Preliminary Data:** All compounds were scanned in the SRM mode, and the following parent to fragment transitions were monitored: m/z 94→79 for 1-MP, m/z 108→93 for 1,4-DMP, m/z 97→79 for 1-MP-$d_3$ and m/z 111→93 for 1,4-DMP-$d_3$. The matrix effect was investigated not only for the different sources of mice serum but also for plasma with different anticoagulants added, such as heparin, EDTA and citric acid. Both absolute and relative matrix effects were studied, the latter one using two different approaches [2]. The linear answer of the detector was observed for the concentrations from 50 to 5000 ng/mL for both analyzed compounds. The validation parameters such as accuracy and inter and intra-batch precision were within the limits set by the FDA guidelines. Presented method was used for the pilot study of pharmacokinetic behavior of the 1,4-DMP and 1-MP after intravenous administration to mice.

**Novel Aspects:** There is no available method to quantify 1,4-DMP in biological matrices such as mice serum.

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# Development of an Automated Ion-Trap MSn-Based Screening Method for Clinical and Forensic Toxicology

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Screening applications in clinical and forensic environments require rapid and unambiguous results that can be generated even by unskilled users. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) combined with library search is an emerging screening technology in these fields. It provides more valuable information than LC-UV detection while covering a broader and in some respect complementary range of analytes when compared to GC-MS. This study focuses on applying and challenging a robust and easy-to-use Ion-Trap-based solution for the detection and identification of common drugs, drugs of abuse and their metabolites in shortest time as possible in a fully automated environment.

**Method:** Serum and urine extracts are separated using a UHPLC-system (Dionex RSLC) connected to an amaZon X ion trap MS instrument generating data dependent $MS^2$ and $MS^3$ spectra in both positive and negative mode in a single run. Identification of the generated spectra was performed using SmileMS library search software (GeneBio). For method development several chromatographic columns were tested for resolution and peak shape using a mixture of 10 substances covering the desired mass range (100-800 amu) and both polarities. Additionally, the effects of eluent composition and eluent buffer concentration in positive and negative mode were examined to find the most effective method.

**Preliminary Data:** A fast 8 minutes gradient using formic acid, ammonium acetate and acetonitrile as eluent and a Dionex Acclaim RLSC C18 100 × 2.1 mm column were used for chromatographic separation to generate a mass spectral library of about 800 compounds. A subset of 200 compounds were defined and spiked into human serum matrix at 3 different concentration levels (low therapeutic, therapeutic and elevated). All compounds were combined into mixtures containing 5-10 substances each and then analyzed. It has been shown, that low therapeutic doses (low ng/ml range) of commonly used and miss-used benzodiazepines like diazepam could be successfully detected and identified in all three MS-stages. Also low-dose benzodiazepines like flunitrazepam could be detected and identified in spiked serum samples. The final results will show statistical evaluation of the data listing identifications at different concentration levels, false positive and false negative rates. The presented library screening method offers a fast, reliable and sensitive procedure for clinical and forensic analysis. The combination of $MS^2/MS^3$ spectra and retention time allows identification of drugs and metabolites at therapeutic levels.

**Novel Aspects:** A Ion-Trap-based solution for the detection and identification of common drugs, drugs of abuse and their metabolites by library search.

# Production of energy-rich triterpenoids in Euphorbia lathyris, a potential crop for third generation biofuels

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*Euphorbia lathyris* has a high potential to be used as a source of biofuel providing an alternative to fossil resources for petrol, already suggested more than 30 years ago [1]. The plant is prominent for the accumulation of energy rich triterpenoids in the latex, produced by specialized cells named laticifers. However, still little is known about the biosynthesis and accumulation of triterpenoids and the formation of laticifers. Proteomic approaches with mass spectrometry give more insights to the protein pattern and enzymes of these cells and help to identify enzymes involved in triterpenoid biosynthesis and development of laticifer cells. The novel insights into mechanisms of triterpenoid metabolism are pre-requisites for the development of strategies to increase latex triterpenoid content.

**Method:** Proteome patterns of tapped latex and derived fractions thereof are analyzed by 1D&2D denaturing gel electrophoresis and LC-based separation of proteins or peptides. Identification of proteins is performed by ESI-MS/MS as well as MALDI-TOF-MS/MS. Protein identification is done by public database as well as self generated EST database from E. lathyris, because of the lack of sequence information in this plant species. For purification and concentration of proteins involved in triterpenoid biosynthesis, affinity based chromatography for Ac-CoA using enzymes are used for fractionation. The further analysis of these fractions is done by 1D-SDS-PAGE and LC-MS based analysis of tryptic peptide digestion.

**Preliminary Data:** First results showed a very distinct protein pattern from the specialized laticifers compared to the other cells. Furthermore latex cell sap is present in higher amount in younger parts of the plant. Comparison of latex from these different parts and also different stages showed various protein levels and composition. First results reveals a higher protein complexity in the younger stages combined with a lower amount of triterpenoids. Overall the proteome complexity of latex appears lower than that of leaves.

**Novel Aspects:** Improvements of protein alasys from latex samples with high amount of interfering sunstances.

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# Increased confidence of biomarkers identification in EBC samples using LC-MS/MS

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Oxidative stress (OS) is a state of imbalance between continuously generating reactive oxygen species (ROS) during normal cell metabolism and mechanism of detoxifying oxygen radicals through a network of antioxidative enzymes. Exhaled breath condensate (EBC) is considered as a promising source of biomarkers for the real-time analysis of oxidative stress in the lower respiratory tract airways. It is a simple matrix in which biomarkers may be identified, in a way equivalent to blood, sweat, tears, urine and saliva.

**Method:** Samples of EBC were divided into two parts. 100ul were direct infused for determination of amino acids, second 900 ul were concentrated by lyophilization for determination of 3-nitrotyrosine and isoprostanes. HPLC analyses of isoprostanes and 3-nitrotyrosine were performed on Agilent 1200 HPLC system equipped with a Zorbax Eclipse XDB. The mobile phase contained 0,01% (v/v) formic acid with a gradient of solvent A (methanol/acetonitrile 1:1) and solvent B (ultrapure water) at a flow rate 1ml/min. For analysis of amino acids Ascentis® Si column was used with gradient conditions of phase A) 10 mM ammonium acetate and B) acetonitrile. Negative ionization mode was used for selective monitoring of four isoprostanes ions, positive for twenty one amino acids and 3-nitrotyrosine.

**Preliminary Data:** In this study, we have investigated potential usefulness of LC-MS/MS procedure for accurate qualitative and quantitative analysis of four isoprostanes, 3-nitrotyrosine and 21 amino acids in exhaled breath condensate samples. Liquid chromatography coupled with tandem mass spectrometry was used mostly in order to circumvent various problems in the conventional assay methods such as laborious and time-consuming pretreatments in GC–MS and possible cross-reactions of the target compounds in enzyme immunoassay (EIA) as well. The method for amino acids determination is characterized by speed, sensitivity, selectivity and eliminates the need of amino acids derivatization. The most abundant ions using positive ionization mode for all the amino acids and 3-nitrotyrosine was the protonated molecule $[M + H]^+$. For this reason, these ions were chosen as precursors for fragmentation in tandem mass spectrometry. The most abundant fragment ion was selected for MRM mode. Isoprostanes are diastereoisomers and two pairs of amino acids have identical MRM ion transitions, therefore complete chromatographic separation with excellent peak shape was essential for their accurate quantitation. Described methods have a detection limits of 3 pg/mL for isoprostanes and 3-nitrotyrosine and 0,03-1 ng/mL for amino acids. The method was used to assess the presence of 3-nitrotyrosine, isoprostanes and amino acids in exhaled breath condensate samples as a potential biomarkers of oxidative stress in respiratory system.

**Novel Aspects:** Identification and quantitation of oxidative stress biomarkers in exhaled breath condensate samples.

# Organochlorine pesticides and their metabolite analysis in soil samples and microbiological cultures by GC-MS

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Organochlorine pesticides were dominant insecticides in the middle of 20th century. They are very effective but strongly toxic to the environment and were systematically replaced by less hazardous, improved, cheaper or more easily utilized products. Out of use products were often stored in pesticides burial sites which concrete housings, in the course of time, became leaky. In many cases toxic substances from these containers penetrate into the soil and underground waters causing serious contamination of environment. The primary goal of the project is to analyze microorganisms living in pesticide contaminated soils coming from suroundings of pesticides burial sites. It is expected that soil bacteria exposed on large amounts of chemicals for decades acquired abilities to metabolize pesticides.

**Method:** In this part of the project we have analyzed the soil for the presence of organochlorine insecticides like DDT, HCH or Lindane using GC-MS and we are at the stage of analyzing the microbiological cultures fed with pesticides for the changes of their chemical composition. In our lab we have analyzed about 80 samples of soil and a few samples of water for presence of the pesticides most commonly used in Europe using HPLC-MS and GC-MS. GC-MS was used for analyses of the polychlorinated pesticides and other non-polar compounds using EI ionization source and single quadrupole analyzer. Samples were extracted from soil using solid phase-liquid extraction to the ACN/water solution and liquid-liquid extraction to DCM solution for GC-MS.

**Preliminary Data:** The most popular organochlorine pesticides like DDT's, γ-HCH, Methoxychlor and Lindane were found in the samples in the largest amounts. Their possible metabolites like DDD were also found in samples. The results were confirmed by comparison with the commercially available pesticide standards. Preliminary attempts to quantitate the amounts of pesticides in the analyzed samples were performed. In most cases we were able to determine the concentration of pesticides at the microgram levels using GC-MS. Samples of microbiological cultures including blank samples of pure pesticides and soil extracts were prepared for analysis by the extraction to chloroform. Up to now no significant changes in key components concentration have been found. Presence of new compounds, possible metabolism markers, haven't been confirmed, but biological experiments are continued and we are looking forward to successful analyses in the near future.

**Novel Aspects:** Analyses of the soil samples from suroundings of the pesticides burial sites.

# Screening and Determination of more than 250 Pesticides in Fruits and Vegetables with GC/TQ

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Nowadays multi-methods for the determination of pesticide residues in fruit and vegetable extracts are state-of-the-art. Because of emerging time and cost issues a change in methodology can be seen within the last 5-6 years. The QuEChERS method got more and more popular even prior to gas chromatographic separation. To be able to deal better with stronger matrix influences in relation to much more labor intensive clean up approaches using gel permeation chromatography (DFG S19, LUKE method) QuEChERS extracts carry a lot of matrix out of the samples.

**Method:** A fast scanning triple quad instrument with 14,400 Da/s and the ongoing development of duty cycle administration by the software – it still can be controlled by user settings – improves sensitivity and statistical security by having reasonable numbers of datapoints *via* a chromatographic peak. Recently introduced software features allow method setup and maintenance as easy as known for single quads as the compound is in the center of interest. A database covering more than 1000 compounds even reduces time consuming tuning approaches. This compound based scanning also allows compound handling *via* Excel if needed.

**Preliminary Data:** The increased sensitivity and excellent dynamic range of at 5 orders of magnitude allows reliable quantitation of even low amounts of pesticide residues. The improved reliability in the results due to measurement of MRM transitions in relation to single ion monitoring reduces need for re-run of samples and manual data interpretation. The reporting features make the data mining less tedious; saving time for evaluating the data and deliver results as fast as needed.

**Novel Aspects:** Determination of more than 250 pesticides with a fast scanning TQ and database supported method setup.

# Comprehensive metabolomic profiling of Zucker rat plasma using LC and GC ultra high resolution time-of-flight mass spectrometry and GCxGC-TOFMS

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Metabolomic profiling in normal and diseased animals is a challenging and rapidly growing area of research. Rat models in studying diseases are common. The Zucker rat is an example of a breed specifically used for this purpose. A comprehensive analytical approach to the characterization of the metabolome of the plasma for these animals is undertaken with the intent to define differences in metabolite levels in three disease states.The approach uses HPLC and GC coupled to high resolution time-of-flight mass spectrometry and evaluates the capability of GCxGC time-of-flight mass spectrometry to screen for analytes which can be utilized in the comparative analysis of metabolomic profiles of plasma from three strains of Zucker rats. The metabolite profiles are differentially compared and statistically-significant features are identified.

**Method:** Plasma from three strains of rats were obtained – Lean, Fatty and Obese. Protein was removed from the samples by acidification of the samples followed by centrifugation. UHPLC/MS analysis was performed using a LECO Citius™ LC-HRT mass spectrometer based on FFPTM technology. It was operated in the High Resolution mode (50,000 FWHM) in positive ion mode using electrospray ionization. Pools of the disease groups were derivatized and analyzed utilizing a LECO Pegasus™ GC-HRT system and a GCxGC separation followed by TOFMS with electron impact ionization at acquisition rates up to 150 spectra/sec.The data mining strategy compared the combined peak tables, mass spectral similarities, and overlaid chromatograms to discover significant metabolite variations between the lean, fat and obese sample pools.

**Preliminary Data:** The current study provides identification of several analytes which differ in their relative amounts in the plasma of LEAN, FATTY and OBESE Zucker rats. The evaluations employed a comprehensive approach with LC, GC and GCxGC analyses providing different and complementary information on the samples and disease states. Consistent among the findings are changes in the fatty acid composition and changes in the processing of fatty acids. In the case of HPLC-TOF analysis the utility of accurate mass analysis and relative isotope abundance in the identification of compounds are shown with butyryl carnitine as a representative compound. The value of accurate mass is specifically demonstrated in the selective extraction of signal for carnitine and related compounds including the use of accurate fragment masses to monitor changes in the family of acyl carnitines and to characterize butyryl carnitine as an unknown. Similar capabilities are leveraged in the GC-HRT analyses.Together the three techniques provided a complementary set of 41 non-redundant compounds which changed with the phenotype and provide a marker of the metabolic state of the animal. The techniques also provided redundant analytes which helped to verify the three experiments. GC and LC with high performance MS provided powerful tools for exhaustive studies of appropriate statistical power. GCxGC with TOF-MS provides a tool for the deep evaluation of changes to systems which can then be followed up on using less data intensive approaches.

**Novel Aspects:** The benefit of the complementary nature of the GC and LC data from ultra high resolution MS was demonstrated.

# Development of a LC-MS/MS-method for the simultaneous quantitation of sunitinib and its active metabolite N-desethylsunitinib in human plasma and blood

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Sunitinib (SU11248) is an oral multi-target tyrosine kinase inhibitor that was approved by the FDA for the treatment of renal cell carcinoma (RCC) and imatinib-resistant gastrointestinal stromal tumor (GIST) in 2006. It is metabolized to N-desethylsunitinib (SU12662), which is equipotent to the parent compound and has an exposure that is between 23% and 37% of the total exposure [1, 2]. To extend the scope of application of sunitinib, an academic prospective single-arm phase II clinical trial was accomplished where 37.5 mg sunitinib, administered orally on a continuous once-daily dosing regimen, was given to patients with recurrent or progressive glioblastoma multiforme. To monitor the drug levels in blood and plasma therapeutic drug monitoring (TDM) was performed by LC-MS/MS.

**Method:** 157 blood and 115 serum samples were collected from overall 40 patients in German and Austrian hospitals and stored at −20°C until analysis. Sample preparation was accomplished by liquid-liquid-extraction with the addition of 0.4 ml of plasma or blood to 0.8 ml tert. butylmethylether and 0.01 ml of ammonium hydroxide using 50 µg/ml bunitrolol as internal standard. After vortexing the organic layer was separated, evaporated and reconstituted in 100 µl 0.05% aqueous HFBA solution containing 50% acetone. The processed samples were chromatographed on a Si-C18 reversed-phase column with a gradient of 5% to 95% acetone in 0.05% aqueous HFBA solution in 10 min. The compounds were detected by tandem mass spectrometry with multiple reaction monitoring (MRM) in positive ion mode.

**Preliminary Data:** A sensitive and specific method was developed using LC-MS/MS for determination of sunitinib and its metabolite N-desethylsunitinib in human plasma and blood. The linear calibration curves in human plasma and blood were generated in a range of 5.0-200 ng/ml ($r^2$ = 0.997) for sunitinib and 2.5-125 ng/ml ($r^2$ = 0.994) for N-desethylsunitinib. The determined concentrations of sunitinib varied from 19-186 ng/ml and for N-desethylsunitinib from 4-125 ng/ml. For the majority of the samples the sunitinib-to-N-desethylsunitinib-ratios were in the expected range of 23% to 37%. Three patients showed an increased metabolic transformation rate. The sunitinib to N-desethylsunitinib ratios of this patient ranged from 73-121%. Using LC-MS/MS a sensitive and specific method could be established to monitor blood concentrations of sunitinib and its metabolite N-desethylsunitinib in humans undergoing treatment with the oral tyrosine-kinase inhibitor sunitinib which may serve for further optimization of active drug regimens in cancer treatment.

**Novel Aspects:** A LC-MS/MS method was developed for the quantitation of sunitinib and its active metabolite N-desethylsunitinib in human blood and plasma.

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# Profiling and structural analysis of phenolic secondary metabolites present in Mexican lupines using LC/ESI/MSn technique

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Flavonoid glycoconjugates constitute an abundant class of secondary metabolites that are ubiquitous in the plant kingdom. These natural products are diphenylpropanes (C6C3C6) with different numbers of hydroxyl groups attached to the ring structures. Another substitutions on the phenyl ring are different glycosidic, alkyl or acyl moieties, giving rise to more than 9000 metabolites. Profiling of secondary metabolites is a challenging task from an analytical point of view. The application of LC/ESI/MSn technique allows to separate and identify numerous flavonoid glycoconjugates. The aim of these work is profiling and structural analysis of flavonoid secondary metabolites in the complex extracts obtained from leaves and roots of different European and Central America lupine species.

**Method:** Application of two systems, LC/IT-MS (low resolution) and LC/qToF-MS (high resolution), allowed profiling flavonoid conjugates in extracts obtained from roots and leaves of eight Mexican lupine species (*Lupinus exaltatus*, *L. mexicanus*, *L. montanus*, *L. rotinduflorus*, *L. stipulates*, *L. elegans*, *L. hintoni, L. sp.*) and three European lupine species (*L. albus*, *L. angustifolius*, *L. luteus*). Seedlings of plants were grown in greenhouse under controlled conditions. For LC/MS analysis, the frozen tissues were homogenized and extracted in methanol. For the separation and identification of isomeric flavonoids compounds a chip-based nanoelectrospray ionization source (TriVersa NanoMate system) and LC/MS Fraction Collection mode were used.

**Preliminary Data:** Tandem mass spectrometry connected with liquid chromatography, is the dominant technique used for identification and structural analysis of flavonoids. Hyphenation of separation techniques with mass spectrometers, especially those equipped with collision-induced dissociation capabilities (CID MS/MS), allowed to separate and identify numerous isomeric and isobaric flavonoid glycoconjugates. The registered high resolution spectra permited to establish elemental composition of the glycoconjugate molecules from exact m/z values of protonated/deprotonated molecules ($[M + H]^+$/$[M - H]^-$). The elucidation of the MSn spectra enabled location of substituents on different parts of the molecule and the identification of the flavonoid aglycone on the basis of its fragmentation pattern. High resolution mass spectra registered in both positive ion and negative, at different collision energies, allowed to obtain information about the structure of aglycone, the type of subunit of sugar and place of its substitutions, the type of interglycosidic linkages, or acyl substituent. Profiling of flavonoid glycoconjugates in mexican lupine species was reported earlier only two times [1, 2]. In our present work over hundred flavonoid and isoflavonoid glycoconjugates were detected in extracts of lupines. In the extract samples were present glycosides of isoflavones (2`-hydroxygenistein, genistein, biochanin A, luteone, wighteone) flavones (apigenin, chrysoeriol), flavonols (keampferol, quercetin), flavanones (naringenin). There were observed differences in the glycosylation and sugar acylation pattern in profiles of flavonoid between roots and leaves. Profiles of phenolic compounds present in Mexican lupines were compared with profiles these recorded for the European lupine species (*L. albus*, *L. angustifolius*, *L. luteus*). The closest species to Mexican lupines from the point of few of flavonoid glycoconjugates composition is Lupinus luteus due to presence of C,O- diglucosides or C,C- diglucosides or C-glucosides and presence of biochanine derivatives.

**Novel Aspects:** Information obtained from the flavonoid conjugates profiles will be used as a tool for chemotaxonomic analysis of different lupine species.

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# RP x IP-RP HPLC-MS analysis of the presynaptic active zone

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Although the proteome of the presynaptic active zone is only of medium complexity compared to other subproteomes, its dynamic range in the abundance of proteins and its high amount of hydrophobic proteins requires an extended fractionation strategy prior to MS analysis. Previously it could be shown that RP x IP-RP-HPLC is of high separation efficiency and gives a sufficiently homogeneous distribution of peptide elution [1]. The aim of this work was to establish a 2D-LC setup for the proteomic analysis of the murine presynaptic active zone employing reversed phase chromatography at high pH in the first and at low pH in the second dimension.

**Method:** For evaluation of the chromatographic system, five standard proteins were digested with elastase to create a peptide mixture of limited complexity. The presynaptic active zone was isolated in similar manner as previously described [2]; the eluate was purified by MetOH/CHCl3-precipitation before chromatographic separation. The first-dimension separation was carried out with a MicroPro™ Micro/nano HPLC system (SunChrom) at pH 10 and the second with an Easy nLC II (Proxeon Biosystems) at pH 2. Columns were packed in-house with C18 material. Subsequent mass spectrometric analysis was performed using a 4800 MALDI TOF/TOF analyzer (AB Sciex) and an ESI micrOTOF analyzer (Bruker Daltonics). Peptides were identified by Mascot search engine (MatrixScience) and data were processed via an in-house developed program.

**Preliminary Data:** A simple and robust 2D-LC method could be established and it could be confirmed, that RP-LC at high pH in the first and IP-RP-LC at low pH in the second dimension provides a high separation efficiency. The RP x IP-RP chromatographic system showed adequate orthogonality and gave a sufficiently even distribution of peptides over the fractions. Compared to RP-LC more proteins of the presynaptic active zone could be identified, in most cases associated with higher sequence coverage and more peptides.

**Novel Aspects:** An extended analysis of the murine presynaptic active zone could be performed with the adapted RP x IP-RP HPLC-MS system.

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# Liquid chromatography/mass spectrometry assay for direct monitoring of albumin lysine-525 N-homocysteinylation in human serum

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Homocysteine (Hcy) and Hcy-thiolactone are intermediary metabolites that are implicated in the pathology of human cardiovascular and neurodegenerative diseases. Because Hcy-thiolactone is chemically reactive, it readily modifies protein lysine amino groups to form stable isopeptide bonds (N-Hcy-protein). This reaction alters protein structure and function, causes protein damage by a thiyl radical mechanism, and can lead to pathological consequences such as an autoimmune response and thrombosis. Approximately 70% of circulating Hcy is N-linked to blood proteins, mostly albumin and hemoglobin. In human plasma, albumin is the major target for N-homocysteinylation by Hcy-thiolactone both *in vitro* and *in vivo*.

**Method:** Lysine-525 (Lys525) is a predominant site of N-homocysteinylation in human serum albumin in vitro and in vivo, as shown by the identification of 525K*QTALVELVK534 peptide carrying N-linked Hcy at Lys525 (525K*) in both Hcy-thiolactone-modified and native albumin. Analyses of site-specific N-homocysteinylation in vivo require extensive sample workup and enrichment procedures. To overcome these limitations, we have developed a new liquid chromatography/ mass spectrometry (LC/MS) method for monitoring the levels of albumin N-Hcy-Lys525 peptide directly in human plasma samples subjected to tryptic digestion.

**Preliminary Data:** We found that the normalized levels of the m/z 651.3 peptide (containing N-Hcy-Lys525) were significantly higher in cystathionine b-synthase (CBS)-deficient patients compared with healthy individuals ($0.0399 \pm 0.0260$ vs. $0.0102 \pm 0.0121$, $P = 0.0007$). These values suggest that approximately 1% and 4% albumin molecules are N-homocysteinylated at Lys525 in healthy individuals and CBS-deficient patients, respectively. The higher levels of N-homocysteinylation at Lys525 in albumin from CBS-deficient patients reflect higher total Hcy and N-Hcy-protein levels in these patients compared with healthy individuals, that were measured previously using chemical assays. There was a significant correlation between the normalized levels of the N-Hcy-Lys525 peptide and plasma total Hcy levels. In conclusion, the LC/MS assay that we developed, allows monitoring of albumin Lys525 N-homocysteinylation directly in trypsin digested human plasma.

**Novel Aspects:** The LC/MS assay that we developed, allows monitoring of albumin Lys525 N-homocysteinylation directly in trypsin digested human plasma.

# Fast catalytic tests using an automated catalyst test system coupled to a quadrupole mass spectrometer

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A commercial automatic chemisorption instrument was revamped to carry out catalytic reactions. Thus, inlet systems for liquids and gases were attached. The standard thermal conductivity detector was replaced by a quadrupole mass spectrometer. Chloride-free Cu-impregnated zeolite Y, SAPO-5 and SAPO-37 were tested as catalysts for the oxidative gas phase carbonylation of methanol to dimethyl carbonate. Several catalytic test routines may be performed at short times including controlled catalyst pretreatement procedures.

**Method:** The experiments were carried out in an Autochem 2910 (Micromeritics) chemisorption system under atmospheric pressure. A gas mixer was used to introduce feed gas mixtures of CO, $O_2$, He and Ar as reference. Liquid compounds were introduced via a syringe pump followed by an evaporator. This allowed different mixtures of liquid samples to be added to the feed gas. A second syringe pump was used for additionally gaseous compounds like dimethyl ether at calibration procedures. Catalytic tests were performed in a temperature range of 100°C up to 320°C. A single quadrupole mass spectrometer (Omnistar, Pfeiffer Vacuum) was used to analyze the products. Both the detectors (Faraday cup and multiplier) were used to detect compounds over a wide range of concentrations.

**Preliminary Data:** In all test runs the formation of dimethyl carbonate due to an oxidative carbonylation of methanol was observed. Methylformate, dimethoxymethane and dimethyl ether were identified as other main products.. Characteristic ions could be found to determine the concentration of these compounds in the product mixture. Some mass interferences showed only a significant influence at large excess of one compound. Such interferences were taken into account at quantification. Catalytic data such as conversion and selectivity from the oxidative carbonylation of methanol at different Cu impregnated zeolites and alumophosphates were obtained. The catalysts did not need long times to reach a steady state. This allowed temperature ramps between 1 and 5 K/min for a fast determination of catalytic data. The investigation of the catalytic behaviour as described completes the different characterisation methods and allows the fast evaluation of catalytic properties. Thus, the relationship between between catalyst composition and prepreparation methods on one hand and several characterisation data on the other hand could be correlated with the real catalytic performance

**Novel Aspects:** Combination of an automated catalyst test system with a quadrupole MS allows fast catalytic test procedures at high temperature resolution.

# Medicago truncatula full-size ABCG transporter modulates isoflavones transport

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A major function fulfilled by ATP binding cassette (ABC) proteins is membrane translocation of a wide range of unrelated molecules. Recent data point to full size ABCG transporters as important players that influence plant interactions with the environment. Previously we identified 20 genes, coding full size ABCG proteins in Medicago truncatula, a model legume plant (1). Certain of them like MtABCG10 were particularly expressed in roots.

**Method:** Application of a high-resolution mass spectrometer helped to evaluate the elemental composition of protonated Medicago isoflavones. Structures of these natural products were described according to the registered mass spectra and literature data.

**Preliminary Data:** Here we demonstrate that silencing expression of MtABCG10 resulted in a significantly lower amount of various isoflavones. The analyses were conducted with root isolates as well as the media. The LC/MS profiles of target natural products obtained from M. truncatula silenced and control plants differed substantially. The differences were even more pronounced upon elicitation with PAMPs, and concern precursors of medicarpine – a phytoalexin of Medicago sp. The data presented are the first glimpse of evidence concerning full size ABCG transporters in this model legume plant thus providing a scaffold for further studies of their physiological function and possible role in the secretion of defensive molecules. Activity of ABC transporters might be crucial for modulation of plant interactions with other organisms.

**Novel Aspects:** Contribiution of ABCG transporter in Isoflavones transport.

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# Direct extraction of metabolites from adherent growing cells for GC-EIMS analysis can replace time consuming traditional sampling procedures

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Metabolite profiling of adherent growing cells using GC-EIMS is an emerging application of mass spectrometry e.g. in cancer research or to investigate the metabolic response of cells to environmental stimuli. In contrast to suspension cultures, sampling of adherent growing cells within metabolite profiling is especially challenging since cells have to be separated from the medium and detached from the bottom of the cultivation well for counting as fast as possible to avoid changes in intracellular metabolite profiles. Traditional sampling procedures, i.e. harvesting the cells using cell scrapers or trypsinization of cells, are time consuming and tend to change intracellular metabolite pattern through metabolite leakage and continuing metabolism during the sampling time [1].

**Method:** We compared profiles of parallel cultivated cells harvested with the traditional methods trypsinization or cell scraping with profiles generated after direct extraction of metabolites using methanol for snap frozen cells in well plates without prior detachment. After evaporating the extraction solvent, methanolic cell extracts of the three different sampling methods were derivatized by methoxamination and trimethylsilylation and subjected to GC-EIMS measurement on a ThermoFinnigan Trace GC coupled to a double focusing sector field MS. Data analysis was performed with AMDIS 2.65 [2] using a customer library for peak identification. Following normalization of single metabolite areas to the TIC, metabolite patterns were evaluated by mean area comparisons and PCA.

**Preliminary Data:** Direct extraction of cells resulted in an about twofold enhanced overall intensity compared to the other two sampling methods. In contrast, the total amount of detected metabolites was only slightly increased. Principal component analysis showed a clear separation of directly extracted samples from the two other methods and suggested further different metabolite patterns for trypsinizated samples and samples harvested using a cell scraper. The main differences between the directly extracted and the traditionally harvested cell samples could be ascribed to an enhanced amount of sugar compounds like glucose and fructose in the directly extracted samples compared to a relative increase in fatty acids using the other two extraction procedures. Medium contamination as a reason for the enhanced glucose content in directly extracted samples was excluded by washing the cells two times after medium removal with 0.9% NaCl. A comparison of cellular metabolite profiles with metabolite profiles of cultivation medium confirmed that medium removal was indeed achieved. Thus, we suggest faster quenching of cellular metabolism as reason for the high glucose concentrations in directly extracted samples. Using organic solvents for simultaneous quenching of otherwise rapid metabolite turnover for inactivation of the majority of cellular enzymes and extracting cellular metabolites, could present an alternative method to traditional sampling, avoiding time consuming harvesting procedures. We provide evidence that the sampling procedure impacts cellular metabolite pattern and show that direct extraction could be an improvement with respect to extraction efficiency, sampling time and preserving the "true" metabolite pattern of a cell. However, at least half of the metabolites remained unaffected by the sampling procedure.

**Novel Aspects:** Analysis of changes in metabolite patterns after application of different sampling methods to an adherent growing cell line.

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# Quantitative proteomics of differentiating the HL/LL and the AR/AN response in Chlamydomonas reinhardtii

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The response of algae to different conditions has been researched intensively. Nevertheless a clear dissection of the high light response from the anaerobiosis response is still relevant. Attempting to understand the impact of these conditions on the level of the whole proteome of *Chlamydomonas reinhardtii*, quantitative proteomics by using LC-MS was applied. An experiment was designed in order to determine the specific response to high light and anaerobic conditions and dissecting these responses to ascribe them unambiguously to the both conditions. The newly developed computational enhancement procedure (Barth & Fufezan in prep.) allowed the total peptide quantification events to be increased by 130%. This procedure is based on RT and Intensity alignments of the 132 FASP LC-MS/MS runs.

**Method:** *Strains and cultivation*: The *C. reinhardtii* strain CC124 (CW15) was used in all experiments. Cells were grown under standard conditions [1]. *Sample preparation. C. reinhardtii* was cultivated in HL/AR, HL/AN, LL/AR, LL/AN conditions for 8 h. A metabolic labeling was applied using heavy nitrogen (N15) and samples were mixed to allow a quantitative comparison at the peptide/protein level between the HL/LL response under AR and AN conditions and the AR/AN response under HL and LL conditions (N15: HL/AR and LL/AN, N14 : LL/AR, HL/AN).Unlabeled and labeled protein raw extracts were mixed on equal protein amounts, digested with Trypsin and separated using the FASP method [2]. Peptide samples were analyzed using LC-MS/MS analysis (LTQ Orbitrap XL (Thermo, Bremen, Germany)).

**Preliminary Data:** *Bioinformatical analysis*: Using OMSSA [3] 3500 fractionated peptides in MS/MS spectra were identified. An RT alignment was performed for all MS runs (132 mzML files) using a knowledge-based approach (Barth & Fufezan in prep.). Quantification of peptides was performed using M-pyQuant (Niehues & Fufezan in prep., Poster). All computational modules are a) based on the Python scripting language, b) part of the MS-informatics Tool Box (Jaeger et al., Poster) and c) requiring the pymzML module as interface to mzML files [4]. 3449 peptides mapping to 1483 proteins and protein groups could be identified within the experiments. Peptide quantification using the normal method resulted in 9144 peptide quantification events representing 1106 peptides and 559 proteins. Using the enhanced approach a total of 21 030 peptide quantification events, representing 1506 peptides or 731 proteins, was achieved. Hence an improvement in quantified peptides of 36%, in quantified proteins of 31% and in peptide quantification events of about 130%, was achieved. These results show an enhancement in quantification efficiency and accuracy in terms of the number of peptide quantification events. *Dissection of responses*: Considering the dissection of the HL/LL from the AR/AN response, some proteins involved in or related to the citric acid cycle, like malate dehydrogenases (MDHM and MDH6 (putative)) or pyruvate dehydrogenase (PDH2) were found to be up-regulated in AR compared to AN conditions, but not in HL compared to LL conditions. The up-regulation was even more pronounced regarding the AR/AN response under HL conditions. Photosynthesis related proteins like lhcb4, lhcb5, lhca8, PSBO, PSBP showed also an up-regulation in AR compared to AN conditions. This effect was equally more pronounced when observing the AR/AN response under HL conditions.

**Novel Aspects:** Using this experimental setup a dissection of the HL/LL from the AR/AN response in C. reinhardtii is possible.

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# Changes in the metabolites profile of Fusarium isolates depending on growing conditions

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Fungi belonging to genera Fusarium are very important cereals pathogens. They are able to produce toxic secondary metabolites, mainly trichothecenes, which are toxic not only for plants but also for animal and human consumers. Since there are about 150 of known trichothecenes thus finding the volatiles markers for early detection of trichothecenes in grains is reasonable. Previous studies demonstrated the presence of trichodiene and others sesquiterpene hydrocarbons in cultures of toxigenic Fusarium [1, 2]. The presence of trichodiene in naturally contaminated grain of wheat was also reported although in that experiments no other sesquiterpenes were present [3]. The current study focus on comparison of profile of trichothecenes and volatile organic compounds (VOCs) produced by isolates Fusarium culmorum depending on growing conditions.

**Method:** Seven Fusarium culmorum isolates (ZFR-124, ZFR-112, ZFR-110, ZFR-118, UWM-1, KF-350, KF-846) were cultured on autoclaved wheat grain, for 2 weeks at 25°C, in the dark. In further experiment two of them (UWM-1, and KF-350) were used for field inoculation of wheat. Analyses of VOCs and group B trichothecenes were performed in fungal cultures and in inoculated wheat grain. VOCs were extracted by mean the SPME method using 200 mm of 53/30 mm DVB/Carboxen/PDMS fibre. The analyses were run on a gas chromatograph mass spectrometer (Hewlett Packard, GC 6890, MS 5972 A), using an HP-5MS (0.25 mm × 30 m) capillary column. The same GC-MS system was used for trichothecenes analysis after extraction with acetonitrile-water (82:18, v/v), purification on charcoal colums and trimethylsililation.

**Preliminary Data:** Fusarium culmorum isolates analyzed in present experiment were previously tested for aggressiveness to wheat and triticale and used for resistance screening under field conditions. All of them were toxigenic isolates able to produce deoxynivalenol (DON) and 3-acetyldeoxynivalenol (3Ac-DON) or nivalenol (NIV) and DON (isolate KF-350 and KF-846). During experiment profile and concentrations of trichothecenes in examined isolates of F. culmorum varied. In cultures the predominant toxin was DON which mean concentration was 47,36 µg/kg followed by 3-AcDON (41,85 µg/kg) and NIV (35,32 µg/kg). Fusarenon – X was stated only in two isolates (mean 0,93 µg/kg) whilst 15-acetyldeoxynivalenol was not detected. Concentration of DON in isolate UWM-1, which was DON producing chemotype and was used in field experiment, equals 19,46 µg/kg and in culture condition the predominan toxin was 3-AcDON – 114,59 µg/kg. Isolate KF-350, which also was used in field experiment, in spite of be NIV-producing chemotype, produced mainly 3-AcDON (27,43 µg/kg) and equals concentrations of DON and NIV – 13,71 µg/kg. The same isolates in field conditions cumulated significantly higher concentrations of toxins. Isolate UWM-1 cumulated 3115 µg/kg of DON, 154 µg/kg of 3-AcDON, 2 µg/kg of 15-AcDON and 50 µg/kg of NIV. In case of isolate KF-350 there was respectively: DON – 87 µg/kg, 3-AcDON – 1 µg/kg, FUS-X – 3 µg/kg and NIV – 3060 µg/kg. Analyzing VOCs in culture samples there was stated about ten times lower level of trichodiene than in inoculated grain. However in culture samples were present other terpenes with mass 204 which were not stated in field inoculated samples.

**Novel Aspects:** New aspect of performed experiment is comparison of VOCs and mycotoxins profiles of Fusarium culmorum isolates depending on growing conditions.

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# Mass spectrometric analysis of antibiotics from bacteria

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Antibiotics are indispensable agents for human live. To combat resistant pathogenic bacteria there is always medical need to explore new antibiotic substances. The naturally occurring thiazolyl peptides (e.g. thiostrepton, sulfomycin, thioplabin) are some of the most potent *in vitro* growth inhibitors of Gram-positive bacteria and are produced widespread among Actinobacteria. However, poor water solubility of thiazolyl peptides complicates their application as antibiotic drugs mad chemical modifications of thiazolyl peptides can improve it. For that reason it is very promising to explore new thiazolyl peptide structures. Previous searches of antibiotics were often performed under inappropriate conditions; more precisely natural living conditions have not been simulated. To reinvestigate the search at modified conditions a fast, sensitive, and cost-effective screening method is required.

**Method:** We use thiazolyl peptide producing Actinobacteria to establish a high throughput infusion mass spectrometry method implementing these demands. Thereby liquid extraction surface analysis using Triversa Nanomate technology is coupled with the high mass accuracy and resolution available on LTQ-OrbitrapXL tandem mass spectrometer.

**Preliminary Data:** The first obtained data suggest that this technique can be a powerful tool for antibiotic discovery and other microbiological approaches, e.g. investigations of signal molecules at surfaces directly between different microorganisms.

**Novel Aspects:** Establishment of a fast, sensitive and cost-effective high throughput bacterial screening for new antibiotics using Triversa Nanomate technology and LTQ-OrbitrapXL.

# Quantitation of endothelin-1 using a high-resolution accurate mass spectrometer-LTQ XL Orbitrap Discovery

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Endothelin (ET-1) is a 21 amino acid peptide that is produced by the vascular endothelium from a 39 amino acid precursor, big ET-1, through the actions of an endothelin converting enzyme found on the endothelial cell membrane. ET-1 formation and release are stimulated by angiotensin II, antidiuretic hormone, thrombin, cytokines and reactive oxygen species. ET-1 is known as a potent vasoconstrictor and elevated blood ET-1 levels are associated with heart, renal and respiratory diseases. The aim of this study was to develop an analytical method using a micro liquid chromatography coupled to a high-resolution hybrid linear ion trap instrument with Fourier transformation mass spectrometry for the quantitative determination of ET-1 in rat plasma.

**Method:** A LTQ-Orbitrap linear ion trap high-resolution mass spectrometer (Thermo Scientific, Bremen, Germany) with a nanoelectrospray ion source connected to a Dionex 3000 (Dionex Corporation, USA) micro flow system was used. Preconcentration of samples was carried out on a C18 trap column in an isocratic mode using 2-propanol and water with 0.005% of TFA. Chromatographic separation was performed on an Acclaim PepMap100, 3 um 100 A column (75 um i.d. × 15 cm, Dionex Corporation) using uncoated PicoTip emitters Silica Tip 30 um. A gradient of 2-propanol and water with 0.005% of TFA was used for 70 min. The flow rate of the mobile phase was set at 400 uL/min, and the eluent was split via a pre-column splitter to 400 nL/min.

**Preliminary Data:** We have developed and evaluated a method based on an accurate mass measurements of extracted target ions in full scan mode (range m/z 200-2000) using micro LC-LTQ-Orbitrap. The method was validated by studies of spiked recoveries, linearity, matrix effect, intra-assay precision, sensitivity, limit of detection and limit of quantification. The intra-assay precision and accuracy of the data obtained from analysis of ET-1 plasma samples are in the agreement with FDA guidelines. The sensitivity of the method in rat plasma is 10 ng/mL. For full scan performed in the orbitrap the ion injection time used was 50 ms, with the automatic gain control (AGC) target set to $1 \times 10^6$, and data were acquired in centroid mode with a mass resolution of 30000. For MS/MS settings scans were performed in the ion trap at a normalized collision energy of 35% with an AGC setting of $1 \times 10^4$. Ionization voltage was set to 1.6 kV, the capillary voltage and temperature were 40 and 200°C, respectively. All other parameters were programmed as suggested by the manufacturer. Using this strategy, coeluting matrix compounds or noisy peaks can be easily excluded, facilitating the identification and quantification of known or novel analytes in a single run analysis [1]. The data demonstrate that the LTQ-Orbitrap in full scan mode provides linear dynamic range. The full scan approach is more efficient, it is truly generic, and the analysis of reasonably behaved compounds does not require the operator to select a scan range and acquire data using generic settings [2].

**Acknowledgments:** This work was supported by the European Union from the resources of the European Regional Development Found under the Innovative Economy Programme (grant coordinated by JCET-UJ, No POIG.01.01.02-00-069/09).

**Novel Aspects:** Using full scan HRMS it is possible to obtaine data not only for endothelin-1, but also for other components [3].

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# Identification of acylated homoserine lactones (AHLs) produced by food spoilage microflora with the using of HPLC/DAD/ESI-TOF-MS technique

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N-acyl homoserine lactones (AHLs) are important intercellular signaling molecules used by many bacteria to monitor their population density and control a variety of physiological functions in a cell-density-dependent manner by the process called quorum sensing [1-4]. Quorum sensing involves synthesis and detection of extra cellular signals termed as autoinducers. Many Gram-negative bacteria like *Pseudomonas aeruginosa*, *Vibrio fischeri*, *Agrobacterium tumefaciens* etc., use acyl homoserine lactones (AHLs) as cell-cell communication molecules. When a threshold bacterial density (and corresponding AHL concentration) is reached, AHLs interact with transcriptional activators to trigger the expression of target genes. The objective of this study was to confirm the presence of signaling molecules in isolated culture medium, earlier identified by another method.

**Method:** Analyses of appropriate extracts (containing signaling molecules) were performed on an Agilent 1200 series HPLC equipped with a diode array detector followed by a time of flight mass spectrometer with an electrospray interface. A 150 × 2,1 mm i.d. ACE C18 column with a proper precolumn was used for separation. Elution was performed using mobile phase A (0,1% aqueous solution of formic acid) and mobile phase B (acetonitrile). Chromatograms were recorded at 194 and 210 nm. Column effluent was monitored in positive mode of the MS. Major mass spectrometer parameters were as follows: capillary voltage – 3500 V, scan acquisition – from 50 to 1700 m/z, fragmentor voltage – 150 V.

**Preliminary Data:** Initial screening of obtained isolates for AHL-production was performed according to the methods of Ravn et al. [2]. In the method *Agrobacterium tumefaciens* ATCC 51350 and *Chromobacterium violaceum* ATCC 31532 were used as AHL monitor strains to screen for AHL-producing microflora. The tested strains were streaked in parallel to the monitor strains on LB agar plates. *Agrobacterium tumefaciens* carries a lacZ fusion to a traI and produces a blue color in a presence of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) in response to AHL [3]. *Agrobacterium tumefaciens* have a high affinity for long chained AHL (≥ C8). The CviR of *Chromobacterium violaceum* regulates the production of a purple pigment when induced by AHL. *Chromobacterium violaceum* is induced by C4-C8 unsubstituted AHL [4]. All plates were incubated for 3 days at 15°C. Subsequently, HPLC-ESI-MS technique was used to identify in details signaling molecules (present in bacterial supernatants). High-resolution mass spectrometry connected with the fragmentation in the ion source could be a useful tool during structural studies of compounds.

**Novel Aspects:** The HPLC-ESI-MS technique with fragmentation in the ion source could be used during detection of molecules occurring in complex matrix.

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# High Resolution LC-MS for Qualitative and Quantitative Mycotoxin Analysis in Complex Food and Feed Extracts

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Mycotoxins in agricultural products do have the potential to serious affecting the health of human and cattle. Therefore, there are strict official regulations in most of the countries with regards to the presence of these compounds in feed and food. Due to the complex matrices and time saving demands, the development of highly selective and sensitive multiresidue methods is necessary to ensure reliable results.

**Method:** A high resolution accurate mass (HRAM) approach on a benchtop Ortbitrap LC-MS/MS instrument was used. The method using full scan analysis mode is simple to set up and provides the ability of retrospective data analysis [1]. Precursor selection was used in combination with higher energy collision induced dissociation (HCD) to confirm the full scan results by MS/MS spectra in the same HPLC run.

**Preliminary Data:** In this work, the approach was used to screen for mycotoxins as well as for pesticides in high complex matrices like wheat and sow feed extracts. The use of different resolving powers (R = 35 k, R = 70 k and R = 140 k FWHM @ m/z 200) is compared with regards to the possibility to resolve the compounds of interest from matrix compounds. Quantitation was done by accurate mass extraction of the pseudomolecular ions from the high-complex matrix background. For confirming the identity of the screened substances a HRAM MS/MS was done.

**Novel Aspects:** Multiresidue HRAM screening methods on a benchtop Orbitrap LC-MS/MS instrument.

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# Mass Spectrometry Based Analysis of Blood Lipidome in Cancer Patients Treated with Radiotherapy and in Irradiated Mice

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Phospholipids (PLs) are the major cell membrane lipids. Besides structural role they are also involved in regulation of cell, apoptosis and proliferation. Malfunction in phospholipid homeostasis may become a serious issue for the whole organism leading to heart disease or cancer. Depending on the structure of polar head group PLs are divided into six major classes, but hundreds of PLs exist differing by non-polar groups linked to the sn-1 and sn-2 positions of glycerol backbone. In general PLs can be analyzed by mass spectrometry (MS) [1]. However, different classes of PLs ionize differently hence different MS tools might be optimal for analysis of different PLs. Here we aimed to implement MS-based analyses to identify radiation-induced changes in profiles of PLs present in blood.

**Method:** Two types of blood samples were analyzed. (i) Human serum samples were collected from 10 patients with locally advanced NSCLC (1) before, (2) during and (3) after radiotherapy. (ii) Mouse plasma samples were taken from 22 animals (1) one week and (2) twenty weeks after heart irradiation. Total lipids were extracted according to modified Folch method [2]. MS analyses of lipids were conduct on GC-ToF and MALDI TOF/TOF. For GC-MS analysis two step derivatization (O-methoxyamine for amine groups and MSTFA for silylation of hydroxyl and carboxyl groups) was performed. For MALDI-MS total lipid extracts dissolved in chloroform were registered directly in positive ion mode using DHB as matrix.

**Preliminary Data:** PCs and PEs were mainly analyzed. Preliminary data confirmed that ionizing radiation induced several changes in composition of blood lipidome.

**Novel Aspects:** Blood lipidome may be successfully analyzed by mass spectrometry tools.

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# Profiling of phospholipids in Acanthamoeba castellanii by LC-MS technique

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*Acanthamoeba castellanii* is a small protozoan living in various natural environmental sources (soil, fresh water, air) as well as in anthropogenic ecosystems. It has been increasingly recognized as human pathogens which causes serious, even life-threatening, infections such as granulomatous amoebic encephalitis and amoebic keratitis. Furthermore, amoebae feeding on bacteria have a significant effect on the control of a bacterial population. On the other hand, some microorganisms have evolved to become resistant to amoebae, since they are able to survive and growth inside free-living amoeba after internalization. In this way, amoebae act as reservoirs for pathogenic microorganisms (e.g. *Legionella*, *Mycobacterium*) [1, 2]. Phospholipids (PLs) are major components of cell membranes and the composition of phospholipid molecular species can affect membrane fluidity and stability.

**Method:** Normal phase (NP) and reverse phase (RP) liquid chromatography methods coupled with mass spectrometry technique were tested for identification of phospholipids isolated from *A. castellanii* cells. Precursor ion scan (PI) and neutral loss scan (NL) techniques were used to determine selectively phospholipid classes in the mixture. On the basis on fragmentation spectra of individual phospholipid species the structure of analyzed compounds were characterized.

**Preliminary Data:** In A. castellanii the following pattern of PLs was obtained: PC, PE, PS, PI, PA and DPG by HPLC-ESI-MS analysis. PC and PE were identified as main polar lipids in the phospholipid mixture isolated from the cells. Our results showed also that the certain long chain FAs of analyzed protozoan were associated with specific polar lipids. The presence of the 28- and 30-carbon acids were confirmed in PE, PS, PA and DPG classes of phospholipids.

**Novel Aspects:** The 28- and 30-carbon acids, identified in A. castellanii, were associated with specific classes of phospholipids (PE, PS, PA, DPG).

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# Antraquinones discoloration – identification of photodegradation products based on MS/MS spectra

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The identification of natural dyes in work of art is often difficult because of chemical changes in dyes composition. The color change is caused by dye ageing. This processes depend on environmental and conservation conditions. Photodegradation, due to light radiation is one of the most important factors of ageing.

**Method:** The present study concerns discoloration of anthraquinones. Degradation products were identified based on the MS/MS spectra obtained in the positive and negative ion modes of electrospray ionization. The technique was combined with capillary high performance liquid chromatography, which enabled restriction of sample volume to 1 μL and still effective separation of analyzed compounds. Spectra registered for each of the degradation product allowed to identify its lost fragments, what in consequence made possible reconstruction of their molecular structure.

**Preliminary Data:** In this study, compounds based on 9,10-anthracenedione skeleton were aged by exposure for light. The major products of degradation, phtalic acid and/or anhydride and their esters, were identified through capillary-HPLC ESI MS/MS analysis. Depending on chromophore groups in the molecule, the lost fragments of the compound involved 28, 32, 44 Da.

The study was partially supported by the Ministry of Science and Higher Education grant no. N N204 247040

**Novel Aspects:** Application of capillary HPLC coupled with ESI MS/MS for identification of products of natural colorants degradation obtained during artificial ageing

# Combined use of planar chromatography and DART mass spectrometry for characterization of propolis samples by multivariate data analysis

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Sophisticated statistical tools are very helpful for extracting the full analytical power from analytical methods. In particular, the newly developed prompt ambient mass spectrometry technique, employing Direct Analysis in Real Time ionization, is perspective for sample characterization and differentiation by means of multivariate data analysis, but needs studies due to the methods novelty. Its combination with high-performance thin-layer chromatography (HPTLC) fingerprints has not been used until now. However, such a combination is especially promising, as it is a superposition of two ultrafast but information-rich analytical methods, giving the extended information on the nature of the samples. In the current study propolis samples differentiation was performed using HPTLC, Direct Analysis in Real Time mass spectrometry (DART-MS) and multivariate data analysis.

**Method:** Propolis extracts in ethyl acetate were obtained from the Apicultural State Institute (Stuttgart, Germany). The newly developed HPTLC method was used for determination of flavonoids and phenolic compounds in propolis extracts [1]. For registration of DART mass spectra of propolis extracts, the *DART-SVP A* ion source (IonSense, Saugus, MA, USA) was equipped with a motorized rail and the 12 DIP-it® sampler, and coupled to the G1956B MSD single quadrupole mass spectrometer (Agilent) via the Vapur vacuum interface (IonSense). The mass spectrometer was operated in negative ion mode.

**Preliminary Data:** The results obtained by the chemometric evaluation of HPTLC and DART-MS data provide complementary information. The complexity, expense, and analysis time are significantly reduced due to the use of statistical tools for evaluation of fingerprints. When the propolis samples are previously analyzed by chemometric evaluation of HPTLC data, the DART mass spectra of the same samples may be analyzed more effectively and give more reliable information as compared to using these methods separately. The developed approaches allowed categorizing 91 propolis samples from Germany and other locations by their flavonoid and phenolic acid profiles. HPTLC with selective post-chromatographic derivatization provided information on the functional groups in marker compounds, while DART-MS delivered information on possible molecular masses of the principal components (flavonoid and phenolic markers).

**Novel Aspects:** It is a first study on the combined use of HPTLC and DART-MS for characterization of propolis samples by MVA.

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# Mass spectrometric analysis of anthocyanins and metabolites in complex biological samples

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Anthocyanins are one of the main classes of flavonoids in plants and responsible for red, blue and purple colors in fruits, vegetables and flowers. Anthocyanins have antioxidant and antiviral properties and are therefore the focus of numerous studies. Epidemiological studies sparked interest in them because of a positive correlation of flavonol intake and reduced mortality from cardiovascular disease [1]. In the context of the ANTHONIA study (funded by the BMBF) the effects and metabolism of anthocyanins are investigated in detail. Cell culture, animal model and human studies are performed to investigate the bioavailability and metabolism of anthocyanins.

**Method:** The analysis of anthocyanins and their metabolites in complex samples (fruit extract, biological fluids) requires a dedicated preparation protocol and highly sensitive detection system. The first steps are purification and preconcentration by solid phase extraction (SPE). Fast chromatographic separation of samples was achieved by using a RSLC (Rapid Separation Liquid Chromatography) system (Ultimate 3000, Dionex) with column pressures up to 600 bar. The RSLC system was coupled via an electrospray ionization source (ESI) to a LTQ FTICR Ultra or an Exactive Orbitrap (Thermo Fischer Scientific GmbH, Bremen) allowing measurements with high mass accuracy (< 2 ppm). With the Exactive Orbitrap fast switching between positive and negative ion mode is possible in one LC run.

**Preliminary Data:** The main focus of ANTHONIA are glucosides of malvidin, peonidin, petunidin, delphinidin and cyanidin in anthocyanin-rich beverages. We quantified the intact anthocyanins in 1000+ plasma, intestinal content, urine and faeces samples. High mass accuracy proved to be essential in our HPLC-MS measurements for the correct identification and quantification in complex biological samples. Furthermore a group of metabolites occurring after intake of anthocyanins were detected. Identification of compounds was verified by fragmentation experiments. Anthocyanin fragmentation pathways ($MS^n$) were studied in detail allowing the differentiation of isomeric structures. This was facilitated by HCD (higher-energy collisional dissociation) which provided efficient fragmentation and an extended mass range compared to CID (collision induced dissociation). The high mass accuracy also allows for quantification without the need for MS/MS experiments. Consequently metabolites (e.g. products of glucuronidation and sulfation) can be analyzed retrospectively. We developed a software tool to do this in a semi-automatic way for a large number of samples.

**Novel Aspects:** Accurate mass measurements of anthocyanins and metabolites in complex biological samples.

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# Implementation of HPLC-MS in analysis of phenolic compounds of fodder grasses

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Fodder grasses are important for agriculture, especially in livestock feeding. Phenolic compounds in these plants play role as a taste ingredient and bring benefits for animal health. High Performance Liquid Chromatography integrated with Mass Spectrometry (HPLC/MS) may be successfully used for profiling of phenolic compounds in extracts of plant. However, sensitivity of this apparatus limits the detection of compounds appearing in the smallest amounts. Therefore preparative flash chromatography on reversed phase column combined with the preparative HPLC can be applied for purification of phenolic compounds from about 1000 grams of plant material. This approach allowed to detect more phenolic compounds than it was possible during metabolite profiling using HPLC/MSn.

**Method:** Grasses were grown under greenhouse conditions. Flash chromatography: Phenolic compounds were extracted from 900 g of plant tissues using 80% (v/v) methanol and purified using C18 silica gel flash chromatography column (1.5 × 80 cm). The studied compounds were eluted with water-methanol solutions with increasing methanol contents. Fractions containing the same compounds have been pooled, evaporated and submitted to re-chromatography at the preparative HPLC using Beckman System Gold . HPLC/MSn: Agilent 1100 HPLC-DAD instrument with the XBridge C18 column (Waters) link to ion trap mass spectrometer Esquire 3000 (Bruker Daltonics). Each fraction of a single compound was evaporated to dryness and submitted to Nuclear Magnetic Resonance (NMR) analysis (Chemistry Department, Adam Mickiewicz University in Poznań).

**Preliminary Data:** Two systems, UV-HPLC LC/IT-MS (low resolution) and LC/qToF-MS (high resolution), were applied to analyze phenolics in leaves and root of 17 cultivars of grasses belonging to 6 species. There were 146 compounds identified which were O- and C-glycosides of flavones: apigenin, chrysoeriol, tricin, as well as flavonols: quercetin, kaempferol and isorhamnetin. The sugar moieties can be acylated with hydroxycinnamic, glucuronic or malonic acid. Other phenolic metabolites such as chlorogenic acid and its isomers and other quinic acid derivatives were also detected. Substantial differences in profiles of flavonoids between roots and leaves were observed. Flash chromatography combined with preparative HPLC was applied to isolate and purify compounds from 900 g of leaves of Phleum pratensis cv. Obra. Such obtained fractions were analyzed with HPLC/MS. As results, 14 additional compounds were identified not detected during direct analyses of the leaf extracts. Glycoconjugates of dihydroxy-methoxyflavone and naringenin were among the currently identified compounds.

**Novel Aspects:** Combination of the two systems mentions above extend the quantity and quality of information in research on metabolites in plants.

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# Carbohydrate Analysis in Urine by Liquid Chromatography and Tandem Mass Spectrometry as Non-invasive Method for Establishing Permeability of Digestive Tract

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Developed method with usage of high-performance liquid chromatography coupled with tandem mass spectrometry (HPLC-MS/MS) is able to identify and determine simultaneously mannitol, lactulose and sucrose concentration levels in excreted urine after oral administration of solution of selected non-digestable carbohydrates. HILIC was a method of choice due to its advantages during separation of polar compounds. Mobile phase is mainly organic (≥70%) with amount of polar/aqueous solvent. High amount of organic solvent due to its volatility enhances the response of ESI-MS/MS. Nowadays columns (like ZIC-HILIC or ZIC-pHILIC) offer electrostatic interaction and hydrophilic partitioning between analyte and stationary phase [1, 2].

**Method:** The HPLC-MS/MS contained Agilent 1200 series pump, degasser, autosampler, column oven and Q-Trap 4000 triple quadrupole mass spectrometer (Applied Biosystems) was used. Compounds of interest ionise in negative ESI mode. For MS/MS optimization of parameters flow injection analysis (FIA) was done using 1μg/ml solution of each substance respectively. For each carbohydrate specific and unique ion transition was chosen. The main parameters optimized for the ion transition were: declustering potential, entrance potential, collision cell exit potential and collision energy. The chromatographic separation was performed using 250 × 2.1 mm, 5 μm with pore size 200 Å ZIC-HILIC HPLC column. Mobile phase gradient was chosen and set from 75% of ACN in 5 mmol NH4Ac to 40% of ACN in 10 minutes with flow 300 μL/min.

**Preliminary Data:** Parameters for the monitored ion transitions and MS/MS operation parameters were optimized for every substance (mannitol, lactulose, sucrose and raffinose) to obtain maximum intensity and confidence during analysis. Declustering potential is the most important parameter which impacts the response from detector and therefore has the greatest influence to the peak intensity during analysis. The main challenge during analysis of carbohydrates is the fact, that lactulose and sucrose are characterized by the same molecular mass, ionise in similar pattern and the same fragment ions are formed. Only the optimization of HPLC conditions and usage of ZIC-HILIC column gives satisfactory results in separation of these two compounds. Samples of urine were purified by dispersive solid phase extraction using Amberlite MB150 ion-exchange resin. Raffinose was chosen as an internal standard. Within-run precision (CV) measured at three concentrations was 1.22, 0.3 and 0.54% for lactulose; 3.02, 1.52 and 0.67% for mannitol and 0.55, 1.11 and 1.68% for sucrose. Between-run CVs were 1.58, 1.29 and 0.62% for lactulose; 3.55, 1.66 and 2.35% for mannitol; and for sucrose 1.63, 0.19 and 0.17%. Analytical recovery of all three sugar probes was 89.8-110.8%. The detection limits were 0.19 mg/ml for lactulose and sucrose, while for mannitol LOD was at 0.36 mg/ml. Values of correlation coefficients for lactulose, mannitol and sucrose were at 0.9997, 0.9988, 0.9997 respectively. The proposed method is rapid, simple, sensitive and suitable for determination of intestinal and upper digestive tract permeability.

**Novel Aspects:** Simultaneous analysis of three carbohydrates. Quick sample preparation. Estimation of upper digestive tract and intestinal permeability at single analysis.

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# Biopharmaceutical glycoprofiling using pronase in-gel digestion and MALDI-Orbitrap MS

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Up to this day, several methods have been applied to the analysis of glycans attached to biopharmaceuticals. If the glycans are cleaved from the protein part, e.g. using N-glycosidase F, information about the glycosylation site is lost. If glycopeptides are produced using proteolytic enzymes, suppression by coproduced peptides is a major problem. The highly efficient digestion with pronase yields glycopeptides with short peptide sequences, which are, however, sufficient for glycosylation site identification, whereas the masses of non-glycosylated peptides are below the measured mass range in MALDI MS [1]. In-gel digestion could be advantageous compared to the established in-solution digestion as purification of the sample before and after digestion is not necessary.

**Method:** A commercially available monoclonal antibody (Cetuximab, Merck Serono GmbH) was subjected to SDS-PAGE separation using a Mini-Protean 3 system (Bio-Rad) in two different concentrations and under both reducing and non-reducing conditions (with or without the addition of DTT to Laemmli buffer). After Coomassie blue staining, bands stemming from native antibody as well as from separated heavy and light chains were cut out for further analysis. Pronase in-gel digestion and extraction of glycopeptides were performed using a Microlab Star liquid handling workstation (Hamilton Bonaduz). Extracts were analyzed by MALDI-Orbitrap mass spectrometry (Thermo Scientific) in positive and negative ion mode using DHB as matrix. Identification of glycopeptides was conducted semi-automatically by a software developed in-house.

**Preliminary Data:** Cetuximab is a recombinant monoclonal antibody with two glycosylation sites positioned in the conserved and the variable parts of the heavy chains. Therefore, a separation of glycosylation sites by SDS-PAGE is not possible under reducing conditions. Nevertheless, the addition of DTT seemed to increase the accessibility towards the proteases. Glycopeptides extracted from heavy chain digests could be assigned to both glycosylation sites and contained the major glycoforms already reported for Cetuximab [2]. The highly accurate MALDI Orbitrap mass spectrometric measurements and the self-written software facilitated the assignment to specific glycopeptides. The comparison with in-solution digestions of the same sample revealed very similar glycopeptide profiles. However, in-gel digestion automatically removes detergents and salts from the biopharmaceutical formulation before digestion, so that the usual purification step performed before in-solution digestion, e.g. using molecular weight cut-off membranes, is not necessary. Moreover, heavy and light chains can be examined separately without additional separation steps. Therefore, in-gel digestion using pronase is a valuable alternative to in-solution digestion due to fewer sample handling steps and ease of automation.

**Novel Aspects:** In-gel digestion with pronase and highly accurate MALDI Orbitrap mass measurements enables detailed glycoprofiling of biopharmaceuticals.

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# New sensitive LC-MS/MS method for amoxicillin determination in kidney and liver pork tissue

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Amoxicillin is well known drug, widely used in human and animal antibacterial therapy. It may seems that analysis of amoxicillin is easy, but due to its highly hydrophility (XlogP-2,0) amoxicillin analysis provides problems with separation from matrix and sample purification. Therefore for investigation of amoxicillin in animals tissues new and selective and sensitive method with MS/MS detection for determination of amoxicillin in pigs kidney and liver in reference to internal standard deuterium labeled amoxicillin (IS) was developed.

**Method:** Amoxicillin and IS were extracted from pig tissue with 5% trichloroacetic acid and purified with SPE system. The analyses were performed on Alliance HPLC system with MS/MS detector (Waters Micromass Quattro micro™) and chromatographic column Waters Atlantis HILIC Silica 3 mm, 10 cm × 2,1 mm. With chromatographic separation of analytes accomplished by isocratic elution of 0.01 M formic acid and acetonitrile, and flow 0.2 ml/min, the retention times for amoxicillin and IS were 6.5 min. The ESI source was working in positive mode, producing protonated molecular ions [M+H]+. Precursor and daughter ions of amoxicillin and IS were analyzed (m/z 366.4→208.1 and 370.3→212.4 respectively).

**Preliminary Data:** According to GLP requirements for bioanalytical methods the following validation parameters were estimated: linearity (linear model y = a + bx, range from 20.0 to 250 μg/kg), precision, accuracy and limit of the quantitation. Summary results of validation for liver and kidney tissue are presented in table 1. Despite the fact that results of parameters obtained during validation were very similar for kidney and liver tissue, result of matrix effect was different. This effect was estimated as decrease of signal for amoxicillin, for both tissues and equal 20.1% in kidney and 6.1% in liver. Decrease of signal for IS was on the same level in both matrixes and equal 25.1% and 29.3% respectively. Those data illustrated that matrix effect is a parameter that has big impact on bioanalyses and results obtained during studies.

**Novel Aspects:** New LC-MS/Ms method for amoxicillin determination in animals tissue.

# In depth examination of fragmentation reactions of thiourea- and urea-compounds important as CID-labile reagents for sensitive and effective chemical crosslinking

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We investigate the characteristic fragmentation reactions upon collision induced dissociation (CID) of thiourea- and urea-compounds, which proved to be promising reagents for chemical crosslinking [1,2]. The fragmentation patterns, which have been extensively investigated [3] are now examined by qualitative threshold CID experiments in a quadrupole ion trap [4]. As model compounds labeled thiourea- and urea-derivatives are synthesized to allow unambiguous characterization of competing fragmentation pathways. We report on energy-dependent CID experiments to get at least semi quantitative information on the threshold energies of the fragmentation pathways of interest [4, 5].

**Method:** Tandem-MS CID-experiments with monoisotopic precursor ion selection (conducted in the BE part) were carried out in the octapole or in the quadrupole ion trap (QIT) of a *Finnigan* MAT 900 mass spectrometer. Additional exact ion mass measurements of precursor and product ions were conducted in the orbitrap analyzer of a LTQ Orbitrap XL instrument (Thermo Fisher, Bremen, Germany). The energy dependent CID experiments were carried out in the quadrupole ion trap of a Bruker Daltronic Esquire 3000 (BrukerDaltronics, Bremen, Germany) using an isolation width of 2.0 and an accumulation time of 20 ms. All experiments were carried out with acidified (addition of 0.1% CF3COOH) analyte solutions in methanol.

**Preliminary Data:** The results of our set of CID-experiments document the subtle competition of the fragmentation pathways of the XL-compounds. The energy-dependent dissociation experiments conducted at the quadrupole ion trap instrument yield threshold energies for fragmentation reactions of a number of urea- and thiourea-compounds. We investigate fragmentation reactions of a set of structurally very similar precursor ions leading to a set of corresponding product ions which are all 7-membered ring heterocycles with comparable functionalities and therefore ring strains. Interestingly, we find similar threshold energies for the nucleophilic attack of an amide oxygen onto the adjacent thiourea carbonyl as well as for the reversed attack of the thiourea sulfur onto the amide carbonyl. As the product ion of the former fragmentation reaction is found with predominant abundance we assume that the two competing reaction mechanisms must have distinctly different entropic demands.

**Novel Aspects:** Energy-dependent CID experiments deliver semi-quantitative threshold energies of characteristic dissociation reactions of a set of thiourea- and urea-compounds.

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# Chip-Based Nanoelectrospray Mass Spectrometry of Fructooligosaccharides Produced by Levansucrases from Pseudomonas Syringae Pv. Tomato and P. Chlororaphis Subs. P. Aurantiaca

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Levansucrases (EC 2.4.1.10) are bacterial extracellular enzymes belonging to family 68 of glycoside hydrolases according to the database of carbohydrate active enzymes, CAZy. They carry out sucrose hydrolysis as well as transfructosylation reactions with sucrose molecule or fructooligosaccharide chain as acceptors. The pattern of produced fructans depends on specific features of the levansucrase. Levansucrases of Pseudomonas syringae pv. tomato DC3000 (Lsc3) and Pseudomonas chlororaphis subsp. aurantiaca (LscA) have 73% identity of protein sequences, similar sub-strate specificity and kinetic properties. Both enzymes produce levan and fructooligosaccharides (FOS) of varied length from sucrose, raffinose and sugar beet molasses. A novel high-throughput chip-based nanoelectrospray mass spectrometric method was applied to screen alternative fructosyl acceptors for levansucrases.

**Method:** For mass spectrometry analysis of synthesized FOS, 460 µg of LscA were incubated in 1 ml McIllvaine's buffer with 1200 mM of sucrose or raffinose at 37°C for 20 h. The reaction mixtures and control samples were diluted three times in deionized water/methanol/formic acid (4:4:1, v/v/v) and centrifuged (20 min) at room temperature. Thereafter the samples were completely dried in a SpeedVac Concentrator (Thermo Electrone Corporation, USA) and dissolved in 50 µl of $H_2O$. The solutions were diluted 10 times in deionized water/methanol/formic acid (4:2:1, v/v/v) followed by centrifugation. Fully automated chip-based nanoelectrospray mass spectrometry was performed on a NanoMate 400 robot (Advion BioSciences, NY, USA) coupled with a high-capacity ion trap mass spectrometer (HCT MS) from Bruker Daltonics (Bremen, Germany).

**Preliminary Data:** The ability of d-sorbitol, xylobiose, d-galacturonic acid, d-mannitol, xylitol and methyl-d-glucopyranoside to serve as fructosyl acceptors for levansucrases is shown for the first time. As most interesting outcome of this work we emphasize the ability of Lsc3 and LscA to transfructosylate various nonconventional acceptors producing heterooligofructans. These products can have novel or enhanced physiological effects and thereby applications of their own, but they can also serve as starting material for the synthesis of new oligosaccharidic products. A simple and rapid chip-based mass spectrometry analysis method applied in this work is certainly feasible to test levansucrases and other enzymes regarding the ability to transfructosylate/transglycosylate various new acceptor substrates.

**Novel Aspects:** The ability of sorbitol, mannitol, and xylitol to serve as fructosyl acceptors for levansucrases is shown for the first time.

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# Mass spectrometric identification of ribonucleoside adducts originating from 1-methoxy-3-indolylmethyl glucosinolate in phenol-chloroform isolates of murine liver DNA

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Glucosinolates are plant secondary metabolites present primarily in Brassica vegetables such as broccoli and cabbage. Recently, we have reported on 1-methoxy-3-indolylmethyl (1-MIM) glucosinolate for being a potent genotoxicant in bacterial and mammalian cells after activation by the plant enzyme myrosinase [1]. Thereby, the induction of mutations could be correlated with the formation of DNA adducts. Consequently, we have identified and synthesized the major DNA adducts $N^2$-(1-MIM)-dG and $N^6$-(1-MIM)-dA. Moreover, it was shown that the stable breakdown product 1-MIM alcohol forms identical DNA adducts after activation by sulfotransferases. We have developed an isotope dilution UPLC-ESI-MS/MS method for highly sensitive and selective MRM quantification of those adducted 2'-deoxynucleosides. Currently, we apply this method to several animal experiments and in vitro studies.

**Method:** The first step of the sample preparation procedure for the DNA adduct analysis is the DNA isolation (e.g. from animal tissue). For that we applied the commonly used phenol-chloroform extraction protocol, which includes the pre-treatment of the sample with an excess of RNase to avoid ribonucleic acid contaminations. The isolated DNA (25 µg) was then subjected to enzymatic hydrolysis and purification by solid phase extraction prior to UPLC-ESI-MS/MS analysis. We used three mass transitions each for the detection of $N^2$-(1-MIM)-dG and $N^6$-(1-MIM)-dA: the loss of the 2'-deoxyribose ($N^2$-(1-MIM)-dG: m/z 427 > 311), the cleavage of the 2'-deoxynucleoside ($N^2$-(1-MIM)-dG: m/z 427 > 160) and the secession of the 2'-deoxynucleoside and an additional methyl group ($N^2$-(1-MIM)-dG: m/z 427 > 145).

**Preliminary Data:** In various tissues (especially liver) of mice, which were treated with 1-MIM alcohol, we could observe an intense MRM signal chromatographically nearby that of $N^2$-(1-MIM)-dG. The corresponding compound showed the 1-MIM adduct-specific transitions m/z 427 > 160 and m/z 427 > 145, but not the loss of the 2'-deoxyribose (m/z 427 > 311). For recording a product ion mass spectrum of the desired compound, we hydrolyzed and pooled a total of 750 µg highly adducted murine liver DNA. The resulting mass spectrum revealed four analogous fragments as $N^6$-(1-MIM)-dA. However, the molecular ion showed a positive mass shift of m/z 16. We assumed that the unknown compound is the $N^6$-bound 1-MIM adduct of the ribonucleoside adenosine (Ado). To confirm our hypothesis, we conducted in vitro incubations of 1-MIM glucosinolate, myrosinase and Ado or RNA, respectively. The observed signals identified in murine liver DNA and that obtained in vitro exhibited identical chromatographic profiles and product ion mass spectra. Thus, our presumption that $N^6$-(1-MIM)-Ado is responsible for the unknown peak in the MRM channel of $N^2$-(1-MIM)-dG was confirmed. Furthermore, we could detect 1-MIM adducts of guanosine (Guo) and cytidine after incubating these single ribonucleosides or RNA with 1-MIM glucosinolate and myrosinase. First semi-quantitative adduct analyses of phenol-chloroform DNA extracts of murine liver tissues revealed relatively high RNA contaminations. While the amounts of $N^2$-(1-MIM)-dG and $N^2$-(1-MIM)-Guo were comparable, the concentration of $N^6$-(1-MIM)-Ado even exceeded that of $N^6$-(1-MIM)-dA about 4-fold. In contrast, no double peak was detected in pure commercially purchased DNA from herring sperm, which was adducted synthetically. In conclusion, we could identify RNA adducts originating from 1-MIM glucosinolate *in vitro* and *in vivo* for the first time using UPLC-ESI-MS/MS. However, this also shows that there is still a need for optimization of the phenol-chloroform DNA extraction protocol.

**Novel Aspects:** Identification of RNA adducts originating from the *Brassica* ingredient 1-MIM glucosinolate *in vitro* and *in vivo* by UPLC-ESI-MS/MS.

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# Molecular Expression and Structure of Gangliosides in Brain Metastasisof Lung Adenocarcinoma by NanoMate-QTOF MS and MS/MS

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Three years following surgery for lung tumor removal, a male patient (73-y-old) reports neurological symptoms such as sudden occipital headaches, dizziness, nausea, and lack of coordination. The sample of normal human cerebellum (male, 79-y-old) served as a control; it was obtained from the Department of Forensic Medicine, School of Medicine, University of Zagreb, Croatia. Ganglioside extraction was performed according to Vukelić et al. [2]. Ganglioside mixtures isolated and purified from brain metastasis of lung adenocarcinoma and normal cerebellar brain tissue used as control were analyzed in parallel. MS was performed on a QTOF Micro instrument (Waters, Manchester, UK). Fully automated chip-nanoESI was performed on a NanoMate 400 robot (Advion BioSciences, Ithaca, NY, USA) mounted to the QTOF MS.

**Method:** Three years following surgery for lung tumor removal, a male patient (73-y-old) reports neurological symptoms such as sudden occipital headaches, dizziness, nausea, and lack of coordination. The sample of normal human cerebellum (male, 79-y-old) served as a control; it was obtained from the Department of Forensic Medicine, School of Medicine, University of Zagreb, Croatia. Ganglioside extraction was performed according to Vukelić et al. [2]. Ganglioside mixtures isolated and purified from brain metastasis of lung adenocarcinoma and normal cerebellar brain tissue used as control were analyzed in parallel. MS was performed on a QTOF Micro instrument (Waters, Manchester, UK). Fully automated chip-nanoESI was performed on a NanoMate 400 robot (Advion BioSciences, Ithaca, NY, USA) mounted to the QTOF MS.

**Preliminary Data:** Qualitative analysis of GG pattern from brain metastasis sample using HPTLC showed the fractions migrating as GM3, GM2, and less or no visible fraction. In the sample obtained from brain metastasis, the GG fraction with migration properties of GM3 was the major one, accounting for 52.27% of the total GG content followed by GM2 with 34.81%, while proportions of more complex structures (GM1, GD1a, GD1b, and GT1b) were lower compared with healthy brain tissue. While in MS of brain metastasis sample only singly charged ions are present, MS profile of normal cerebellum GG extract is characterized by the presence of singly, doubly, and even triply charged ions. Healthy cerebellar tissue was found to contain a higher variety of GG structures differing in their sialylation degree, from short, monosialylated (GM) to large, polysialylated carbohydrate chains (GH) and also ganglioside chains modified by O-acetyl (O-Ac) and fucosyl (Fuc) attachments. In contrast to healthy cerebellar tissue, the ganglioside mixture extracted from brain metastasis of lung adenocarcinoma exhibits mostly species of short oligosaccharide chains and reduced overall sialic acid content. More than a half, from the total of 59 different ions detected and corresponding to 125 possible structures in brain metastatic tissue, represent monosialylated species of GM1, GM2, GM3, and GM4-type. GG composition of brain metastasis sample was found to be highly altered in comparison to the composition of healthy human brain [3]. Chip-nanoESI QTOF MS and CID MS/MS were able to provide compositional and structural characterization of native ganglioside mixtures from secondary brain tumors with remarkable analysis speed and sensitivity [4]. For all these reasons, the bioanalytical platform demonstrated here for determination of GSL molecular markers in brain tumors has real perspectives of development into a routine, ultrafast, and sensitive method applicable to other types of cancer and molecular markers.

**Novel Aspects:** First optimization and application of chip-based nanoelectrospray mass spectrometry for the investigation of gangliosides in a secondary brain tumor.

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# GC-APCI-QTOF-MS: An innovative Technique for Metabolite Profiling Studies

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GC-MS-based metabolite profiling of crude plant extracts reveals more than 100 compounds. Thereof, more than half can be identified using comprehensive mass spectral libraries, the rest remains unknown. In order to identify these we applied GC-APCI-QTOF-MS in succession to our metabolite profiling studies since this technique allows for the determination of elemental compositions of quasi-molecular and fragment ions. In addition, assumptions can be made about biochemical substance classes while recording collision-induced dissociation (CID) mass spectra. Its soft ionisation manner, high mass accuracy and resolution make it feasible. Thus, GC-APCI-QTOF-MS can complement GC-EI-MS-based metabolite profiling.

**Method:** *Arabidopsis thaliana* and *Piriformospora indica* were co-cultivated in a hydroponic system and roots analysed for metabolic differences in an untargeted manner using GC-EI-QUAD-MS. Then, all differential peaks (t-test: control vs. co-cultivated) were annotated with the help of comprehensive mass spectral libraries [1, 2] and the remaining peaks characterised by means of GC-APCI-QTOF-MS (Fig. 1). We also analyzed a complex synthetic mixture (50 components) with both instrumental platforms (GC-EI-QUAD-MS and GC-APCI-QTOF-MS) and compared both with respect to limit of detection and linear range.

**Preliminary Data:** Metabolite profiling via GC-EI-QUAD-MS revealed 29 differential compounds, of which 15 could be identified. These include amino acids (Ala, Asn, pyro-Glu, Ser, Thr), sugars (Glc, Fru, Suc, Trh, myo-Ino, αMDG), amides (urea) and organic acids (lactic acid, meglutol). The other 14 peaks, which could not be annotated by database search, were provided with elemental compositions obtained by accurate mass measurements of quasi-molecular ions. Some could be identified using electron impact (EI) spectra and atmospheric pressure chemical ionisation (APCI) spectra in parallel. During that process, CID mass spectra yielded beneficial information concerning functional groups. These resulted from the application of different collision energies (10 eV, 20 eV, 30 eV). In contrast, EI spectra offered a comprehensive molecular fingerprint, which can be used for structure elucidation. In addition we could show that GC-APCI-QTOF-MS is much more sensitive for most substance classes relevant for metabolite profiling studies than GC-EI-MS (up to 100 fold) and can not only be used for structure elucidation purposes of so-far unidentified compounds, but also for the profiling of low-abundant trace compounds.

**Novel Aspects:** GC-APCI-QTOF-MS can complement GC-EI-MS-based metabolite profiling studies.

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# Mass spectrometric analysis of caramelization products derived from disaccharides

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Caramel constitutes one of the mankind's oldest and most important dietary materials, have been widely used for coloring and flavoring of foods and beverages, for instance bear, soft drinks, soups and candies [1]. The volatile compounds formed in caramelization, represented by furans such as hydroxymethylfurfural (HMF) and hydroxyacetylfuran (HAF), furanones such as hydroxydimethylfuranone (HDF), dihydroxydimethylfuranone (DDF) and the pyranones have been thoroughly studied [2, 3]. The high non-volatile products have been divided into three classes of Caramelans ($C_{24}H_{36}O_{18}$), Caramelens ($C_{36}H_{50}O_{25}$) and Caramelins ($C_{125}H_{188}O_{80}$) [4]. The chemistry and structure of the non-volatile components of caramel have been investigated on few occasions and a dramatic lack of knowledge exists on these important dietary compounds consumed annually of several millions of tons.

**Method:** All carbohydrates samples were heated in an oven for 2 h at 180°C (sucrose, maltose) and at 200°C (lactose). High resolution mass spectra were recorded using a Bruker Daltonics micrOTOF Focus instrument. The separation was achieved on a 250 × 4.6 mm i.d. column containing diphenyl 5 µm and 5 × 4.6 mm i.d. guard column (Varian, Darmstadt, Germany). Solvent was water/formic acid (1000:0.05 v/v), and delivered at a total flow rate of 850 µL/min by 25 min isocratic. Tandem mass spectra were acquired in an Auto-MSn mode using a ramping of the collision energy. MALDI-TOF-MS spectra were recorded using an Ultraflex MALDI TOF mass spectrometer.

**Preliminary Data:** Caramel is composed from several thousands of compounds derived from a small number of unselective reactions. Products formed in the caramelization of sucrose, maltose and lactose include oligomers with up to six carbohydrate units, dehydration products of oligomers loosing up to a maximum of eight water molecules, hydration products of sugar oligomers, disproportionation products. The usefulness of an analytical strategy such as the van Krevelen and the Kendrick plot analysis to study the composition of dietary compounds, such as caramel have been demonstrated.

**Novel Aspects:** The innovative analytical strategies for complex mixture analysis used, provides a comprehensive account of the chemical composition of caramel.

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# Extracting Homologous Series from Fingerprint Bio-Oil Mass Spectra: A Complement to PCA

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Identifying critical chemical compound classes in complex mixtures such as petroleum or bio-oils is a challenging issue. New development of biomass derived oils, such as the Lignin-to-Liquid (LtL) oils, challenge the classical protocols for analogue crude oil analysis, due to both compositional differences as well as the lack of comparable product volumes with which to carry out a full characterisation. Focus on fingerprint analyses on low resolution equipment aim to give a fast, cheap and easy access to some of the most critical compositional traits and trends in these and many other products or process lines.

**Method:** Fingerprint Electrospray Ionisation – Mass Spectrometry (ESI-MS) has proved to be a powerful tool for detecting homologous series of chemical compounds in bio-oils, especially in combination with supporting statistic analysis. To improve the accessibility of this information, we have developed a new method to decompose mass spectra on the basis of these equally spaced series and use these as orthogonal basis vectors similar to principal components in Principal Component Analysis (PCA).

**Preliminary Data:** Results comprise our recently published method of decomposing fingerprint mass spectra on the basis of 14 Da spaced signals, enabling a more in-depth understanding of the complex reaction network in biomass conversion processes. We highlight further potential of the new method, and relate the strengths and weaknesses of the new procedure to conventional PCA.

**Novel Aspects:** A new model-based decomposition of mass spectra into homologous series is presented in complement to PCA.

# Characterization of oil by ESI, APPI and GC/APCI FTMS

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Crude oil as well as oil fractions represent an extremely complex mixture of organic compounds consisting of various elemental compositions and chemical structures. The composition of many compounds is not exactly known. Oil consists mostly (>95%) of hydrocarbons. However, the remaining part is composed mainly of hetero atomic compound classes containing oxygen, sulfur and nitrogen. These compounds are polar and most of them can be detected by mass spectrometry using atmospheric pressure ionization. The composition of the hetero atomic compounds is a fingerprint. Therefore, the oil industry is highly interested in qualitative and quantitative information of compound classes to optimize refining and catalytic processes by avoiding emulsion formation, coke formation and corrosion.

**Method:** Polar compounds can be detected by electrospray ionization mass spectrometry [1]. Direct infusion experiments can be carried out using an instrument with an ultra-high resolving power and mass accuracy to achieve mass peak separation and correct annotation of the molecular formula of all peaks in the mass spectrum. Several studies have been made in positive and negative ion mode by Fourier transform ion cyclotron resonance mass spectrometry (FT-ICR MS) using electrospray ionization [2]. An alternative to ESI, APCI and APPI [3] with direct infusion is GC/APCI. Isomers can be separated by GC and the molecular ions are detected by APCI mostly without any fragmentation in contrast to GC/MS instruments with an EI source.

**Preliminary Data:** Two different oil samples were analyzed by ESI, APPI and GC/APCI using a solarix 12T FT-ICR mass spectrometer (Bruker Daltonics, Billerica, MA). A few thousand elemental compositions have been detected and identified for each sample in positive and negative ion mode. Nearly all peaks in a mass spectrum can be assigned with exactly one molecular formula by FT-ICR mass spectrometry with a resolving power of more than 400,000 (200,000 with GC/APCI) and a mass accuracy better than 0.5 ppm. The classification has been done by intensity plots of compound classes and DBE (double bond equivalents) vs. C plots. The oil samples could be distinguished by the relative abundances of their compound classes. However, a detailed analysis by DBE vs. C plots of specific compound classes improves the confidence for the oil characterization. GC/APCI can be used to compare samples by their relative abundances of specific isomers, for instance alkylated benzothiophenes and dibenzothiopenes as well as PAHs.

**Novel Aspects:** FT-ICR-MS can be used to distinguish very similar oil samples on the molecular level with various ionization techniques.

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# Mass spectrometric behaviour of new naphth[1,2-e][1,3]oxazino[3,2-c]quinazolin-13-ones

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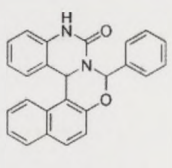
This work relates to a continuation of the studies on the synthesis and conformational analysis of new differently substituted naphth[1,2-e][1,3]oxazino[3,4-c]quinazoline derivatives [1], and naphth[1,2-e][1,3]oxazino[3,2-c]quinazolin-13-ones (see Scheme 1) [2]. Latter compounds were characterized by NMR spectroscopy and molecular modelling. The mass spectrometric behaviour of the compounds was studied by electron ionization (EI) and electrospray ionization (ESI) mass spectrometry combined with collision induced dissociation (CID). The influence of the different substituents (H, Ph, $\alpha$- and $\beta$-naphthyl, $p$-$OCH_3$-Ph and $p$-Cl-Ph) at the 1,3-oxazine ring system on the fragmentation was investigated. The phenyl substituted derivatives consisted of one pair of regioisomers. The mass spectra of these regioisomers were analyzed to make regio chemical conclusions. The preferred conformers, obtained by molecular modelling were compared with the mass spectrometric results.

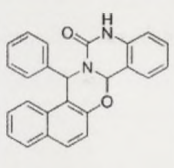
**Method:** The low resolution EI mass spectra were obtained using a Thermo Trace DSQII instrument (Axel Semrau).The elemental compositions of principal fragment ions were verified with a MicromassGC-$TOF_{micro}$ mass spectrometer (Micromass, Waters Inc., Wythenshawe, UK). The ESI and CID spectra were recorded using a ESI-Q-TOF mass spectrometer maXis (Bruker Bremen). Density functional theory calculations were carried out at the B3LYP/6-31G** level of theory using Gaussian 09 program package. Compounds were studied in all the configurations to find the preferred conformers and conformational equilibria

**Preliminary Data:** The fragmentations depend on the regiochemistry and the substituents of the compounds. The mass spectra of the regioisomeric 8-phenyl naphth[1,2-*e*][1,3]oxazino[3,4-*c*]quina-zolin-10-one (**1a**) and 15-phenyl-naphth[1,2-*e*][1,3]oxazino[3,2-*c*]quinazolin-13-one(**1b**) were different. For example the relative abundances of the molecular ion was higher for 1a than for **1b** and the $[M\text{-}OH]^+$ and the $[M\text{-}NH\text{-}CO]^+$ ions were only observed for **1a**. The fragmentation pathways proved to be quite similar for all the compounds studied (except for 1a). The EI mass spectra are dominated by fragmentation such as ring cleavage reactions to build up the ions $[M\text{-}C_8H_7N_2O]^+$. These ions formed the base peak of all compounds (except for **2** and **6**). The ions related or similar to their complementary ions were also observed. A possible mechanism for the required multiple bond cleavage to form the ion $[M\text{-}C_8H_7N_2O]^+$ is described earlier for naphthoxazine-derivatives [3]. The occurrence and the abundances of characteristic fragment ions in the mass spectra of the compounds will be discussed with respect to their global energy minima structures and were correlated with appropriate parameters obtained by *ab initio* calculations.

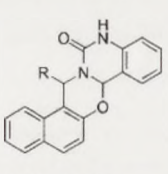
naphth[1,2-e][1,3]oxazino[3,2-c]quinazolin-13-ones



Compound **1a**



Compound **1b**



Compound **2** R=H
Compound **3** R=α-naphthyl
Compound **4** R=β-naphthyl
Compound **5** R=$p$-$CH_3O$-Ph
Compound **6** R=$p$-Cl-Ph

Scheme **1**

**Novel Aspects:** Mass spectrometric behaviour of new compounds compared with results from molecular modelling

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# Enrichment of Cross-Linked Peptides for Improved MS Analysis

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Chemical cross-linking in combination with mass spectrometry is suitable for determining low-resolution protein structures and for mapping protein-ligand interactions [1, 2]. The low amounts of protein required are one of the great advantages of this strategy. Additionally, cross-linking reactions can be performed in solution under almost native conditions. However, despite the simplicity of the cross-linking approach, data interpretation is often tedious due to the enormous complexity of the reaction mixtures obtained after enzymatic cleavage of cross-linked proteins. Therefore, we intended to improve existing enrichment strategies using strong cation exchange chromatography (SCX) for cross-linked peptides based on their higher charge states compared to unmodified peptides [3] with bovine serum albumin (BSA) and glutathione S-transferase (GST) as model proteins.

**Method:** Cross-linking reactions were carried out with 13.1 µM BSA (in 26.3 µM HEPES, pH 7.5) and 10 µM GST (in 20 µM HEPES, pH 7.5) using the cross-linker $BS^3$-$D_0/D_4$ (Thermo Fisher Scientific) at a protein-to-cross-linker molar ratio of 1:100. The results of the cross-linking reactions were analyzed by 1D-SDS-PAGE and linear MALDI-TOF-MS (Ultraflex III, Bruker Daltonik). Samples were proteolyzed with trypsin and the resulting peptide mixtures were analyzed by nano-HPLC (RP C-18 column, 75 µm × 250 mm, Dionex Corporation)/nano-ESI-LTQ-Orbitrap-MS (Thermo Fisher Scientific). Peptide mixtures were additionally separated by SCX chromatography (1 mm × 250 mm, Hypersil-Keystone, Thermo Fisher Scientific). Several KCl gradients were evaluated and elution fractions were analyzed by nano-HPLC/nano-ESI-LTQ-Orbitrap-MS/MS.Cross-linked products were analyzed by the StavroX software [4].

**Preliminary Data:** The approach of using SCX chromatography to selectively enrich interpeptide cross-linked peptides is based on their higher charge state compared to unmodified peptides, peptides with hydrolyzed cross-linker and intrapeptide cross-linking products [3]. Under acidic conditions, the majority of cross-linked peptides exhibit charge states $\geq 4^+$ due to the sum of two independent peptides with a total of two basic tryptic C-termini (Arg or Lys). Tryptic peptides usually have lower charge states and will elute earlier during SCX chromatography. To separate these differentially charged species, we applied several continuous KCl gradients to obtain as many interpeptide cross-linked peptides as possible. The improvement of this additional separation step for cross-linked products of BSA with the cross-linker $BS^3$ allowed the identification of 154 interpeptidal cross-linking products after LC/MS analyses, whereas LC/MS analyses without a prior enrichment lead to the identification of only 20 cross-linked products. The application of the SCX enrichment strategy for the analysis of cross-linked products of glutathione S-transferase (GST) with $BS^3$ allowed the identification of 26 interpeptidal cross-linking products compared to 16 without enrichment by SCX chromatography. Thus, for both proteins (BSA and GST) investigated herein, the introduction of an SCX-chromatography-based enrichment step prior to LC/MS analyses of cross-linking products lead to a considerable gain in structural information.

**Novel Aspects:** Improve enrichment of cross-linked peptides for protein structure analysis.

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# Investigation of Laminin/Nidogen Interactions by Chemical Cross-Linking and ESI-LTQ-Orbitrap-MS

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The scaffold architecture of basement membranes is created by a network of covalently attached type IV collagen and non-covalently attached laminin. The connection between collagen and laminin is provided by nidogen 1 and 2. Consequently, laminin/nidogen interaction is crucial for a stable assembly of basement membranes. The complex structure between the C-terminal G3 domain of nidogen 1 and LE modules 3 to 5 of laminin γ1 subunit has been published [1], yet, the interactions of full length nidogen 1 and 2 have not been studied on a molecular level so far. In this work, we report the investigation of nidogen 1 and 2 interaction with different laminin γ1 fragments by chemical cross-linking and ESI-LTQ-Orbitrap mass spectrometry.

**Method:** Cross-linking reactions were performed using the homobifunctional, amine-reactive cross-linker $BS^2G$ (*bis*[sulfosuccinimidyl]glutarate). Nidogen 1 or 2 was incubated with the laminin γ1 fragment (LE 3-5, LE 3-5 N802D mutant, short-arm, short-arm N802D mutant or short-arm ΔLE 4) in 20 mM HEPES, 100 mM NaCl, 5 mM $CaCl_2$, pH 7.4. After adding $BS^2G$ in 200-1000-fold molar excess cross-linking reactions were conducted for 30 min at room temperature and quenched by adding $NH_4HCO_3$. Cross-linking reaction mixtures were separated by 1D-gel electrophoresis (SDS-PAGE) and *in-gel* digested with trypsin and Glu-C. Analysis of the peptide mixtures was done by nano-HPLC (Dionex)/nano-ESI (Proxeon)-LTQ-OrbitrapXL (Thermo Fischer Scientific) mass spectrometry. The in-house software StavroX [2] was used to identify cross-linked peptides.

**Preliminary Data:** The interactions of full length nidogen 1 with laminin γ1 fragments were investigated using a combination of chemical cross-linking and high-resolution mass spectrometry. The cross-linker $BS^2G$ reacts with amine groups of lysine residues, which are up to ca. 19 Å apart. The C-terminal globular G3 domain of nidogen 1 is known to interact with the epidermal growth factor-like modules LE 3 to 5 in the laminin γ1 subunit since this complex has been crystallized ([1], pdb entry 1NPE). Therefore, we chose the complex between nidogen 1 and laminin γ1 LE 3-5 as a system to validate our cross-linking approach. Initial results indicate that laminin γ1 LE 3-5 does not exclusively interact with the G3 domain, but also with N-terminal regions of nidogen 1. Moreover, the analysis of intramolecular cross-links within nidogen 1 yielded a number of cross-links between *N*- and *C*-terminal regions suggesting a globular structure of nidogen 1 rather than a linear domain arrangement. Since the crystal structure of the nidogen 1 G3 domain/laminin γ1 LE 3-5 complex indicates that Asn-802 within laminin γ1 LE module 4 is crucial for the assembly of the complex, the N802D mutant of laminin γ1 LE 3-5 was also probed as a potential interaction partner of nidogen 1. Furthermore, we conducted cross-linking experiments with an elongated form of laminin γ1 LE 3-5 (laminin γ1 short-arm fragment), its N802D mutant, and a laminin γ1 short-arm variant lacking the LE 4 module. Preliminary data suggest that laminin γ1 LE 4 module is essential for complex formation between laminin and nidogen 1. In order to elucidate differences in the binding behavior of both nidogen isoforms, identical cross-linking experiments will be carried out with nidogen 2.

**Novel Aspects:** Identification of novel interaction sites between laminin γ1 and nidogen 1 and 2.

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# Dual labeling of biomolecules using MeCAT and DOTA derivatives

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The quantification of low abundance proteins in biological samples is a major challenge in proteomics. The recent introduction of bi-functional chelating agents (such as MeCAT and DOTA derivatives) that can harbor a metal and covalently interact with specific groups of the target biomolecules, allows monitoring the labeled compounds by highly sensitive elemental MS techniques as ICP-MS. Furthermore, the structural information of the labeled species can be obtained easily by molecular MS techniques as LC-ESI-MS. Despite the inherent high sensitivity of ICP-MS, even more sensitivity is usually required and hence methodological approaches like the selective enrichment of target species and increasing the number of introduced metal ions can contribute to overcome this problem. Both approaches were combined in the presented work.

**Method:** Cysteinyl peptides were selectively captured using thiopropyl sepharose 6B affinity resin [1]. After washing and removal of non-captured peptides, the free N-termini and lysine primary amines of the captured cysteinyl peptides were labeled with DOTA-NHS-ester then metallated with a lanthanide ion. Immobilization of peptides allowed washing the peptides and removing the excess of reagents. The immobilized cysteinyl peptides were then released from the resin with a reducing agent (TCEP). Finally thiol groups of cysteines were labeled with a lanthanide-containing MeCAT reagent [2] after a necessary clean step to remove TCEP excess. The structural information of the labeled peptides was achieved by nanoLC-ESI-FTICR (Thermo), whereas LC-ICP-MS (Element XR, Thermo) was used for the quantification of the labeled species.

**Preliminary Data:** The efficiency of the method was optimized using cysteine-containing standard peptides and then applied to bovine serum albumin (BSA) and human serum albumin (HSA) to demonstrate qualitative and quantitative aspects of this strategy. The efficient capture/wash/release process of cysteinyl peptides was demonstrated by *nano*LC-ESI-FTICR-MS comparing the identified peptides from digested BSA samples directly measured and after resin capture and elution. The new on-resin labeling of amino groups showed quantitative amino labeling for standard peptides and for most of the peptides from BSA and HSA digestion. The effect of excess TCEP on the MeCAT labeling of thiols was investigated concluding that excessive amounts of TCEP in the reaction medium competitively hinder the labeling reaction. Pierce® C18 Spin Columns were tested as the best option to remove excess TCEP. Complete dual labeling of the standard peptides was demonstrated by *nano*LC-ESI-FTICR-MS, whereas more than 80% of the detected peptides of BSA and HSA were completely dual-labeled with no evidences of incomplete labeling for non-detected peptides. Parallel detection by LC coupled to ICP-MS delivered reliable quantitative information such as the amino:thiol ratio in peptides analysed. Thus, the versatile lanthanide choice in both labeling steps allowed estimating primary amino and thiol stoichiometries for the studied samples using different lanthanides. On the other hand, enhancement of ICP-MS signal was achieved as expected when all positions were labeled with the same lanthanide. Although further improvements must be carried for absolute quantification of proteins, relative quantification was demonstrated through the successful linear calibration of the signal in standard addition experiments, which also showed the pre-concentration capability of the resin.

**Novel Aspects:** Selective dual labeling of primary amino and thiol groups of peptides with lanthanide-containing DOTA derivatives after efficient cysteinyl peptides enrichment.

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# Mass-spectrometric epitope mapping with polyclonal antibodies directed against the Ro52 autoantigen

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Ro52 is an autoantigen mostly associated with Sjøgren's syndrome (SS), systemic lupus erythematosus (SLE) and other autoimmune diseases, and it triggers autoreactive antibody production (anti-Ro52) in patients [1]. In our study by using mass-spectrometry [2], we first aim at determining the main epitopes in SS and SLE patients and second at stratifying these patients according to the epitope pattern upon correlation of mass spectrometric results with clinical data such as severity, onset of disease and response to treatment, to finally enable targeted therapy. At first a polyclonal Rabbit antibody serum shall serve as a model for the patient analyses in order to set-up the system.

**Method:** Using the SouBl21 E. coli strand, we expressed rRo52 that contained the human Ro52 sequence plus flanking amino acids. Expression was confirmed by 1D gel electrophoresis (12% T) and Western blot analysis using primary Rabbit antiRo52 antibodies, Mouse antiHis antibodies in conjunction with secondary mouse antibodies labeled with IRDy. Next, rRo52 was digested with Trypsin, LysC and degraded with BrCN, and peptide mixtures were analyzed by mass-spectrometry for peptide mapping and sequencing.

**Preliminary Data:** Our results show that rRo52 has been successfully expressed as a full-length protein together with some truncated forms. Protein concentration ranged from 950 µg/ml to 1100 µg/ml. Trypsin *in-gel* digestion was performed with 8 bands from the SDS gel where the main band was confirmed to contain the full length rRo52 sequence and sequence coverage was 40%. By comparing the Ro52 sequence parts that were contained in the bands that were decorated with anti-His and anti-Ro52 antibodies with those from the bands that were postively stained with anti-His but not with anti-Ro52, we could restrict the Ro52 epitope region to the amino acid sequence between position 255 and 326. This epitope region shall be confirmed and further narrowed with subsequent analyses using a combination of *in-solution* and *in-gel* digestions followed by mass spectrometric investigations together with Western blot analyses on the proteolytic rRo52 fragments.

**Novel Aspects:** Determining the main Ro52 epitopes in SS and SLE patients to enable targeted therapy.

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# Monitoring Conformational Changes in PPARα by Photo-Affinity Labeling and High-Resolution Mass Spectrometry

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Chemical cross-linking combined with an enzymatic digestion and mass spectrometric analysis of the reaction products has evolved into an alternative strategy to identify protein-protein and protein-ligand interactions. Peroxisome proliferator-activated receptors (PPARs) belong to the superfamily of nuclear receptors. One subtype, PPARα, plays a crucial role in lipid metabolism and presents an important target for designing antidyslipidemic drugs for the treatment of the metabolic syndrome [1]. Conformational changes in PPARα upon ligand binding were investigated by photo-affinity labeling (PAL) studies combined with mass spectrometry.

**Method:** PPARα variants were purified on an ÄKTA FPLC system (GE Healthcare) using 1ml-HisTrapFF and 1ml-HiTrap Q HP columns. Protein expression and purification were monitored by 1D-gel electrophoresis (SDS-PAGE) and separated gel bands were analyzed MALDI-TOF/TOF-MS/MS mass spectrometry (Ultraflex III, Bruker Daltonik) and nanoHPLC/nano-ESI-MS/MS using a nano-HPLC system (Dionex, RP C18 column 75 µm × 250 mm) coupled to an LTQ-Orbitrap XL mass spectrometer (Thermo Fisher Scientific) equipped with a nano-ESI source (Proxeon). Cross-linking product mixtures were analyzed using the *in-house* Software StavroX [2]. Identified cross-links were checked manually and visualized by PyMol.

**Preliminary Data:** Expression und purification of PPARα variants were optimized to obtain maximum protein yields. For PAL studies, the photoreactive amino acid *para*-benzoylphenylalanine (Bpa) was incorporated at positions Leu-258 and Leu-460 in PPARα. The integration of Bpa at position 258 was confirmed by MS/MS experiments. PPARα variant L258Bpa was employed for PAL studies both in the absence and presence of PPARα antagonist GW6471 verifying the conformational changes in PPARα that had already been suggested in previous studies [3]. In the presence of GW6471 a higher number of cross-linked products were identified than in the absence of GW6471, particularly in the PPARα domain comprising amino acids 215 to 226. Upon ligand binding, a conformational change ("mouse trap" principle) is induced in PPARα as indicated by several cross-links identified between Bpa and specific amino acids, such as leucine, phenylalanine, valine and arginine residues. A number of cross-links were identified between Bpa-258 and methionine residues in PPARα underlining the preference of benzophenone for methionines [4]. By a detailed inspection of MS/MS data, we were able to confirm a mixed cross-linked species between Bpa-258 and Phe-218 / Arg-226. Further PPARα variants are currently under investigation in order to confirm the conformational changes induced by ligand binding in future PAL experiments.

**Novel Aspects:** Incorporation of Bpa in PPARα

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# Characterization of Different Immunoglobulin G for Defined DNA Adducts and their Complexes by Mass Spectrometry under Native Conditions

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Since antibodies have been increasingly used in the diagnostic and therapeutic medicine new analytical tools for their detection are getting more and more important. In recent years especially soft ionization techniques of mass spectrometry like electrospray ionization (ESI) were further developed for the detection of high mass molecules. This allows the investigation of large proteins like antibodies in their native state without any digest. In order to understand structural aspects of native non-covalent complexes the analysis of a whole antibody-antigen-complex would be a big challenge. Our work present the first steps in accomplishing that topic.

**Method:** As model compounds we used different immunoglobulin G (IgG) with an average mass of 150 kDa. We focused on two monoclonal antibodies specific for different cell damaging DNA adducts: An 8-oxo-2'-deoxyguanosine (8-oxo-dG) specific mouse antibody for the detection of oxygen induced DNA damages. And a cisplatin specific rat antibody binding to cisplatin induced DNA adducts. Both types of antibodies were developed by Thomale et al. [1]. The detection of these native proteins was performed by MALDI-TOF-MS and a modified Waters Micromass nano-ESI-TOF-MS (modified by MS Vision) [2]. Synthetic oligonucleotides with different lengths and a known base sequence were used to produce the specific DNA antigens.

**Preliminary Data:** The detection of the different the IgGs was successful performed using MALDI-TOF and nano-ESI-TOF-MS. MALDI measurements resulted in data about the singly charged species under different matrices, concentration and mass spectrometrical conditions. Using nano-ESI-MS we found the antibodies to be 20 to 50 fold charged – extremely depending on the parameters used. The influence of protein concentrations, buffer systems (their kind, concentration and pH) and mass spectrometrical parameters were under investigation. First results indicate that the detection of the whole antibody-antigen-complex is possible using MALDI-MS.

**Novel Aspects:** Detection of antibodies and their complexes under native conditions via MALDI-TOF-MS and nano-ESI-TOF-MS.

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# Epitope Structure of the Asialoglycoprotein Receptor H1-CRD to a Monoclonal Antibody Revealed by Proteolytic Excision Mass Spectrometry

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The asialoglycoprotein receptor (ASGP-R) belongs to the C-type (calcium-dependent) class of lectins and is located on hepatocytes of all mammalian organisms. The carbohydrate recognition domain of subunit H1 (H1-CRD) provides clearance of circulating glycoproteins.with terminal galactose and N-acetylgalactosamine. Recent studies suggest that the carbohydrate recognition domain of the asialoglycoprotein receptor (H1-CRD) is used as entry site into hepatocytes by hepatitis A, hepatitis B, and Marburg viruses. Thus, molecules binding specifically to the CRD might exert inhibition towards these diseases by blocking the virus entry site. In this study, epitope elucidation was carried out by proteolytic epitope excision in combination with high resolution mass spectrometry, using a monoclonal antibody against the carbohydrate recognition domain.

**Method:** The recombinant carbohydrate recognition domain (CRD) employed was expressed in E. coli and purified by affinity chromatography using a galactose-Sepharose affinity column, followed by size exclusion chromatography. ESI-FTICR- MS of the intact protein (17 kDa) was carried out on a 7 T Bruker Apex II FTICR mass spectrometer in acidic solution. LC-MS and LC-MS/MS of tryptic mixtures of the carbohydrate recognition domain provided additional information on the structure of the antigen. For epitope elucidation, antibodies were immobilized on N-hydroxysuccinimide activated Sepharose. The affinity column was used for epitope excision and extraction using trypsin. Affinity bound peptides, eluted under acidic conditions from the column were desalted and analysed by MALDI-FT-ICR-MS.

**Preliminary Data:** Molecular mass determination by ESI-FTICR- MS of the 17 kDa intact protein and LC-MS/MS analysis of proteolytic fragments (trypsin and LysC) provided the identification of 2 intra-molecular disulfide bridges (7 Cys residues), and a Cys-mercaptoethanol adduct formed by treatment with ß-mercaptoethanol during protein extraction from the cell lysate. N-terminal sequence analysis and LC-MS of the digestion mixture produced by LysC showed the absence of the N-terminal Met-1 residue. The antigen was found to bind to the immobilized antibody in both native and alkylated form. Epitope -excision and -extraction with trypsin and FTICR-MS of affinity-bound peptides provided the identification of two specific epitope peptides, (5-16) containing a vicinal disulfide linkage and (17-23). To investigate the possible role of the cysteine residues in the interaction with the antibody, epitope excision-mass spectrometry was additionally performed with the alkylated H1CRD which identified the same epitope peptides (5-16) and (17-23). These peptides identified the location of the epitope at the N-terminal domain (5-23), on the opposite side of H1CRD, remote from the carbohydrate binding site (239-264). Affinity studies of the synthetic epitope peptides revealed independent binding of each peptide to the antibody.

**Novel Aspects:** Proteolytic excision and extraction combined with FTICR-MS of affinity-bound peptides provide the identification of two specific H1-CRD epitope peptides.

# Determination of Sunitinib in human plasma using LC/MS

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Sunitinib is an inhibitor of several receptor tyrosine kinases and is used for treatment of gastrointestinal stromal tumors as well as advanced renal cell carcinoma. Considering the need for low-cost cancer treatments, it is essential to develop reliable and sensitive bioanalytical methods of sunitinib determination in human plasma for conducting pharmacokinetic studies of new generic drug products.

**Method:** Currently available bioanalytical methods are fast and sensitive, predominantly based on protein precipitation and MS/MS detection, providing LLOQs at the level of 0.1 ng/mL [1]. Presented work describes validation of a method developed for sunitinib determination using LC/MS instrument, conducted according to respective FDA [2] and EMA [3] guidelines. In order to achieve required LLOQ, liquid-liquid extraction of 500 µL plasma sample with hexane/isopropanol (90/10 v/v) mixture was used. Chromatographic analysis was carried out using Zorbax SB-C18 150 × 3 mm, 3.5 µm column and isocratic elution with 55/45 (v/v) mixture of 0,1% HCOOH and ACN/MeOH (80/20 v/v). The detector was a single quadrupole setup with ESI (+) probe, operating in Single Ion Monitoring (SIM) mode.

**Preliminary Data:** Applied sample preparation procedure as well as instrumental conditions allowed recoveries of over 80% for both sunitinib and sunitinib-d10 (internal standard) and linear detection range of 0.1-150.0 ng/mL. Presented study was supported by the European Union (European Regional Development Fund) in frame of the Innovative Economy Operational Programme 2007-2013 (project No. UDA-POIG.01.03.01-14-069/08)

**Novel Aspects:** A new LC/MS method of sunitinib determination in human plasma was developed.

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# Electrochemical reduction of disulfide bonds in peptides and proteins followed by on-line MS detection

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Disulfide bonds are one of the most important post-translational modifications of proteins. They are stabilizing protein's 3-dimensional structure and are crucial for their biological function. The reduction of intra- and intermolecular disulfide bonds is necessary for successful characterization and assignment of the bonding sites by MS. Off-line reduction is performed using highly concentrated chemical agent (*e.g.* dithiothreitol (DTT)) that needs to be removed prior LC/MS analysis. Alternatively, thiol – free reducing agents as TCEP (tris (2-carboxyethyl) phosphine) can be used. However, the sample preparation remains laborious and difficult to combine with on-line LC/MS. Moreover, the possibility of on-line disulfide bond reduction can be beneficial for the determination of disulfide bond arrangements or top down proteomics strategy, which relies on fragmentation of intact proteins without enzymatic digestion.

**Method:** Typically 2-20 µM solutions of the target compound (peptide, protein, etc.) in 1% formic acid /acetonitrile (90/10, v/v) were pumped into the electrochemical (EC) cell at a flow rate of 50 µL/min. In EC/MS experiments the reduced sample was directly introduced into the ESI-MS. The cell was operating in pulse mode. The potential between the auxiliary and working electrode was set between 0 and minus 3000 mV to reduce the compounds of interest.

**Preliminary Data:** In this poster, we present electrochemically (EC) assisted reduction of biologically active peptides and proteins containing disulfide bonds followed by on-line mass spectrometric detection. For the first time the reduction of disulfide bonds is performed using a proprietary semi-precious metal working electrode. The unique properties of the working electrode allow for complete reduction of all disulfide bonds of the tested proteins and peptides. Furthermore, a special electrochemical method based on square-wave potential pulses was developed and applied for efficient and stable reduction. Insulin, a small protein of 5733 Da containing 3 disulfide bridges was used as model protein for system evaluation. The results are compared with the previous approach exploiting a conductive diamond working electrode. Reduction of other peptides and proteins such as Somatostatin with one disulfide bond (1638 Da) and α-Lactalbumin with four bonds (14 178 Da) will be shown to demonstrate the power of electrochemical S-S bond reduction. The data were acquired using high resolution Fourier transform ion cyclotron resonance mass spectrometer (FT ICR) that allowed for accurate molecular weight determination of intact protein before and after reduction. Additionally, high mass resolving power was necessary for unambiguous identification of a peak originated from Somatostatin and its reduced form. The electrochemical cell can be positioned before or after the HPLC separation, i.e., in an EC/LC/MS or LC/EC/MS set-up, resulting in a fully automated platform for fast characterization of S-S bonds in protein/peptide based biopharmaceuticals.

**Novel Aspects:** We present the first method free of chemicals to reduce disulfide bonds online following HPLC separation

# Bioprocess Optimization using Untargeted Accurate Mass Metabolomics

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The production of pharmaceuticals, antibodies, nutrients or other commercially interesting molecules is achieved using microorganisms or mammalian cells. Currently, over 2 biollion tons/year MSG (monosodium-glutamate) are being produced by industrial scale fermentation. While those processes are relatively cheap still the need exists to yield higher levels in shorter time with less resources. Metabolomics is the analysis of the complete metabolic composition with in principle one analysis allows to shed light into those fermetnation processes. The gained insight reveals bottlenecks, nutrient limitations and potential toxic media components or by byproducts. Here we describe the application of a world-leading accurate mass metabolomics platform to understand and optimize a fermentation process.

**Method:** Cells and media were samples in a several day long fermentation processes and collected every four hours. Media and cells were analyzed seperately on a well-established metabolomics platform. This plattform employs an Agilent GC-MSD and 6540 accurate mass QTOF for untargeted analysis. A proprietary data exploration platform allows the robust and precise annotation of mass spec data, as well as the identification of metabolites. Bioinformatic tools and multivariate data analysis identifies statistical relevant metabolites and allows modelling of metabolism to understand nutrient limitations and bottlenecks.

**Preliminary Data:** The fermentation process studied with the non-targeted metabolomics platform shows the decrease of nutrients in the media and first an increase of those nutrients in the cells. This goes along with a continues decrease in the cells. At the same time the cells are producing metabolites and desirable endproducts, which some of them are being secreted. Interestingly, the untargeted approach used reveals also the production of other industrial interesting compounds. And as such this platform proofs highly valuable also as a screening tool to identify the capabilities of mciroorganisms for the production of commercially interesting compounds. In this experiment limiting nutrients could be revealed, which can limit the production of the end product. Further, a processing influencing unknown compound synthesized by the cells could be identified by using the mass accuracy of the platform, fragmentation possibilities and novel mass spec analysis tools.

**Novel Aspects:** Metabolomic Discoveries employs accurate-mass metabolomics to optimize and improve products and processes. This project demonstrates the successful application of metabolomics.

# Carbonyl tagging reagents: Are they universal?

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The term "carbonylation" refers to non-enzymatic post-translational modifications yielding reactive aldehydes, ketones or lactams. Carbonylation is universally accepted as a biomarker of oxidative stress (OS) and is one of the most widely studied OS induced protein modifications. Elevated levels of protein bound carbonyls have been found in several pathological states including Alzheimer's disease, Parkinson's disease, and atherosclerosis. Mass spectrometry (MS) is commonly applied to characterize and identify such protein bound carbonyls by using carbonyl tagging reagents to facilitate their enrichment, identification and quantification by MS. Three basic questions, however, remain to be open: (i) the reactivity of the tagging reagents towards different carbonyl functionalities (aldehydes, ketones and lactams), (ii) the labeling efficiency, and (iii) MS behavior of the labeled peptides.

**Method:** Here we studied these questions for four tagging reagents with different carbonyl-specific groups (hydrazine, hydrazide, hydroxyl amine and amine) using a set of model peptides, containing aldehyde (glutamic semialdehyde, hydroxynonenal adduct of lysine and N-formylkynurenine), ketone (2-amino-3-ketobutyric acid, pyruvic acid adduct of lysine), and lactam groups (glyoxal and methylglyoxal adducts of arginine and oxindolylalanine). The reactions were qualitatively (MALDI- and ESI-MS) and quantitatively (RP-HPLC) monitored. Furthermore, the fragmentation-characteristics were studied on an LTQ-Orbitrap-MS using CID (collision-induced disassociation), HCD (high energy collision-induced dissociation) and ETD (electron transfer dissociation).

**Preliminary Data:** The hydrazine reagent derivatized all studied carbonyl groups, whereas the biotin conjugated hydrazide and hydroxyl amine derivatives reacted only with aldehydes and ketones. The amine derivative was specific for aldehydes. Lactams were difficult to derivatize, due to the resonance stabilization of the carbonyl group. None of the tested reagents was able to quantitatively derivatize all different carbonyl groups, but hydrazine appeared to be the best choice. The ionization efficiency in ESI depended on both the peptide sequence and the tagging reagent. The fragmentation behavior of peptides labeled with biotin conjugated carbonyl tagging reagents was best in ETD, as the derivatization was more stable and biotin less fragmented than in CID. Hydrazine tagged peptides were favorable for CID or HCD fragmentation, as the tag was stable under these conditions. In conclusion, hydrazine functionalized tagging reagent can be used for one-pot derivatization of all carbonyl groups, though quantitative derivatization of lactams cannot be achieved. Considering the fragmentation techniques CID or HCD can be employed, depending upon the type of application and the availability of the mass spectrometer

**Novel Aspects:** Detailed MS-based study of carbonyl tagging reagents and model carbonylated peptides allowed development of specific methods to examine protein carbonylation.

# Combined Electrochemical Oxidation/Ionization of a Selenoxanthene by On-Line Electrospray-High Resolution Mass Spectrometry

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9-Phenyl-2,3,4,5,6,7,8,9-octahydro-1*H*-selenoxanthene (SEL) was recently introduced in order to find use in the manufacture of food supplements, pharmaceutical and cosmetic products. It is claimed to exhibit antioxidant, detoxifying, hypolipidemic, immunomodulating and immunocorrecting, anti-atherogenic, anti-atherosclerotic and anabolic properties [1]. This work discusses the electrochemical (EC) investigation of SEL with regard to the ionization efficiency of the nonpolar compound and also a first prediction of its possible biotransformation.

**Method:** The oxidation behavior of the antioxidant SEL is studied by means of an instrumental on-line set-up consisting of an electrochemical thin-layer cell coupled directly to the electrospray ionization (ESI) source of a high resolution mass spectrometer (HR-MS). EC coupled on-line to MS enables the identification of products and intermediates of electrochemical reactions. A potential ramp between 0 and 2500 mV vs. $Pd/H_2$ (10 mV/s) was applied in the EC cell in order to screen for emerging oxidation products. Display of the obtained data as three-dimensional mass voltammograms visualizes the dependency of the oxidation/ionization efficiency of the EC cell depending on the applied potential.

**Preliminary Data:** Electrochemically assisted electrospray ionization of the nonpolar compound SEL enabled the detection of the corresponding cation and therefore makes ESI-MS more amenable to a wider group of compounds. Furthermore, EC oxidation gave rise to the formation of eight oxygenated and dimeric oxidation products. Molecular formulae could successfully be assigned for all oxidation products with deviations below 5 ppm between calculated and determined m/z in HR-ESI-MS. Due to the distribution of the naturally occurring isotopes of selenium and the low mass differences of the electrochemical products, overlapping of the signals occurs. Therefore, HR-ESI-MS proves to be essential to resolve and identify the signals of all oxidation products. Electrochemical oxidation allows a first prediction of possible phase-I metabolism reactions of SEL, which might occur *in vivo*. Electrochemically assisted electrospray ionization therefore enables method development for the identification of SEL in food supplements by means of ESI-MS.

**Novel Aspects:** EC coupled online to ESI-MS allows the oxidation/ionization of selected neutral compounds and the prediction of potential in vivo metabolites.

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# Databases as tools for structural elucidation of transformation products?! The example of biological Sulisobenzone transformation

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In recent years, the identification of unknown substances has been a major topic, especially in environmental science. These contaminants often are reaction or transformation products (TPs) of common pollutants. Liquid chromatography coupled to high-resolution mass spectrometry (LC-HR-$MS^n$) is state of the art for the analysis and structure elucidation of polar substances. A trend is to combine this method with advanced software solutions for the prediction of transformation products and/or theoretical fragmentation patterns of proposed structures. This study combines an experimental approach on the biotransformation of the far spread UV-filter Sulisobenzone (BP-4) in conjunction with the use of a biotransformation prediction system (*UM-PPS* [1]) as well as a mass spectral interpretation software (*MassFrontier* [2]) for the identification of potential BP-4 TPs.

**Method:** Biological degradation experiments were performed on BP-4 using activated sludge, diluted with effluent from a municipal wastewater treatment plant. The batch experiments were aerated with an air/$CO_2$-mixture to maintain aerobic conditions and a neutral pH. BP-4 was spiked and its fate was monitored by LC-HR-MS (LTQ Orbitrap Velos). Transformation products were predicted by *UM-PPS* and compared to the parent masses of newly emerging HR-MS-peaks during the BP-4 batch experiments. HR-$MS^n$ fragmentation patterns were used for proposing TP-structures based on expert knowledge. *MassFrontier* was afterwards supplied with the proposed structures and generated fragments and fragment-structures. These were checked for consistency with the experimental fragmentation data.

**Preliminary Data:** The 15 *UM-PPS* database-generated TPs on the first three levels of biodegradation showed no consistencies with the nine BP-4 TPs formed in batch systems. Based on expert knowledge of fragmentation characteristics, the chemical structures of the TPs were proposed prior to the use of the fragment-generation tool in the spectral interpretation software. Plausible chemical structures could be suggested for several TPs by interpreting HR-$MS^n$-experiments. All proposed structures were subjected to *MassFrontier* fragment-generation. For one potential TP-structure, *MassFrontier* generated 31 possible fragments. On the stage of $MS^3$/$MS^4$ two consistencies were identified. No further analogies could be found between analyzed and calculated fragments in this case, although the proposed chemical structure of this TP could unequivocally be confirmed by preparative synthesis of an authentic standard. These preliminary results indicate that databases are of limited support in the structure elucidation of environmental transformation products so far. Bearing in mind that biodegradation is strongly dependent on the substance itself and the experimental setup, there still is a lack of systematic studies, which results could provide a far-spread base for the building up of databases.

**Novel Aspects:** Combination of experimental biodegradation with prediction of transformation products and a mass spectral interpretation software for structure elucidation.

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# Electrochemical oxidation and protein adduct formation of aniline: A liquid chromatography/mass spectrometry study

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Aniline is a common substance in chemical industries and it has remarkable electrochemical properties [1]. First studies date back to the 19th century. Aniline is known to be a skin sensitizer. As sensitization results from the modification of skin proteins, mostly caused by electrophilic agents, the use of electrochemistry in combination with liquid chromatography and mass spectrometry provides an interesting option to simulate this process. It has been shown that the generation of electrophilic species can be achieved by electrochemistry. It is known from literature that aniline forms electrophilic species after electrochemical oxidation. However, the electrochemical approach has not been employed in terms of protein modification experiments so far.

**Method:** The oxidation products of aniline were generated by electrochemical oxidation in a thin-layer cell, which was coupled online to electrospray ionization-mass spectrometry. Without any pretreatment, the oxidation products were identified by electrospray ionization-time of flight mass spectrometry (ESI-ToF/MS) using their accurate masses. A mass voltammogram was generated by plotting obtained mass spectra against the applied potential. Protein modification was performed subsequently. Aniline was oxidized at a constant potential and the products were allowed to react with β-lactoglobulin A (β-LGA) or human serum albumin (HSA), respectively. Generated adducts were analyzed by liquid chromatography (LC) coupled to ESI-ToF-MS.

**Preliminary Data:** Oligomerization states with up to six monomeric units in different redox states of aniline were observed using this setup. Moreover, oxidation products other than only aniline oligomers were found. Mono- and dihydroxylated products, which have not been reported so far, were generated and identified. In protein modification experiments, the formation of two aniline adducts with both β-LGA and HSA was observed. For both β-LGA and HSA, the adducts were successfully identified and attributed to the modifying species. Primarily, this study was intended to develop a tool for the evaluation of potential chemical skin sensitizers, which are known to initiate their detrimental effects by binding to proteins. This qualitative analysis demonstrates that this approach could be a valuable tool for assessment of skin sensitization potential of chemicals [2].

**Novel Aspects:** The modification of proteins was simulated in an approach using electrochemistry/liquid chromatography/mass spectrometry, allowing the assessment of potent skin sensitizers.

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# Estimation of individual exchange rate constants for H/D exchange experiments

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Hydrogen/Deuterium exchange, combined with mass spectrometry (HXMS) is a technique to study the conformation of small biomolecules. It exploits the fact that the accessibility of a hydrogen atom is controlled by the distinctive structure adopted by the biomolecule and by the protection extent of the hydrogen in this structure. Therefore, determination of the exchange rates and the number of exchanged hydrogen atoms makes it possible to draw conclusions about the conformation and dynamics of proteins. We present a statistical method that allows estimating the individual exchange rate constants of exchangeable hydrogens and drawing conclusions about the conformation of biomolecules.

**Method:** We present a method that is similar to the three component method of Zhang and Smith (1993) and the global rate constant estimation method of Fattahi (2010). Based upon the mass shifts in the isotopic distribution of the biomolecule, due to the exchange of hydrogen with deuterium, our method determines the number of exchangeable hydrogens and their exchange rate constants by means of Newton-Raphson based optimization, which utilizes the known isotopic distribution of the non-deuterated biomolecule.

**Preliminary Data:** The proposed method is evaluated on two simulated datasets. In general, the estimated exchange rate constants match the true exchange rates, especially when taking standard error into account. However, a non-identifiability issue is present: our method does not return one unique solution but several equivalent solutions. The same issue is also present when applying the method of Zhang and Smith (1993). We show that this problem can partially be solved by a carefully chosen design of experiment.

**Novel Aspects:** Our method allows the identification of individual H/D exchange rates

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# Highly Sensitive and Robust Analysis for Lipophilic Marine Toxins in Shellfish

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Marine biotoxins are increasingly threatening the human health in many parts of the world. While the toxins are formed by microscopic planktonic algae of several genera usually at very low concentrations, they can be accumulated in bivalve molluscs to reach toxic doses. The consumption of contaminated shellfish or fish can lead to human poisoning or even death. In animals and humans there are four recognized symptom types of shellfish poisoning: In the last two decades the number and intensity of harmful algae blooms has increased and a bigger number of toxic compounds have been found in the marine food chain [3]. For the determination of the lipophilic marine biotoxins (lipMBT) ASU §64 method L 12.03/04 4 is used in germany.

**Method:** The separation of the lipMBT is performed on an Agilent Infinety 1290 HPLC-System. A Luna column (5 µm, C18(2), 100 Å, 150 × 2.00 mm) from Phenomenex is used for all lipMBT than YTX an a Zorbax Eclipse Plus (C8, 5 µm, 4.6 × 75 mm) is used for YTX. A gradient elution is used (negative mode: solvent A: 2 mM ammonium acetate in H2O, solvent B: MeOH; positive mode: solvent A: 0.1% formic acid in water, solvent B: MeOH). The mass spectrometric detection is performed with an Agilent 6460 triple quadrupole mass spectrometer equipped with an electrospray JetStream (ESI) interface operating in negative ionization mode for YTX and in positive ionization mode for all other lipMBT.

**Preliminary Data:** Modern methods for the analysis of marine biotoxins, in this case lipMBT, are based on techniques like LC-MS/MS. Until July 2011 the mouse bioassay (MBA), a biological testing method, was the official reference method in the EU for the detection of lipMBT. This situation has changed by the Commission Regulation (EU) No 15/2011 of 10 January 2011. After the 1st July 2011 the MBA is not longer the official reference method for the detection of lipMBT. The MBA were replaced by a LC-MS/MS method validated under the coordination of the EU-RL on marine biotoxins. For routine analysis in our Laboratory we use the international validated [1] official method on the basis of §64 German Food and Feed Act [2]. This method is comparable to the method from the EU-RL. Triple quadrupole mass spectrometric detection is the technique of choice for the control of the regulated lipMBT. The used method for routine analysis is robust and applicable for official control of lipMBT. All toxins regulated in Regulation (EC) No 853/2004 Annex III Chapter V are implemented in this method although there is a lack of commercial available certified standard substances. Only okadaic acid, azaspiracids 1, 2 and 3; pectenotoxin 2 and yessotoxin are available. In addition to these toxins it is possible to determine 13-desmethyl C spirolide, gymnodimine (both not regulated) and domoic acid (ASP poisoning). For the future there is a need for more commercial available standards especially for the quantification of marine biotoxins in the food chain. And additionally there have to implemented more specific methods using LC-MS/MS for the investigation of not yet regulated toxins like pinnatoxins, ciguatoxin, other isomers of the azaspiracid-group or the pectenotoxin-group. As well as a LC-MS/MS method that is easier to use as the actual used methods for the routine control of PSP-toxins.

**Novel Aspects:** The described method is robust and expandable for other toxin groups like ASP and fit for use in routine.

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# HOCl-induced phospholipid oxidation products as biomarkers of oxidative stress in inflammatory diseases

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The interest in phospholipid (PL) analysis is continuously increasing ("Lipidomics") because compounds such as lysophospholipids represent important disease and cellular integrity markers. Inflammatory diseases are associated with pronounced oxidative stress. At inflammatory loci, hypochlorous acid (HOCl) is generated by myeloperoxidase. HOCl reacts with a variety of biomolecules and induces the generation of lysophosphatidylcholine (LPC) from polyunsaturated phosphatidylcholine (PC). As many tissues and cells contain huge amounts of polyunsaturated PC species enhanced LPC concentrations are normally detectable under inflammatory conditions. However, human (e.g. liver) samples contain also major amounts of polyunsaturated phosphatidylethanolamine (PE). Unfortunately, it is so far widely unknown, if PE oxidation leads to LPE generation in the same manner as LPC is derived from PC.

**Method:** An aliquot of the phospholipid of interest (in chloroform) was evaporated to dryness. Multilamellar liposomes were prepared in 50 mM phosphate buffer (pH 7.4). NaOCl (as source of HOCl) was diluted with 50 mM phosphate buffer (pH 7.4) immediately prior to use. Liposomes (2 mM phospholipid) were incubated with varying concentrations of NaOCl/HOCl for 1 h at pH 7.4. To extract the lipids the same volume of a chloroform/methanol mixture (1:1, v/v) was added and the chloroform layer isolated. The PL oxidation products were analyzed by positive and negative ion MALDI-TOF MS (Bruker Autoflex) as well as $^{31}$P NMR (Bruker Avance 600). Dihydroxy benzoic acid (DHB), 9-aminoacridine (9-AA) and 4-chloro-α-cyanocinnamic acid (ClCCA) were used as MALDI matrices [1].

**Preliminary Data:** LPC and LPE formation could be detected by 31P NMR spectroscopy and MALDI-TOF MS in the extracts of liver biopsies from patients suffering from inflammatory diseases [2]. Both lysophospholipids are assumed to be massively generated under the influence of the enzyme phospholipase $A_2$ ($PLA_2$) although previous work provided evidence that LPC is also generated by HOCl oxidation. It could be shown by MALDI-TOF MS that PC as well as PE give the expected chlorination/oxidation-products of the double bonds in the unsaturated fatty acyl residue under chlorohydrin, dichloride and epoxide formation. In contrast, PC (but not PE) is also characterized by subsequent lysophospholipid formation upon treatment with HOCl [2]. In contrast, PE is also converted into mono- and dichloramines that are, however, only detectable in the presence of ClCCA [1]. Summarizing, it is obvious that the LPC formation from unsaturated PC and the accumulation in inflammatory liver tissue can be caused by both, $PLA_2$ activity and HOCl oxidation whereas LPE results exclusively from $PLA_2$ activity. It is concluded that LPC represents a useful biomarker of oxidative stress (generated by $PLA_2$ and HOCl) that can be easily determined by MS – even in complex mixtures and without the need of previous separation. LPE might be a reliable in vivo biomarker of $PLA_2$ activity.

**Novel Aspects:** Lysophosphatidylethanolamine might be a reliable in vivo biomarker of $PLA_2$ activity.

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# Investigating protease activities by mass spectrometry in body fluids and tissues

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We developed a mass spectrometry based enzyme screening (MES) assay for monitoring proteolytic activities [1, 2], which is helpful for the detection and identification of peptide processing and metabolizing enzymes. This assay includes the incubation of immobilized proteins with a defined peptidic probe. Presence of many different proteases in blood plasma or tissues often leads to the generation of various reaction products from the peptidic probes. MALDI-MES is ideal for detecting these reaction products, but since quantification with MALDI-MS is challenging it is not optimal for kinetic measurements. Hence we developed a selected reaction monitoring (SRM) based MES method for measuring kinetics of the generation of proteolytic reaction products that will be demonstrated for angiotensin- and endothelin-generating and metabolizing proteases.

**Method:** For investigating protease activities in complex protein fractions by the MES method proteins from human plasma and placental tissue were covalently immobilized onto affinity chromatographic material. Unbound molecules like buffer components that might interfere with mass spectrometry were removed by washing. Immobilized proteins were incubated with a reaction specific probe, here with an angiotensin I or big endothelin-1. Aliquots were removed from the reaction mixture after defined incubation times and analysed by MALDI-MS and SRM-MS for identification of reaction products as well as determination of kinetics of generation of different reaction products.

**Preliminary Data:** MALDI-MES gives a quick overview about the presence of peptide hormone generating and metabolizing protease activities in body fluids and tissues. The MALDI spectra are easily interpretable since the signals of the substrate and its proteolysis products are the only signals present. After incubation of placental proteins with big endothelin-1 the endothelin peptides ET-1 (1-21) and ET-1 (1-31) were detected. Incubation of plasma proteins with angiotensin I (AI) resulted in the reaction products A 1-9, A 1-7 and AII. Analysis of the kinetics by SRM-MS of the generation of these peptides showed that ET-1 (1-31) in comparison to ET-1 (1-21) was generated earlier than ET-1 (1-21) by placental membrane proteins. A 1-9 was generated more quickly than A 1-7 and AII by human plasma proteases for the slope of its kinetic curve was steeper in comparison to the other angiotensins, but the kinetics also show that it was degraded soon, indicating that A 1-9 is an important intermediate for the generation of A 1-7 and AII in human plasma. In summary MALDI-MES combined with SRM-MES is a powerful tool for detecting peptide-hormone generating and metabolizing protease activities and for measuring their kinetics in complex protein mixtures like blood plasma.

**Novel Aspects:** Qualitative and quantitative analysis of proteolytic activities in complex biological samples.

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# Ion Formation Mechanism and Charge Localisation in ESI of an Antenna-Like Ferrocene-Porphyrin-Fullerene Dyad

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ESI is used to investigate a novel dendritic $C_{60}$ derivative of the composition: $C_{60}$-$H_2P$-(ZnP-$Fc_3$)$_3$ with $H_2P$ = porphyrin, ZnP = zinc porphyrin and Fc = ferrocene. The main focus of this study is the elucidation of the ion formation mechanism and the identification of the charged sites (charge localization).

**Method:** ESI is carried out with an ion trap mass spectrometer which is use for the bulk material of the work. A large-scale TOF mass spectrometer is used for high resolution confirmation of the key protagonists.

**Preliminary Data:** Spraying from $CH_2C_{12}$ solution, a charge state envelop is obtained, reaching from three up to six positive charges. Isotopic pattern analysis reveals that the molecule is ionised entirely through oxidation, i.e. by removal of electrons rather than via multiple protonation. The fact that the ESI source can function as an intrinsic electrochemical cell is obviously very efficient with the molecule under study. The molecule is then synthetically disassembled into its building blocks. These individual sub-units were investigated separately to elucidate their role in the oxidation process. It turns out that neither $C_{60}$ nor the different porphyrin units (which were studied with or without central metal atom) are essentially involved with the ion formation. It is evident that the peripheral ferrocene units are the main protagonists in the multiple oxidation process. Square wave and cyclic voltammetry measurements support these findings, as the ferrocene units show with only 0.20-0.24 V (vs $Fc^+$/Fc) the lowest oxidation potentials of all other sub-units. In summary, the ligand on $C_{60}$ functions as a massive oxidation-antenna, allowing – at its periphery – the facile oxidative ionisation into highly charged states, thereby producing one of the most highly charged (true) molecular ions ever produced by ESI.

**Novel Aspects:** Highly charged molecular ions are obtained by oxidations during ESI.

# LC/MS-based validation of bioanalytical methods according to new European Medicines Agency guideline

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Results of pharmacokinetic studies directly influence therapeutic efficiency of drugs and safety of patients. Therefore, reliability of bioanalytical methods must be proven by detailed validation. Novel European Medicines Agency (EMA) [1] guideline describes mostly known and widely applied requirements, but also introduces some novel recommendations, e.g. incurred sample reanalysis.

**Method:** EMA guideline is dedicated to toxicokinetic and pharmacokinetic studies in all phases of clinical trials and specifies requirements on both small and macromolecules. In case of small analytes, liquid chromatography coupled to mass spectrometric detection (LC/MS) is a method of choice. Specific recommendations concerning LC/MS-based bioanalytical methods include matrix effect evaluation, internal standard selection (preferably stable isotope-labeled) and assessment of back-conversion of analyte to its metabolite in ionization source.

**Preliminary Data:** Currently, the guideline is the most complete and up-to-date document on bioanalytical issues. EMA and U.S. Food and Drug Administration (FDA) [2] requirements in this field are compliant to a large extent, which is very important for pharmaceutical industry.

**Novel Aspects:** EMA Guideline is comming into effect 1 February 2012. Novel requirements include specific recommendations concerning LC/MS-based bioanalytical methods.

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# Lipid- and protein-bound carbonyls: development of new MS-based analytical techniques

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Nowadays, mass spectrometry (MS) is the leading technique to discover disease-specific biomarkers. Oxidative stress, an imbalance between the generation of reactive oxygen species (ROS) and their elimination by antioxidants, has been linked to the pathophysiology of many disorders, such as atherosclerosis and neurodegeneration. ROS-driven oxidation of proteins and lipids yields oxidized biomolecules with altered structures and functionalities. One of the most abundant and hazardous modifications are reactive carbonyls (aldehydes/ketones), which are considered a major hallmark of ROS-related diseases. Protein carbonylation results either from direct oxidation of amino acid residues or from the reaction between proteins and secondary products of glycation and lipid-peroxidation. Lipid peroxides are formed during oxidation of polyunsaturated fatty acids, which further react with nucleophilic amino acid residues.

**Method:** Here we present different novel MS-based strategies developed in our laboratory to assess the changes in protein and lipid structures leading to "carbonyl stress" phenomena. To specifically map protein carbonylation sites and optimize the analytical parameters, we have synthesized first a panel of carbonylated standard peptides. Furthermore, complex mixtures of protein- and lipid-derived carbonyls were obtained from several *in vitro* and *in vivo* models of oxidative stress. Using this set of samples we evaluated several derivatization techniques for (i) superior separation in chromatography (RPC, HILIC), (ii) high ionization efficiencies (MALDI or ESI), (iii) favorable fragmentation (CID, ETD), and (iv) the shelf stability of the products. The studies were conducted on MALDI-TOF/TOF, ESI-QqTOF, and ESI-LTQ-Orbitrap mass spectrometers.

**Preliminary Data:** In the study of protein carbonylation, derivatization with dinitrophenylhydrazine (DNPH) was favorable, as the reagent excess provided a selective matrix to specifically analyze DNP-modified peptides by MALDI-MS. Therefore, the derivatized peptides could be enriched (MALDI MS-based enrichment) and identified by RPC-ESI-MS (using inclusion list function). This approach was further optimized on BSA oxidized *in vitro*, and then applied to the proteome of HeLa cells treated with Cu(II)-ions. This robust and effective strategy allowed us to identify several hundred carbonylated proteins representing different functional groups and subcellular localization. In the study of lipid-derived carbonyls, the derivatization using hydrazide/hydrazine chemistry allowed a shotgun lipidomics approach on an ESI-LTQ-Orbitrap-MS equipped with an TriVersa NanoMate ion source. The superior ionization efficiencies and the identification of several specific reporter ions allowed us to identify more than hundred carbonylated lipid peroxidation products in the mixture of four *in vitro* oxidized phospholipids. Biological relevance of detected carbonylated lipid species was proved by identification of the corresponding adducts on ApoB-100 protein of LDL complex isolated from the blood of healthy donors. Based on obtained results, several "carbonylation hot spots" were detected in ApoB-100, especially in the lipid-bound domains.

**Novel Aspects:** Presented novel proteomics and lipidomics strategies allow a robust and sensitive analysis of carbonylated species in qualitative and quantitative terms.

# Mass spectrometric analysis of caramelization products derived from disaccharides

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Caramel constitutes one of the mankind's oldest and most important dietary materials, have been widely used for coloring and flavoring of foods and beverages, for instance bear, soft drinks, soups and candies [1]. The volatile compounds formed in caramelization, represented by furans such as hydroxymethylfurfural (HMF) and hydroxyacetylfuran (HAF), furanones such as hydroxydimethylfuranone (HDF), dihydroxydimethylfuranone (DDF) and the pyranones have been thoroughly studied [2, 3]. The high non-volatile products have been divided into three classes of Caramelans ($C_{24}H_{36}O_{18}$), Caramelens ($C_{36}H_{50}O_{25}$) and Caramelins ($C_{125}H_{188}O_{80}$) [4]. The chemistry and structure of the non-volatile components of caramel have been investigated on few occasions and a dramatic lack of knowledge exists on these important dietary compounds consumed annually of several millions of tons.

**Method:** All carbohydrates samples were heated in an oven for 2 h at 180°C (sucrose, maltose) and at 200°C (lactose). High resolution mass spectra were recorded using a Bruker Daltonics micrOTOF Focus instrument. The separation was achieved on a 250 × 4.6 mm i.d. column containing diphenyl 5 µm and 5 × 4.6 mm i.d. guard column (Varian, Darmstadt, Germany). Solvent was water/formic acid (1000:0.05 v/v), and delivered at a total flow rate of 850 µL/min by 25 min isocratic. Tandem mass spectra were acquired in an Auto-$MS^n$ mode using a ramping of the collision energy. MALDI-TOF-MS spectra were recorded using an Ultraflex MALDI TOF mass spectrometer.

**Preliminary Data:** Caramel is composed from several thousands of compounds derived from a small number of unselective reactions. Products formed in the caramelization of sucrose, maltose and lactose include oligomers with up to six carbohydrate units, dehydration products of oligomers loosing up to a maximum of eight water molecules, hydration products of sugar oligomers, disproportionation products. The usefulness of an analytical strategy such as the van Krevelen and the Kendrick plot analysis to study the composition of dietary compounds, such as caramel have been demonstrated.

**Novel Aspects:** The innovative analytical strategies for complex mixture analysis used, provides a comprehensive account of the chemical composition of caramel.

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# Mass spectrometric analysis of renal sulfatides

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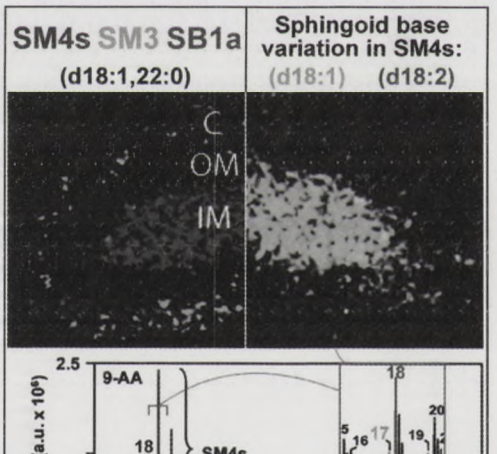
Sulfatides, a class of acidic glycosphingolipids, are highly expressed in mammalian myelin and in kidney. These molecules are known to be important components for membrane stability and furthermore for osmotic stability in renal cells. We analyzed this class of molecules by different kinds of mass spectrometric methods.

**Method:** MALDI-TOF mass spectrometry using the matrix 9-aminoacridine for the detection of complex renal sulfatides either from extracts on stainless steel target or in situ from frozen murine kidney sections [1]. In situ high resolution mass spectrometry of complex renal sulfatides from frozen sections using 9-amioacridne as matrix and a MALDI-LTQ Orbitrap mass spectrometer. NanoESI- tandem (QqQ) mass spectrometry to classify subgroups of renal sulfatides [1].




**Preliminary Data:** Using 9-aminoacridine as matrix in the MALDI process, we were able to enhance the sulfatide specific sensitivity of the method leading to the detection of complex sulfatides [1]. Using MALDI in situ mass spectrometry, we analyzed the tissue-distribution of sulfatides with regard to their carbohydrate head group and their ceramide anchor composition in kidney revealing distinct localization patterns for sulfatide subgroups either according to their head group or according to their type of ceramide anchor [1]. Analysis of extracts with a MALDI-LTQ Orbitrap mass spectrometer enabled clear assignment and separation of sulfatide peaks with identical nominal mass. Performing in situ analysis of frozen kidney sections on the MALDI Orbitrap instrument, we found completely opposite tissue distributions for some sulfatides with the same nominal mass (differing by 37 ppm), which could not be resolved on the MALDI-TOF instrument and hence resulted in a false distribution pattern. Comparing control kidneys with those of a mouse model of the human inherited disease metachromatic leukodystrophy, we found different subtypes of sulfatides to accumulate with different speed. Isobaric sulfatides with an additional double bond in either the acyl chain or the sphingoidbase of their ceramide anchor could be differentiated by analyzing extracts in the precursor ion scan mode of a nanoESI-QqQ mass spectrometer [1].

**Novel Aspects:** Detection of complex renal sulfatides by MALDI IMS and identification of a new subclass of sulfatides in mouse kidney.

## References

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# Mass spectrometric analysis of the catalytic degradation of organosolv lignin

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The development of renewable fuel sources has become increasingly important as the world looks towards alternatives to the use of fossil fuels. Lignocellulosic biomass has quickly become a popular source of renewable biofuels. Due to the highly recalcitrant nature of lignin found in this biomass, there is a growing interest in the catalytic conversion of lignocellulosic biomass to viable biofuels. The need for reliable characterization of mixtures and molecular composition determination has led to the development of new mass spectrometry techniques. The ability to characterize the molecular composition of biomass degradation products and biofuels is crucial for the rational development of biofuels. We report here a method for the characterization of lignin degradation products by using an Orbitrap mass spectrometer.

**Method:** Organosolv lignin was treated with Raney Ni under pressure and at different temperatures. Experiments were performed in a Thermo LTQ-Orbitrap Elite mass spectrometer. Ionization was performed via atmospheric pressure chemical ionization (APCI). Samples of organosolv lignin were dissolved in methanol.

**Preliminary Data:** Lignin samples treated with the catalyst show a higher abundance of peaks when compared to lignin samples treated in the absence of the catalyst. As expected, treatment at higher temperatures led to more lignin degradation, however, at the highest temperature, 300°C, the advantages seen for catalyst-based degradation at 200 and 250°C decreased. High-resolution mass spectrometry is used to investigate the mechanisms by which lignocelluolosic material is broken up during catalytic treatment by determining the class distribution present as well as providing a detailed comparison between different experimental conditions regarding ligin degradation.

**Novel Aspects:** Use of high resolution mass spectrometry to determine composition of renewable biofuels.

# Qualitative and quantitative characterization of human plasma glycation patterns

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Glycation (non-enzymatic glycosylation) is a reversible reaction of protein amino groups with reducing sugars. *D*-Glucose, the most abundant monosaccharide in blood, is readily involved in this process yielding relatively stable fructosamines (Amadori products). The latter undergo further reactions producing diverse advanced glycation end-products (AGEs), known to be markers of diabetes complications. Though no direct pathological effects have been described for Amadori products, they are still recognized as universal diagnostic tools. Multiple glycation sites in plasma proteins were described [1], however, their qualitative and quantitative patterns in healthy and diabetic individuals are still not characterized. Here we present a new approach to identify and relatively quantify Amadori peptides after selective enrichment in diabetic patients and healthy individuals.

**Method:** Plasma proteins obtained from diabetic and healthy individuals were separated from low-molecular components by ultrafiltration and, after reduction of disulfide bonds and alkylation of free sulfhydryl groups, digested with trypsin. Glycated peptides were selectively enriched on *m*-aminophenylboronic acid-agarose and subjected to RP-HPLC-ESI-MS/MS analysis [2]. The glycated peptides were analyzed by LTQ-Orbitrap-MS in information-dependent acquisition (IDA)-experiments comprising survey MS- and dependent MS/MS-scans. Amadori-modified tryptic peptides were identified in tandem mass spectra by their characteristic patterns of neutral losses. Quantification was performed by MS-scans using QqTOF and LTQ-Orbitrap instruments using extracted ion chromatograms of signals with annotated m/z. Alternatively, relative quantification was performed with non-enriched plasma protein digests.

**Preliminary Data:** Fructosamine-modified peptides were identified by their fragmentation patterns comprising neutral losses of 18 u, 36 u (oxonium ions), 54 u (pyrylium ions) and 84 u (furylium ions). Both precursor and fragment ions were accompanied by these signals. *m*-Aminophenylboronic acid chromatography was initially optimized for the synthetic sequence H-AGGK$_{Amadori}$AAFL-NH2, that resulted in recoveries of more than 70%. The optimized procedure was applied to a tryptic digest obtained from *in vitro* glycated BSA. Thereby, 30 Amadori peptides, representing 27 modification sites were unambiguously identified by their fragmentation patterns. This established protocol was then used to analyze plasma samples obtained from five diabetic patients. Altogether, 18 Amadori peptides, covering 40% of the HSA sequence, were unambiguously identified. The majority of these species were relatively quantified in all five samples with a high reproducibility among the replicas. Eleven Lys-residues were glycated at similar quantities in all samples, with glycation site Lys549 H-K$_{Amadori}$QTALVELVK-OH being the most abundant [3]. This study was further extended to two groups of diabetic and healthy individuals, each consisting of five persons. The plasma samples were digested with trypsin (n = 3) and subjected to RP-nanoHPLC-MS with and without *m*-aminophenylboronic acid affinity chromatography enrichment. The diabetic samples resembled the previously identified pattern of HSA glycation sites, but revealed also those in some other abundant proteins. The glycation level varied for the modification sites and was lower for healthy individuals.

**Novel Aspects:** The methods for selective enrichment, LC-MS-based identification and quantification were developed. The method proved to be suitable for diabetes research.

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# Simulation of the Oxidative Metabolism of Diclofenac by Means of Electrochemistry/HPLC/Mass Spectrometry

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Diclofenac is a frequently prescribed drug in case of rheumatoid arthritis or muscle pain. In rare cases, liver damage may occur after intake. The metabolism of the drug plays an important role in the toxicity mechanism. During phase I metabolism in the liver, molecules can be bioactivated, resulting in reactive intermediates. These metabolites can be detoxified by conjugation with endogenous thiols like glutathione (GSH). If the GSH level is depleted, however, a reaction with proteins present in the liver may occur, leading to a modification of cellular functions or immunological responses.

**Method:** In this work, a purely instrumental set-up was used to simulate the oxidative metabolism of diclofenac and to assess the reactivity of the generated oxidation products. The drug is oxidized in an electrochemical thin-layer cell (EC) equipped with a boron doped diamond working electrode. By direct coupling of the EC with a mass spectrometer, mass voltammograms can be recorded. The extension of the set-up by a chromatographic separation allows the further characterization of oxidation products and/or adducts.

**Preliminary Data:** For diclofenac different oxidation products could be generated using this approach and their sum formulae could be calculated based on accurate mass measurements with deviations below 2 ppm. In the following, adduct formation with glutathione was investigated and two glutathione adducts of the benzoquinone imine were found to confirm previous results of Madsen et al [1]. In addition, protein adduct formation was studied by using a model protein (β-lactoglobulin A). A tryptic digest of the generated drug-protein adduct reveals that the protein is modified at its only free thiol group through a benzoquinone imine [2].

**Novel Aspects:** Certain phase I metabolites of diclofenac could be generated and investigated regarding their reactivity towards different trapping agents.

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# The Na+ Affinity Sequences of Sodiated Amino Acids and Ligated Fullerenes Obtained with a Quadrupole Ion Trap

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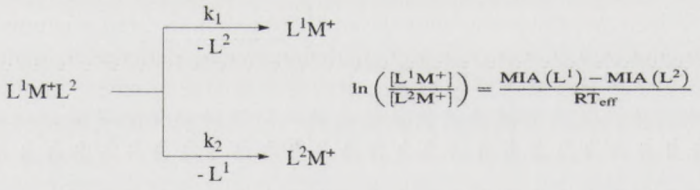
Many different approaches have been established for the determination of thermodynamic information by mass spectrometry. Among these, Cooks' kinetic method emerged as a powerful tool which can be applied to a wide range of systems and often works straightforwardly on a variety of different analysers. In our study we establish the applicability of our ion trap to such investigations by comparison with well-known quantities. Along those lines, the proton affinities of selected amino acids are obtained in excellent agreement with data published by Harrison, by Hunter and Lias and by Tabet and their respective groups. Afterwards we applied the kinetic method to establish the sodium affinity of the sodium salts of the naturally occurring amino acids.

**Method:** The kinetic method is based on the competitive dissociation of hetero-dimeric complexes of two different ligands that are connected by a charge-provider, to which the affinity is to be measured. The affinity is evaluated through the competing dissociation of the complex in MS/MS experiments. Properties like gas phase basicities and acidities or metal cation affinities are among the most popular quantities established by this approach.

**Preliminary Data:** While pure amino acids as such are the topic of numerous studies, the present investigation is the first to cover their sodiated counterparts. The obtained affinity ladder shows similarities with the sodium ion affinities of pure amino acids, yet there are also important differences. We suggest three groups of amino acid ligands showing a markedly affinity progression as a result of their structural features. Finally, we establish the relative sodium affinities of ligated fullerene derivatives (Prof. H. Hungerbühler, Berlin), featuring malonate ligands as the attractive site. The fullerene core and the number of attained ligands are varied. Interestingly, the method is sensitive enough to reveal distinct differences, which are discussed in light of the structural variations.




**Novel Aspects:** Sodium ion affinity sequences have been established for functionalised fullerenes and novel salt-like amino acid complexes.

# Comprehensive and Specific Biological Information at Cellular Resolution by MS Imaging

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Mass spectrometry imaging (MS imaging) has become a method of outstanding importance in life sciences. The information content of the generated MS images greatly depends on the quality of the underlying mass spectral data. We have recently introduced a method that combines the high performance features of Fourier transform mass spectrometers with a spatial resolution in the low micrometer range [1]. We have now optimized our workflow for the 'Exactive Orbitrap' and applied it to the detailed analysis of phospholipids in a number of biological samples. In addition data processing and analysis strategies have been expanded and optimized.

**Method:** Sections of mammalian, insect and plant tissue were prepared with a cryomicrotome. Matrix was applied by a home-built spraying device [2]. MS imaging experiments were performed with a home-built atmospheric-pressure imaging source [1] attached to an 'Exactive Orbitrap' mass spectrometer (Thermo Scientific GmbH, Bremen). Pixel size was between 5 and 10 µm. Mass accuracy was better than 2 ppm (root mean square) under imaging conditions, i.e. compounds were identified with high confidence. MS images were generated with a bin size of dm/z = 0.01, which largely eliminates interferences from neighboring peaks. In all experiments the high resolution and mass accuracy proved to be essential for specific image generation and reliable identification of analytes.

**Preliminary Data:** A dedicated sample preparation protocol was established for the analysis of single cells. We were able for the first time to identify larger metabolites (phospholipids) and investigate their spatial distribution within native single cells in one measurement. A full profile of phospholipids and smaller metabolites such as nucleic acids and cholesterol was obtained from a single 7 µm pixel. Intact phospholipids and their acyl chain fragments were detected simultaneously in all ion fragmentation experiments in a human brain tumor sample. This allowed the (tentative) differentiation of isomeric lipid structures throughout the whole section within one experiment. A complete set of positive and negative ion images was obtained simultaneously by periodically switching the polarity of the ion optics throughout the imaging experiment. This significantly increased the number of lipids that could be identified in a single experiment in mammalian tissue and thus improves the differentiation of tissue types. The effect of microbial infection on plants was studied based on changes in the phospholipid profiles. Pathogen specific signals were detected at a spatial resolution of 10 µm. Detailed distributions of phospholipids were detected within a root section of less than 1 mm in diameter. Whole body-sections of insects (e.g. paederus) were also imaged at 10 µm spatial resolution. Phospholipid patterns were used to obtain morphological information and to assign signals of interest (e.g. insect defense agents) to specific organs. Statistical analysis tools (including PCA and LDA) were adopted and applied for semiautomic assignment of tissue types in mouse and human tissue sections. Additional data analysis tools were made accessible by conversion to the common data format for MS imagingimzML (www.imzml.org). The presented work-flow with its improvements in sample preparation, measurement parameters and data processing significantly improves the biologically relevant information that can be obtained by mass spectrometry imaging.

**Novel Aspects:** Highly reliable biological information from MS imaging for mammalian, insect and plant samples.

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# The metallacrown as a template for Spontaneous Self-Assembly of a Polypeptide into a tetra helical bundle – the mass spectrometric study

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Designing of the stable artificial proteins is difficult task. This problem can be simplified significantly by attaching the short peptides of defined conformational preferences to the template assuring the proper backbone orientation (M Mutter, TASP concept [1]). In the TASP molecules the peptides are covalently connected to the template. The peptide chains may be also connected by noncovalent bonds or by forming labile complexes with metal ions [2]. Such design makes the TASP molecule the reversible system, which can be investigated by dynamic combinatorial chemistry methods. In this work we will presented the mass spectrometric study on new scaffold based on the metallacrown supramolecule, the stable structure formed by aminohydroxamic acids in presence of the cooper ions.

**Method:** *The chemical synthesis*. The peptides containing aminohydroxamic acid moiety were obtained by solid phase synthesis, using CHEMMATRIX® resin derivatized by our method. Obtained conjugates were purified by preparative HPLC and characterized by HRMS. *Mass spectrometric measurements*. MS experiments were performed on an Apex-Qe Ultra 7T and MicrOTOF-Q instruments (Bruker Daltonics) equipped with a ESI source. The measurements were performed in positive and negative ion mode. The samples were prepared approx 30 min before measurement by dissolving in ammonium carbonate buffer, containing acetonitrile.

**Preliminary Data:** *Peptide design.* The literature data showed high stability of complexes formed by aminohydroxamic acids with the transition metals ions. The interaction of $Cu^{2+}$ ions and aminohydroxamates result in formation of supramolecular structures with stoichiometry $L_4Cu_5$. The stability of metallacrowns formed by α, β and γ aminohydroxamic acids and $Cu^{2+}$ ions changes in the order β > γ > α [3]. In our study we decided to combine stable metallacrown formed by β-aminohydroxamic acid (βahx )with amphiphilic peptide sequences similar to that applied by Mutter as the constituents of TASP. To study the influence of side chain charge on the formation of dynamic TASP, besides of peptide basing on the Mutter's sequence **AH** (Ac-KALEKALKEALAKL-βahx) we synthesized also sequence **AHE** (Ac-EALEEALEEALAEL-βahx) and **AHK** (Ac-KALKKALKKALAKL-βahx), composed of only negatively and positively charged side chains respectively. *Mass spectrometric studies*. The interaction of peptides **AH**, **AHK** and **AHE** with $Cu^{2+}$ ions was studied by ESI-MS methods. The mixture of ligands with metal ions was infused directly to the ion source. Obtained spectra showed clearly that in the case of peptides **AH** and **AHK**, the complex with stoichiometry $L_4Cu_5$ was the predominant form. The predominant ionization state was +7 and +8 for **AH** and **AHK** complexes respectively. On the other hand the system **AHE** – $Cu^{2+}$ did not show formation metallacrown in the positive ion mode. In negative ion mode, we detected the system $L_4Cu_5$, but at higher concentration of $Cu^{2+}$ as compared to **AH** and **AHK** complexes. The molecular formula of obtained complexes was confirmed by HRMS and, independently, by MS/MS. In the system containing the equimolar mixture of **AHK** and **AHE**, we detected formation of two species $\mathbf{AHK}_2\mathbf{AHE}_2Cu_5$ (predominant) and $\mathbf{AHK}_3\mathbf{AHE}_1Cu_5$. The MS data correlate well with results obtained in solution by CD method.

**Novel Aspects:** The demonstration of suitability of metallacrowns as templates for assembly of synthetic proteins.

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# MS-Viewer – A Web Based Spectral Viewer for Database Search Results

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Many academic journals now require annotated spectra to be submitted to support manuscripts. As some manuscripts now report upwards of 100 000 phosphorylations site this is now becoming impractical. A spectral viewer has now been added to the Protein Prospector web site which allows the annotated spectra from database search results to be viewed interactively. Once the data has been uploaded and the report formatted other internet users can access the data *via* a keyword. The spectral viewer is also freely available for local installation.

**Method:** The program input is either a single peak list file or an archive of peak list files and a database search results file. The results file is expected to be in tabular form with columns containing peptides, spectrum identifiers and precursor charges. A fraction column is also required if multiple peak lists are uploaded. Columns containing other information may also be present. The individual spectra in the report can be viewed by clicking on entries in the peptide column. There is also a facility for re-searching individual spectra using the MS-Tag program. Data from up to four columns can be used for sorting the table. Reports can be saved and then viewed in the future by entering a keyword.

**Preliminary Data:** The software has already been developed and is available for use on the Protein Prospector web site and for local installation. We will demonstrate the use of the software to view Protein Prospector, Mascot and Xtandem results. Another unique feature of the software that we will also demonstrate is the ability to deal with ambiguous site assignments for modified amino acids.

This work was supported by NIH P41RR001614.

**Novel Aspects:** Web based spectral viewer for database search results.

# Study of pharmaceutical product by HPLC and MS

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Liquid chromatography and mass spectrometry investigation were carried on pharmaceutical product, which solutions were subjected to variety of forced stress conditions. The influence of acidic and basic hydrolyses, UV-radiation, oxidation and reduction, and the effect of temperature and duration of action, were examined. The solvent of pure active substance and medicine - naratriptan in form of tablets were taken for comparative studies. This drug belonging to the triptan group, serotonin agonist is commonly prescribed for migraine headaches in adults. Experiments were performed by using HPLC Shimadzu system and a mass spectrometer MicrOTOF-QII from Bruker Daltonik coupled with an LC Ultimate 3000 system (Dionex) to obtain the electrospray ionization time-of-flight mass spectra (LC-ESI-MS-TOF). Identification was based on the ESI mass spectra, MS/MS experiments and exact mass measurements of registered ions. Structure of compounds detected as impurities and degradation products have been proposed, new structures have been identified. The strong process of degradation of active substance was observed in solvent of oxidizing agent – 3% hydrogen peroxide in high temperature, leading to total decomposition of active substance during 8 hours. A new chromatographic method developed to study naratriptan under forced stress condition was proposed. The structures of some new compounds were presented.

# Polymer modified cyclodextrins – molecular level characterization through LC/MS and MS/MS

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Cyclodextrins (CD) ability to form inclusion complexes and hence to alter the physical-chemical properties of guest molecules has been largely exploited in order increase the water mixing properties of hydrophobic drugs. The structure of native CD molecules can be modified in order to optimize the physical inclusion of a specific guest or to fit various environmental requirements needed for specific pharmaceutical applications. The polymer modifications of these molecules benefited lately of increased attention and as consequence, the products characterization became also an important issue. The characterization of these type of products at molecular level in not straight forward since to the well known polymer chains dispersity one has to deal with the variation of the substitution degrees. Classical characterization techniques like NMR and SEC are providing insufficient answers in this case. Moreover, MS characterization may be sometimes misleading and can provide biased results. Direct injection ESI mass spectrometry is known to be an easy and fast method to achieve cyclodextrin derivatives characterization. In fact, ESI mass spectrometry has been used to characterize CD derivatives like DiMe-β-CD, Me-β-CD, and sulfobutylether-CD. ESI-MS/MS with low-energy CID, when applied to permethylated carbohydrate derivatives, produces copious fragmentation throughout the oligosaccharide molecule, providing structural information. Larger CD polymer conjugates are also characterized either through ESI or MALDI MS. However, their characterization at molecular level via tandem mass spectrometry was attempted only in our group. We present herein the investigation of esterified cyclodextrins through LC/MS and MS/MS on a LC ESI-QTOF instrument. The present study shows that depending on the chosen parent ion type, the cyclodextrin derivatives can be selectively cleaved at the level of the acetal junction or at the level of the ester bond. This distinct behavior in CID fragmentation conditions allows a direct access at the modified cyclodextrin substitution pattern. The compounds used for this study are a standard commercial sample, triacetyl-β-cyclodextrin (TABCD), and a random esterified β-cyclodextrin with 3-OH butyrate (PHBCD) synthesized in our laboratory. The tandem MS experiments, performed on a Q TOF instrument, revealed distinct fragmentation patterns according to the type of adduct submitted to CID fragmentation. The proton charged parent ions were cleaved predominantly at the level the semiacetal and the ester bonds for $[TABCD]^+$. In the case of $[PHBCD]^+$ parent ions the observed fragments issued from the cleavage of the semiacetal bonds. The $Na^+$ cationized samples yielded fragments only at the level of the alkyl ester bonds for both considered compounds. This study reveals the esterified CD substitution pattern, first due to the direct observation of the intact substituted glucopyranosic units and second due to the clear evidence of the esterified functions, demonstrating that a fast evaluation of this type of compounds is possible through tandem MS.

**Acknowledgements:** This research has been supported by Romanian MECTS project PN-II-RU-PD-2011-3-0127 and European Union's Seventh Framework Programme (FP7/2007-2013) under grant agreement n°264115-STREAM.

# The mass spectra fingerprint of 1-phenyl-pyrazolon[3,4-B]quinoxaline dyes

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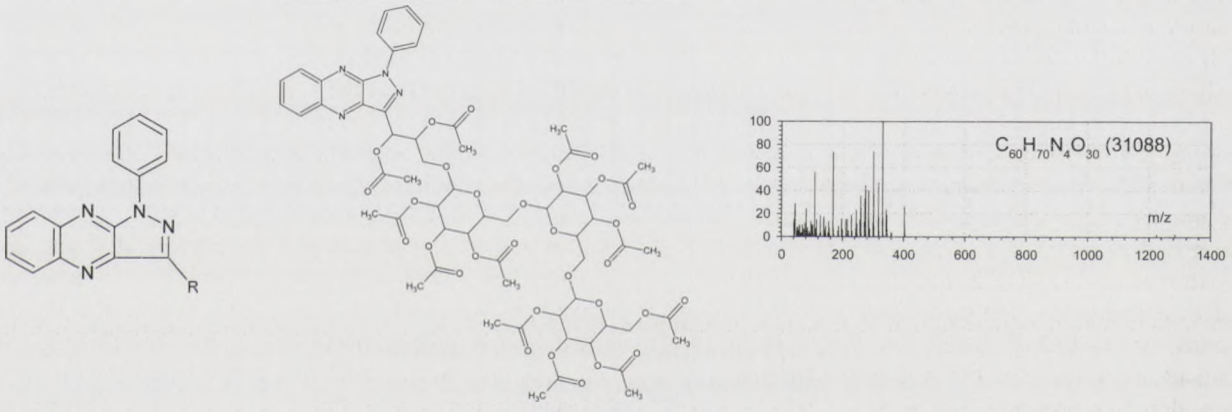
Dyes containing a 1*H*-pyrazolo[3,4-*b*]quinoxaline (PQ) moiety have been investigated since 1941. The PQs show applicable properties in antifungal [1], or antibacterial activity [2], separately in the *in vitro* tuberculosis [3]. The synthesis of 3-methyl-1-phenyl-1*H*-pyrazolo[3,4-*b*]quinoxaline (MPPQ) was first described by Ohle in 1941 [4]. Kolemainen et al. [5] analysed the selectivity of the reaction and descri bed the selected PQs as singlet oxygen sensitisers and efficient photoinitiators of free radical polymerisation [6, 7]. C-Nucleoside analogs have a glycosyl group attached to a nitrogen heterocycle at a ring-carbon atom, instead of a ring-nitrogen atom. This carbon-carbon linkage is more stable than the glycosyl carbon-nitrogen bond of true nucleosides, which makes the compounds useful tools for biochemical investigations and for antimitotic and antiviral research. The mass spectra of 1-phenyl-1*H*-pyrazolo[3,4-*b*]quinoxalines derivatives (PPQs) were presented in literature as supplementary results. Metwally [2] used MS for the synthetic MPPQ-s identification. Dolejš [8] and Sallam [9, 10] described saccharide derivatives of PPQ, using a similar method of synthesis. The authors of these papers explained the fragmentation partially and applied summary formulas only.

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**of Polish Mass Spectrometry Society**
**and German Mass Spectrometry Society**
**Abstracts**

4-7 March 2012, Poznań, Poland

Edited by
**JÜRGEN GROTEMEYER, MACIEJ STOBIECKI**

Contributions are printed as delivered by the authors
without substantial modifications



**ISBN 978-83-7712-064-4**

**Scientific Publishers OWN**
Institute of Bioorganic Chemistry
Polish Academy of Sciences
*e-mail*: own@man.poznan.pl

Printed in Poland (Moś and Łuczak, Poznań)