# ADVANCES IN CHROMATOGRAPHY AND ELECTROPHORESIS & CHIRANAL 2026

## BOOK OF ABSTRACTS AND PROGRAM

CHC
CHIRANAL

JUNE 15th –18th, 2026

OLOMOUC

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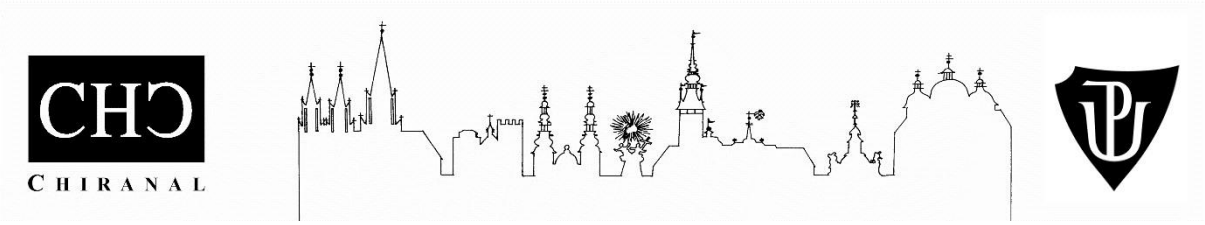


**OLOMOUC, JUNE 15th–18th, 2026**

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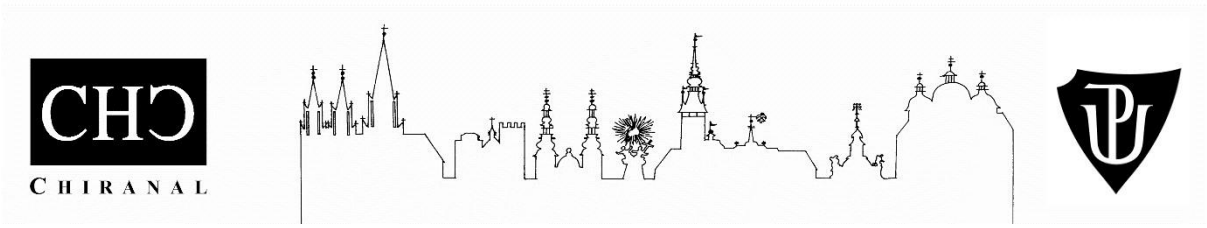


# BOOK OF ABSTRACTS AND PROGRAM

organized by:

Department of Analytical Chemistry

Faculty of Science

Palacký University Olomouc

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Conference venue

*Science Faculty, Palacký University Olomouc*

*17. listopadu 1192/12*

*779 00 Olomouc*

*Czech Republic*

**Advances in Chromatography and Electrophoresis & Chiranal 2026:**
**Book of Abstracts and Program**

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## PREFACE

Dear colleagues,

It is our great pleasure to welcome you to the conference Advances in Chromatography and Electrophoresis & Chiranal 2026 in Olomouc. With this meeting, we proudly continue a long-standing tradition that began in 1997 and has grown into an established international platform bringing together researchers, students, and industrial partners working in separation science.

Over the years, this conference has aimed not only to present current scientific advances, but also to strengthen collaboration across disciplines, institutions, and generations. In the spirit of Chiranal, we continue to foster an open and friendly environment where established experts meet early-career scientists and students, creating space for discussion, inspiration, and new cooperation.

This year's scientific program reflects the remarkable breadth and vitality of contemporary analytical chemistry and separation science. The lectures and poster presentations cover a wide range of topics including chromatographic method development, supercritical fluid separation, capillary electrophoresis, multidimensional separations, mass spectrometry, multiomics, sample preparation, and emerging analytical platforms. We are looking forward to inspiring lectures, fruitful discussions, and new ideas arising from this diverse scientific program.

The scientific program is complemented by a rich social and cultural program, which has become an integral part of our conference tradition. Participants will have the opportunity to meet informally during the welcome evening, social dinner, and accompanying events, including the traditional bowling tournament and a guided tour of Olomouc followed by a concert in the historic Corpus Christi Chapel.

This year, however, our meeting also carries a more personal dimension. We would like to dedicate this conference to the memory of Professor Zdeněk Stránský, who recently passed away. Professor Stránský was an outstanding scientist, respected colleague, and inspiring personality whose work and human approach influenced generations of students and collaborators. His contribution to analytical chemistry and to the academic community remains deeply rooted in our field. We remember him with gratitude and respect.

We would like to express our sincere thanks to all sponsors, exhibitors, and conference partners for their generous support. Without their help, it would not have been possible to organize this symposium at the quality and scale we all value.

Finally, we would like to sincerely thank all members of the organizing committee and colleagues involved in the preparation and realization of the conference. Their dedication, cooperation, and support are deeply appreciated.

Ladies and gentlemen, dear colleagues, welcome to Olomouc. We wish you an inspiring conference, stimulating discussions, and a pleasant stay in our city.

Petr Bednář, Marianna Nytka, Lucie Papoušková, Jakub Slačálek

on behalf of the organizing committee

Olomouc, June 15th, 2026

**ADVANCES IN CHROMATOGRAPHY AND ELECTROPHORESIS & CHIRANAL 2026**

**FINAL PROGRAM**

***Conference venues:***

*Faculty of Science UP, 17. listopadu 1192/12, Olomouc, Czech Republic*

*Fort Science, 17. listopadu 939/7, 779 00 Olomouc, Czech Republic*

## Monday (June 15th, 2026)

14:00–17:30 Registration (Fort Science, foyer, ground floor), individual arrival of participants and accommodation

16:00–16:15 Opening of the conference (Fort Science, Laudon Hall, ground floor), presentation of official representatives of the conference partners Foreword: Petr Bednář

16:15–17:15 *Plenary lecture (Fort Science, Laudon Hall, ground floor), chairmen: Karel Lemr, Juraj Ševčík*

**Michael Laemmerhofer** – Metabolomics and Lipidomics in Biomedical Research

17:30–?? Welcome party + banquet (Fort Science, Laudon Hall, ground floor)

## Tuesday (June 16th, 2026)

08:30–09:00 Breakfast (Faculty of Science, foyer, 2nd floor), installation of posters (Faculty of Science, seminary rooms, 2nd floor)

09:00–11:00 *Lectures I (Faculty of Science, assembly hall, 2nd floor): Innovations in Chromatographic Method Development, Supercritical Fluid Separation; chairmen: Michael Lämmerhofer, Petr Bednář*

09:00–09:30 **Lucie Nováková** – Breaking Boundaries in Natural Product Analysis with SFC-MS

09:30–10:00 **Kevin Schug** – Data Science-Assisted Method Development for On-Line Supercritical Fluid Extraction – Chromatography

10:00–10:20 **Roman Szucs** – Predicting Chromatographic Retention Times: Descriptor Choice, Feature Selection, and QSAR Interpretation

10:20–10:40 **Paweł Świt** – Development of Novel Calibration Methods and Their Application to the Chromatographic Determination of Bisphenols

10:40–11:00 **Kevin Schug (Shimadzu)** – Applications of online SFE – SFC: A Powerful Tool for Small Molecule Extraction and Analysis

11:00–11:30 Coffee break (Faculty of Science, foyer, 2nd floor)

11:30–13:10 *Lectures II (Faculty of Science, assembly hall, 2nd floor): Advances in Separation Selectivity and Stationary Phases; chairmen: Kevin Schug, Petr Barták*

11:30–12:00 **Wolfgang Lindner** – Enantioselective Ion Pairing Systems, my 50 years of Contribution

12:00–12:30 **Michal Kohout** – The Future of Multimodal Chiral Stationary Phases

12:30–12:50 **Pavel Blatný (TAPI Czech Industries s.r.o.)** – Analytical Challenges in the Development of Voclosporin HPLC methods: Application of High Temperature Liquid Chromatography in Pharmaceutical Analysis

12:50–13:10 **Petra Lewits (Merck)** – Next-Gen Zwitterionic HILIC: Particle Morphology Matters

13:30–15:00 Lunch (university cafeteria, Šmeralova 12)

15:00–16:30 ***Poster session*** and coffee break (Faculty of Science, foyer, seminary rooms, 2nd floor)

16:30–18:30 *Lectures III (Faculty of Science, assembly hall, 2nd floor): Advanced Separation Techniques for Complex Sample Analysis; chairmen: Wolfgang Lindner, Tomáš Pluháček*

16:30–17:00 **Marco Mattonai** – Quantification of Polyethylene in Compostable Plastic Formulations by Analytical Pyrolysis-Based Methods with Split Modulation

17:00–17:30 **Petr Vozka** – Untargeted Analysis of a Late Classic Period Maya Femur by Comprehensive Two-Dimensional Gas Chromatography (GCxGC)

17:30–17:50 **Ivan Petřík** – Theoretical Decomposition and Practical Mitigation of Matrix Effects in Quantitative LC–MS Calibration

17:50–18:10 **Daniel Foshag (Agilent Technologies)** – Feed Injection-Enabled 2D-LC/MS for Enhanced Analyses of Lipophilic Drug Formulations

18:10–18:30 **Ondřej Kuchler (ECOM)** – Modern approach for analytical and preparative liquid chromatography by ECOM manufacturer

18:30–19:30 Dinner (university cafeteria, Šmeralova 12)

## Wednesday (June 17th, 2026)

08:30–09:00 Breakfast (Faculty of Science, foyer, 2nd floor)

09:00–11:00 *Lectures IV (Faculty of Science, assembly hall, 2nd floor): Advances in Sample Preparation and Applied Analysis; chairmen: Marco Mattonai, Volodymyr Pauk*

09:00–09:30 **Justyna Płotka-Wasylka** – The Role of Analytical Chemistry in Solving Contemporary Challenges in Biomethane Plants

09:30–10:00 **František Švec** – Centrifugation-Assisted Micro-Extraction Using Functionalized Glass Beads for Rapid HPLC Analysis

10:00–10:20 **Massoud Kaykhaii** – A Simple Microextraction Technique: PT-µSPE

10:20–10:40 **Jitka Široká** – LC-MS/MS Salicylate Profiling in Arabidopsis Thaliana Plants under Infection with Pathogens of Different Lifestyles

10:40–11:00 **Štěpán Dostál (Chromservis)** – Chromline: Performance & Innovation

11:00–11:30 Coffee break (Faculty of Science, foyer, 2nd floor)

11:30–13:30 *Lectures V (Faculty of Science, assembly hall, 2nd floor): Advances in Mass Spectrometry and Multiomics; chairmen: František Švec, Lukáš Kučera*

11:30–12:00 **Hans-Dieter Junker** – Rational Approaches to MALDI-MS Matrix Design

12:00–12:30 **David Friedecký** – Multiomics in Laboratory Medicine

12:30–12:50 **Vojtěch Zemek** – Back to the Old Masters of Lacquerware: Classification of Asian Lacquers Using Atmospheric Solids Analysis Probe High-Resolution Tandem Mass Spectrometry

12:50–13:10 **Alex Muck (Waters)** – Using Charge Detection Mass Spectrometry with an Electrostatic Linear Ion Trap for the Analysis of Viral Vectors and Large Protein Complexes

13:10–13:30 **Daniel Vláčil (Bruker)** – Pioneering 4D Multiomics and Functional Proteomics/Matbolomics 2.0

13:30–15:00 Lunch (university cafeteria, Šmeralova 12)

15:00–17:00 ***Social/sport events:***

- Bowling at Bowland Olomouc
- A Guided Tour of Olomouc followed by a Concert by the Vocal Ensemble Muzikůra in the Corpus Christi Chapel

19:00–?? Social dinner (Faculty of Science, foyer, 6th floor)

## Thursday (June 18th, 2026)

08:30–09:00 Breakfast (Faculty of Science, foyer, 2nd floor)

09:00–11:00 *Lectures VI (Faculty of Science, assembly hall, 2nd floor): Emerging Analytical Platforms and Methodologies; chairmen: Petr Kubáň, Lucie Papoušková*

09:00–09:30 **Christian Neusüß** – Capillary Electrophoresis - Mass Spectrometry for the Characterization of Proteoforms on the Intact Level

09:30–10:00 **Václav Kašička** – Investigation of Acid-Base and Electromigration Properties of Biopeptides by Capillary Electrophoresis

10:00–10:20 **Ondřej Novák** – From Tissue to Single Cells: Targeted Hormonomics in Root Protoplasts

10:20–10:40 **Kateryna Trach** – Three-level Microfluidic Architecture as a Framework for Modular Analytical Instrumentation

10:40–11:00 **Petr Česla** – Optimizing Gradient Conditions in Comprehensive 2D Liquid Chromatography: A Comparison of Chromatographic Response Functions

11:00–11:30 Coffee break (Faculty of Science, foyer, 2nd floor)

11:30–13:00 *Lectures VII (Faculty of Science, assembly hall, 2nd floor): Capillary Electrophoresis: Innovations and Applications; chairmen: Christian Neusüß, Jan Petr*

11:30–12:00 **Petr Kubáň** – Open-Source Capillary Electrophoresis - Democratizing Separation Science: Design Principles and Practical Implementations from Our Laboratory

12:00–12:30 **Marián Masár** – Analysis of Biomedical, Environmental and Pharmaceutical Samples by Microchip Electrophoresis

12:30–13:00 **Jana Lavická** – UV-LED Coupling for High-Sensitivity Glycan Analysis in Commercial Capillary Electrophoresis System

13:00–13:30 Closing of conference and farewell drink (Faculty of Science, foyer, 2nd floor)

13:30–15:00 Lunch (university cafeteria, Šmeralova 12)

## LIST OF POSTERS

(numbers correspond with the panel numbers for the given poster presentation)

1. Comparative TWIMS–MS Analysis of Binary and Ternary Lipocalin–Enterobactin Complexes
   *Summra Ahmed, Josef Chmelík, Alan Kádek, Karel Lemr, Dominika Luptáková, Olda Benada, Kristyna Sloupenská, Michal Krupka, Milan Raška, Vladimír Havlíček*

2. Comprehensive Evaluation of Melanin from Black Barley: Isolation, HPLC-based Quantification, and a Feeding Experiment in Rats
   *Batková V., Frankeová N., Jourová L., Anzenbacher P., Mrkvicová E., Martinek P., Zavřelová M. a Anzenbacherová E.*

3. HPLC-UV determination of quercetin and its glycosides in onion: effect of biotic stress
   *Karolína Benešová, Lea Lojková, Helena Pluháčková, Jhonny Alba Mejía, Radim Cerkal*

4. LC-UV analysis of salbutamol sulfate in an *in-vitro* model of human respiratory tract
   *Zuzana Berkešová, Iva Kapustíková, Juraj Piešťanský*

5. A new screening method for the determination of phytosterols in vegetable oils
   *Róbert Bodor, Róbert Kubinec, Jaroslav Blaško, Michal Fulin, Roksolana Fromel, Ján Janošovský*

6. The application of molecular CD spectroscopy for the chiral analysis of amino acids and oligopeptides
   *Lukáš Cach, Marta Farková, Přemysl Lubal*

7. A novel HPLC method with fluorescence detection for the determination of ozanimod in mouse plasma and its application in a pharmacokinetic study
   *Veronika Frýbortová, Štefan Šatka, Lenka Jourová, Pavel Anzenbacher, Iveta Zapletalová, Michal Kraus, Klára Kostovčíková, Eva Anzenbacherová*

8. Fractionation and characterization of humic acids by a combination of stepwise and linear gradient elution in RP-HPLC
   *Róbert Góra*

9. Online SPE-HPLC-MS/MS determination of urinary DINCH plasticizer metabolites for exposure assessment in Slovak adult population
   *Renáta Górová, Helena Jurdáková, Alexandra Mattová, Róbert Góra, Ľubica Murínová*

10. rDUVLAESCI-MS/MSI: a novel approach for direct analysis of biological surfaces and high-speed mass spectrometry imaging (MSI)
    *J. Grepl, B. Papoušková, F. Gregar, K. Lemr, P. Fryčák, T. Pluháček*

11. Ion Mobility – Mass Spectrometry in Investigation of Nitrile-Imine Cross-Linking Reactions in Gas-Phase Peptide Ions
    *Dominik Halman, Chenxun Dai, Sizhong Shen, Yingxuan Wei, Simona Sedláčková, Karel Lemr, František Tureček*

12. Optimisation of monoclonal antibody analysis by CZE-MS and stability testing
*Jana Havlikova, Katarína Maráková, Peter Mikuš*

13. Water-based Randall extraction of bioactive compounds from plants
*Danylo Holub, Dana Štolbová, Eliška Matúšková, Petr Bednář, Petr Barták*

14. Determination of CBD in Isolates, Drops, and Tinctures by a simple HPLC-UV method
*Patrícia Jackuliaková, Juraj Piešťanský*

15. Electrochemical oxidation opens the aromatic ring of veratrole
*Radek Jerga, Dana Štolbová, Andrea Vojs Staňová, Jaroslav Blaško, Marian Vojs, Jana Skopalová, Petr Barták*

16. A Robust Droplet Microfluidic Platform for High-Throughput Enzyme Kinetics and Inhibitor Screening Studies
*Lukáš Jordán, Michal Sedlák, Didem Orbay, Zdeněk Glatz, Marta Pelcová*

17. Retention and Separation of 3-mer Phosphorothioate Oligonucleotides and Their Diastereomers in RPLC
*Květa Kalíková, Gabriela Panýrková, Tímea Dérerová*

18. A configurable gradient score criterion for targeted LC-MS/MS optimatization via min-max normalization
*Michal Kašpar, Pavlína Musilová, Petr Česla*

19. Use of nano sheath liquid flow CE-ESI/MS for analysis of intact and pepsine digested monoclonal antibody trastuzumab
*Renáta Konášová, Dušan Koval, Milan Vrábel, Simona Horkelová, Václav Kašička*

20. Characterization of Cefiderocol Stability and Degradation Products Using Modern Analytical Methods
*Ivana Kopáčová, Juraj Piešťanský*

21. Multimodal cation exchange-type tyrosine-based chiral stationary phases: Synthesis and applications in high-performance liquid chromatography
*Magdaléna Labíková, Adam Pospíšil, Andrea Carotti, Hubert Hettegger, Wolfgang Lindner, Roccaldo Sardella, Michal Kohout*

22. Hexafluoroisopropanol-induced N-octyl Glucopyranoside Biphasic Coacervate System for Protein Fractionation and Enrichment
*Jonathan B. Thacker, Katarina Marakova, Kevin A. Schug*

23. Influence of Solid Matrices on Signal Behaviour in SS-ETAAS
*Nicolas Milan Michalides, Katarína Chovancová, Massoud Kaykhaii, Radoslav Halko*

24. A Novel Approach to Stir Bar Sorptive Extraction with Natural Deep Eutectic Solvent
*Lucia Mikulcová, Massoud Kaykhaii, Iveta Boháčová, Radoslav Halko*

25. Determination of Residual Urinary Cisplatin in Patients Suffering from Germ Cell Tumors by Triple Quadrupole LC-MS/MS
*Denisa Mládková, Barbora Papoušková, Michal Mego, Lucia Kučerová, Tomáš Pluháček*

26. Arrival Time Distribution Profile Fitting for the Analysis of Isomeric Mixtures by Ion Mobility-Mass Spectrometry
*Marianna Nytka, Lucie Papoušková, Karel Lemr*

27. Replacement of Acetonitrile by Greener Solvents in Reversed-Phase Liquid Chromatography of Intact Proteins
*Iva Kapustíková, Martina Opetová, Katarína Maráková*

28. A Highly Sensitive Headspace Gas Chromatographic Method Fully Optimized for Fast Routine Determination of Carboxyhemoglobin in Postmortem Blood
*Roman Papoušek, Vladimíra Gebauerová, Marie Staňková, Petr Handlos, Edita Červenková, Denisa Ptáčková, Lucie Papoušková*

29. Development of a Python-Based Workflow for Design of Experiment in LC–MS Method Optimization of Plant Peptide Hormones
*Tereza Pavlíčková, Ivan Petřík, Ivo Chamrád, René Lenobel, Ondřej Novák*

30. Mirror vs Native: Who Binds Stronger?
*Jan Petr, Elena Kučerová, Tereza Čagánková, Veronika Ručilová, Tadeáš Herentin, Athanasios Markos, Yong Wu, Daniel W. Armstrong*

31. Apparent pH of the Supercritical Carbon Dioxide-Based Fluids Used in Analytical Chemistry
*Veronika Pilařová, Kateřina Plachká, Pavel Vrága, Barbora Masopustová, Lucie Nováková*

32. Development of Innovative Chiral Donor-Acceptor Stationary Phases for HPLC Enantioseparations *Magdaléna Labíková,*
*Adam Pospíšil, Jiří Tůma, Michal Kohout*

33. Preliminary Design of Enhanced NanoCEasy CE-MS Interface
*Oleksandr Prystopiuk, Kateryna Trach, Petr Bednář, Christian Neusüß, Lena Kruse, Ann-Katrin Schwenzer, Sabrina Buntz, Volker Höfer, Simon Kreß, Constantin Bauer, Florian Plathe, Marcel Hübner*

34. Capillary micellar electrokinetic chromatography – a powerful method for achiral and chiral separations of brefeldin A and its derivatives
*Petra Sázelová, Mikhail Klychnikov, Ullrich Jahn, and Václav Kašička*

35. Applications of Magnetic Particles with Immobilized Albumin in Combination with CE for Plasma Protein-Drug Binding Studies
*Michal Sedlák, Lenka Kohútová, Max Demel, Marta Pelcová, Zdeněk Glatz*

36. Microplastics without borders: A Cross-Regional Study of Microplastic Morphology and Composition
*Ľudovít Schreiber, Andrea Vojs Staňová, Patrik Osuský*

37. Advanced Characterization of New Fossil Resins: From Screening to Molecular Fingerprinting
*Jakub Slačálek, Lukáš Kučera*

38. Determination of the binding constants of complexes of cucurbit[7]uril with aromatic cationic ligands by pressure-assisted affinity capillary electrophoresis
*Veronika Šolínová, Doroteja Lončarič, Jiří Kaleta, Václav Kašička*

39. Investigation of acid-base properties of secondary amides and 2-(4-butoxyphenyl)acetic acid by capillary electrophoresis
*Sille Štěpánová, Anna Kubíčková, Eric Andris, Petr Beier, Václav Kašička*

40. Alternative approach to using diazomethane for GC/MS: derivatization reaction in the gas phase
*Dana Štolbová, Danylo Holub, Jana Skopalová, Petr Bednář, Petr Barták*

41. Advanced Pollen Profiling by ASAP-MS Using Standard and Modified Capillary: Chemical Characterization of Intact and Modified Pollen
*Matěj Tesárek, Kateřina Zbíralová, Petr Bednář*

42. Towards the Structure of Melanin in Legume Seeds
*Kateryna Trach, Jana Balarynová, Petr Smýkal, Petr Bednář*

43. Unravelling Complex Asian Lacquer Matrices using LC-MS/MS and Chemometrics
*Zbyněk Žingor, Vojtěch Zemek, Petr Bednář*

44. Free Drug Concentrations in Plasma: Optimization of Ultrafiltration and Validation of an LC-MS Method for Clinical Purposes
*Marta Pelcová, Aneta Nemčíková, Viktória Ďurčová, Zdeněk Glatz, Jan Juřica*

# LECTURES

# Metabolomics and Lipidomics in Biomedical Research

Michael Lämmerhofer[1], Philipp Seyfried[1] Kristian Serafimov[1], Peng Li[1], Min Su[1], Xiaoqing Fu[1], Kefan Chen[1], Zijing Xu[1], Kristina Dittrich[1]

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## Abstract

Mass spectrometry-based metabolomics and lipidomics are powerful, information-rich tools for molecular phenotyping, widely used in biomedical research to uncover mechanistic insights into biochemical processes, identify diagnostic and prognostic biomarkers, and generate or validate biological hypotheses. These approaches enable the mapping of dynamic changes in molecular networks and signaling pathways across experimental and clinical models, revealing shifts in energy homeostasis, immunometabolism, and the interplay between pro- and anti-inflammatory responses. Correlating metabolic signatures with disease states supports prognosis prediction, guides therapeutic decisions, and monitors treatment response.

In our lab, we have developed robust targeted and untargeted metabolomics and lipidomics platforms. Unfortunately, no single LC-MS/MS method can comprehensively cover the entire metabolome, hence sets of methods are required to cover a reasonable range of metabolites and lipids. This presentation will discuss key methodological considerations in metabolomics and lipidomics for biomedical and clinical applications, with a focus on critical aspects of both targeted and untargeted approaches.

We will highlight untargeted phosphoinositide profiling, targeted steroid analysis, isomer-selective metabolomics, and lipidomics (e.g., sugar phosphates in glycolysis and the pentose phosphate pathway), practical applications in platelet lipidomics for coronary artery disease, pharmacolipidomics, and the power of metabolomics in liver disease.

Our untargeted workflows are based on ultra-high-performance liquid chromatography coupled to high-resolution mass spectrometry (QTOF-MS) with data-independent acquisition (DIA), ensuring broad coverage and reproducibility. For the polar metabolome, we employ targeted methods based on HILIC separations with positive and negative MRM assays. Complementing this, we have developed multiple targeted assays to quantify low-abundance metabolites and lipids with challenging physicochemical properties—such as isomer-specific analysis of sugar phosphates, phosphoinositide network metabolites, steroids, oxylipins, bile acids, glutathione and redox-active metabolites, fatty acyl-CoAs, endocannabinoids, and short-chain fatty acids—often requiring specialized sample preparation.

Challenging analytical questions will be illustrated with selected case studies from our clinical and biochemical collaborators. Topics include isomer-selective analysis, spatially resolved metabolomic patterns, cell-population-specific profiling, subcellular lipidomics, and chiral metabolomics.

One subcellular lipidomics example shows that lysosomes act as metabolic-signaling hubs that coordinate anabolic and catabolic programs; mTORC1 activation at the lysosomal surface is driven by the phosphoinositides PI3P and $PI(3,5)P_2$, whose levels are regulated by the phosphoinositide-3 phosphatase MTM1 at ER-lysosome membrane contact sites.

A further challenge is representative sampling: plasma drawn from different sites in the same cirrhotic patient can exhibit distinct phenotypes, and metabolomics can reveal the underlying molecular basis where flow cytometry and immunological assays fail. Additional topics such as the fatty-acyl code will also be discussed.

# Breaking Boundaries in Natural Product Analysis with SFC-MS

Lucie Nováková, Kateřina Plachká, Veronika Pilařová, Štefan Kosturko, Karel Vraj

*Department of Analytical Chemistry, Faculty of Pharmacy in Hradec Králové, Charles University, Heyrovského 1203, 500 03 Hradec Králové, Czech Republic*

e-mail: *novakoval@faf.cuni.cz*

## Introduction

SFC-MS has emerged as a powerful and green analytical tool for natural product analysis, offering a viable alternative to conventional LC and GC. The use of supercritical $CO_2$ provides high chromatographic efficiency, reduced organic solvent consumption, and eliminates the need for derivatization of thermally labile compounds. Moreover, SFC bridges the polarity gap between LC and GC, enabling separation of analytes ranging from nonpolar volatile terpenes to polar glycosylated flavonoids within a single method, streamlining workflows and reducing the need for multiple analytical platforms.

Natural products frequently contain structurally similar compounds, posing significant challenges in their separation and detection. Here we present selected case studies in plant-based material analysis to illustrate the versatility of SFC-MS across the full analyte polarity range.

## Experimental

Experiments were carried out on an Acquity UPC² SFC system (Waters) hyphenated to a Xevo TQ-XS triple quadrupole mass spectrometer (Waters). In the first case study, fat-soluble vitamin E (eight tocopherol and tocotrienol isomers) was analyzed on a BEH 2-EP column using a $CO_2$/MeOH gradient, with ESI detection and direct injection of hexane LLE extracts.

The second case study focused on cannabis, targeting 12 neutral and acidic cannabinoids (MW 282–360, log P 5.8–8.0) on a BEH 2-EP column with $CO_2$/MeOH–ACN + 5% $H_2O$ mobile phase and $ESI^{+/-}$ detection. The third case study addressed eucalyptus extracts using a complementary two-column approach: a PGC column (150 mm) for volatile terpenes with APCI, and a Torus Diol column (50 mm) with 2% $H_2O$ in MeOH for phenolic acids and flavonoids. The PGC-based method was further evaluated as a direct alternative to GC-MS for volatile terpene profiling in fennel extract. The final case study aimed to develop a truly universal single-injection method covering 62 phytochemicals spanning terpenes, vitamins, triterpenic acids, phenolic acids, and glycosylated flavonoids (MW 134–611, log P 1.5–11). Three stationary phases were screened (Torus Diol, Cosmocore PBr, Kinetex Biphenyl) and three ionization sources compared: ESI, dual ESI/APCI (ESCi), and DBDI (SICRIT, Plasmion GmbH), with MS parameters optimized via design of experiment.

## Results

Baseline separation of all eight tocopherol and tocotrienol isomers, including the critical β/γ pair, was achieved in under 5 min on BEH 2-EP, outperforming UHPLC (12 min) with direct injection of hexane extracts. For cannabis, 12 cannabinoids including isomers (Λ8-THC/Λ9-THC) and epimers were resolved in under 3 min, enabling rapid characterization of both declared and undeclared compounds in commercial hemp products.

For eucalyptus, the PGC column provided excellent retention and separation of 14 volatile terpenes (including structurally isomeric compounds at m/z 136 and 154), while Torus Diol enabled separation of 24 phenolic analytes. UHPSFC-APCI-MS/MS on PGC fully replaced GC-EI-MS for fennel volatile profiling, achieving equivalent separation in 8.5 min vs. 18 min for GC, with superior selectivity for phenylpropanoids.
In the 62-compound universal method, the PBr stationary phase enabled elution of the broadest analyte set, vs. 51 on biphenyl and 40 on diol. Ammonium fluoride 3 mmol/L proved essential for improving peak shapes of polar compounds by reducing secondary interactions with the bromine-functionalized stationary phase. DBDI (SICRIT) allowed ionization of both nonfunctionalized terpenes and polar flavonoids in a single mode. The LLOQs and ionization patterns were compared.

**Conclusion**
SFC-MS proves to be a highly versatile and complementary tool for natural product analysis, delivering high selectivity, efficiency, and adequate sensitivity across the full analyte polarity range. The presented case studies demonstrate that a single SFC-MS platform can replace multiple dedicated LC and GC workflows, from lipophilic vitamin isomers and cannabinoids to volatile terpenes and polar glycosylated flavonoids, offering greener, faster, and more streamlined solutions for routine phytochemical analysis.

**Acknowledgements**
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# Data Science-Assisted Method Development for On-Line Supercritical Fluid Extraction – Chromatography

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## Introduction

On-line supercritical fluid extraction – supercritical fluid chromatography – mass spectrometry (SFE-SFC-MS) is a powerful multidimensional tool for extraction and trace determination of small molecules from solid sample matrices. However, method development can be challenging due to need to optimize a large number of variables, many of which exhibit interactions. Previous work has shown that traditional factorial design and response surface methodologies lack sufficient granularity to capture complex response outputs across the variable space [1]. As an alternative, we are exploring the use of surrogate optimization, a meta-modeling method, that can better handle many variables, while minimizing the number of experimental runs. Multivariate adaptive regression splines (MARS) are used to create the surrogate model; MARS can better capture complex response surfaces relative to traditional polynomial modeling. In addition, we are exploring molecular encoding to provide a quantitative metric for molecular similarity, to help guide multi-analyte optimization.

## Experimental

A set of model pharmaceutical molecules was assembled and encoded using E-state indices and Morgan fingerprints. Molecular similarity was calculated using Hamming distance on molecular feature representation vectors assembled using cumulative binarization [2]. The model analyte mixture was deposited initially on diatomaceous Earth, but ultimately, different sample materials, such as polypropylene and cellulose were also studied. Surrogate optimization was performed to optimize settings for static extraction time, dynamic extraction time, modifier (methanol) percentage, extraction pressure, extraction flow rate, chromatography pressure, and split ratio. Initial efforts were focused on optimization for one analyte, while follow-on efforts considered a sequential surrogate optimization approach to optimize settings for multiple analytes, chosen based on their molecular similarity. Supercritical fluid chromatography was performed on a 2-ethylpyridine stationary phase using supercritical carbon dioxide as mobile phase and methanol with ammonium formate as modifier.

## Results

The results to be presented cover one published [3] and two unpublished manuscripts. Initial work was able to test the development of a composite output function that evaluated extraction efficiency, chromatographic efficiency, peak symmetry, and reproducibility of each of these

parameters while optimizing five extraction variables for the extraction and analysis of hydrocodone from diatomaceous Earth [3]. Optimal parameters were reached in 20 experimental runs, achieving low pg detection limits and good precision (< 5% RSD). Additional work sought to expand the number of variables considered to seven and achieved optimization of a diverse set of analytes in 80 experimental runs. Different analytes exhibited significantly different response surfaces, but conditions were ultimately found where low pg detection limits and good precision (<15% RSD) could be achieved for all model analytes. Encoded molecular similarity was used as a guide for analyte section, to compare results from similar and dissimilar analytes; however, the encoding chosen did not ultimately map well with the progression of optimized performance. The most recent unpublished work examines the extraction of analyte from multiple sample materials. As expected, optimal extraction and separation conditions varied substantially for 10 unique sample mixtures created from three different sample materials using a mixture design.

**Conclusion**

This work recounts the development and first use of surrogate optimization for method development in the context of multidimensional extraction and separation systems. It is well suited to handle the large number of variables and variable interactions that give rise to complex response surfaces for diverse analytes and sample materials. The methodology yields excellent operational conditions under fewer analytical runs than would be needed for more traditional optimization approaches. Future work will focus on the development of alternate similarity metrics that better represent the behavior of analytes in SFE-SFC-MS. Future work will also focus on accommodation of more experimental variables, as well as variations in column chemistries.

**Acknowledgements**

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# Predicting Chromatographic Retention Times: Descriptor Choice, Feature Selection and QSAR Interpretation

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**Abstract**

Accurate prediction of chromatographic retention times is a key challenge in analytical chemistry, with direct implications for compound identification, method development, and high-throughput screening. In this work, we present a systematic quantitative structure–activity relationship (QSAR) framework for retention time prediction, focusing on the comparative evaluation of molecular descriptor sources, feature selection strategies, and model interpretability.

Multiple classes of descriptors were investigated, including traditional physicochemical descriptors, topological and fragment-based representations, and solvation-related descriptors. To address descriptor redundancy and overfitting, a range of feature selection algorithms was evaluated, encompassing filter-based, wrapper-based, and embedded methods. Their impact on predictive performance, model stability, and selected feature consistency was assessed using nested cross-validation and permutation-based validation.

Predictive models were trained using regularized linear and nonlinear regression approaches, and their performance was compared across descriptor–feature selection combinations. Beyond prediction accuracy, particular emphasis was placed on model interpretability. The selected features were analyzed in terms of their physicochemical meaning and chromatographic relevance, enabling mechanistic insights into the factors governing retention behavior.

The results demonstrate that both descriptor choice and feature selection strategy strongly influence model robustness and interpretability, with solvation-aware descriptors offering complementary information to conventional molecular features. This study highlights the importance of integrating rigorous feature selection with interpretable QSAR modeling to achieve reliable and chemically meaningful retention time predictions.

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# Development of novel calibration methods and their application to the chromatographic determination of bisphenols

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## Introduction

Bisphenols (BPs) are a class of synthetic chemical compounds commonly used in the production of plastics, resins, and other consumer products, and commonly found in everyday items (food containers, water bottles, thermal paper receipts, tickets, shipping labels, CDs/DVDs, and electrical device casings). Due to their widespread use, BPs have become ubiquitous in the environment. This raises concerns about their potential impact on living organisms. They can disrupt the hormonal systems of various organisms, leading to reproductive disorders, impaired sexual development, or impaired growth. Exposure to bisphenols is also linked to cardiovascular disease, diabetes, obesity, and some cancers. Restrictions on the use of these compounds are being introduced, primarily concerning the most common member of this class, bisphenol A (BPA). However, the availability of alternative analogs (bisphenol F (BPF) and bisphenol S (BPS)) provides a starting point for the development of new analytical methods to reliably determine compounds in this group. It is necessary to search for solutions based on analytical calibration to overcome various interference effects (i.e., multiplicative/proportional, additive/constant, and nonlinear) arising from the complexity of the sample matrix [1]. The main goal of these studies was to develop new methodologies based on the integrated calibration method (ICM) and the H-point standard addition method (HPSAM) to improve the quality of the results and to test them on the example of the determination of BPs (BPA, BPS, and BPF) using a validated chromatographic method - HPLC-DAD (high-performance liquid chromatography with diode array detection) [2-4]. An additional aspect was the evaluation of the developed methods in accordance with the principles of White Analytical Chemistry and environmental friendliness.

## Experimental

The developed and validated chromatographic method enabled the determination of three compounds (BPA, BPS, and BPF) in 10 min. Isocratic flow was used during the measurements, with a mobile phase consisting of acetonitrile and water (1:1) at a flow rate of 1.35 mL/min. An analytical C18 column, EC NUCLEOSIL 100-5 (250 mm × 4.6 mm, 5 μm) (Machery-Nagel, Germany), with a dedicated precolumn, and an injection volume of 10 μL at 30°C were used. Due to the proposed calibration approaches, five wavelengths (225 nm, 230 nm, 235 nm, 270 nm, and 275 nm) were selected for signal acquisition. The retention times of individual substances were ~3.1 min for BPS, ~4.2 min for BPA, and ~5.5 min for BPF. Several samples of different natures were investigated: Roll type 1 and Roll type 2 (unprinted thermal paper), an exemplary store receipt from local markets, and peas, corn, and jackfruit (canned food). The extraction procedure consisted of weighing the appropriate sample mass, sonicating it in ethanol for 10 min, and filtering the resulting extract. These prepared samples were used to prepare calibration solutions.

**Results**

First, the optimized method was validated for each analyte under each of the five measurement conditions. A wide linear range (0-0.5 mg/mL) was obtained, and the $R^2$ coefficient was greater than 0.994. Limits of detection (LOD) values (based on calibration plot parameters) ranged from 0.0022 to 0.0045 mg/mL. The method also demonstrated good limit of quantification (LOQ) values (0.0066-0.0135 mg/mL). The first methodological approach was based on extending the ICM method. In the basic version, three calibration solutions are prepared, consisting of a sample, a standard, and a diluent. Based on the signals, two two-point calibration plots are obtained, followed by two result estimations: interpolation (compliant with external calibration (EC) and extrapolation (compliant with the standard addition method (SAM)), which allows for the detection and elimination of multiplicative effects. The proposed ICM-based methodology involves adding subsequent dilutions, which allows detection of existing nonlinearities and the use of five measurement conditions (different wavelengths) to apply the HPSAM approach and eliminate additive interferences. This methodology allows the generation of a series of calibration plots and result estimations, free of existing interferences, thereby enabling self-verification. The second approach consisted of expanding the HPSAM method from two to five measurement conditions. This type of consideration also enables the use of HPSAM in the signal increment version (calibration plots built from signal differences) and a generalized version that simplifies the three conditions into two calibration lines with positive and negative slopes. This method yields up to 30 result estimations from HPSAM approaches, considering all possible combinations, and 5 results from the SAM method. This method allows compensation for all types of interference and improves the quality of the results. Fluorescence spectroscopy (FS) was used as a reference method to verify the results [2]. Next, analyses were performed using a validated chromatographic method for a synthetic sample with known concentrations of individual BPs to confirm the effectiveness of the developed calibration methodologies. In the next step, food-related products (store receipts and canned food samples) were tested to verify the effectiveness of both methodologies. The obtained analytical results were characterized by very good accuracy (RE<5%) and precision (CV≤4%). The analytical chromatographic method and methodological approaches were evaluated using two tools: the RGB Additive Color Model for Analytical Method Evaluation and the AGREE - Analytical GREEnness Metric Approach. Based on this evaluation, it was demonstrated that the methods can be successfully adapted to other analytical systems and purposes (classification as white color methods). Furthermore, the method was demonstrated to be environmentally friendly (significance parameter >0.6).

**Conclusions**

The developed methodological approaches enabled obtaining results with increased accuracy and full compensation for interferences arising from co-elution with other substances during analysis. High compliance with the goals of White Analytical Chemistry was demonstrated, and environmental friendliness was confirmed.

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# Applications of online SFE – SFC: A Powerful Tool for Small Molecule Extraction and Analysis

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**Abstract**

Supercritical fluid extraction (SFE) and supercritical fluid chromatography (SFC) instruments are well established for their versatile application scope. Individually, SFE and SFC are used routinely in industrial and academic settings for extraction, purification, and analytical determinations. Supercritical carbon dioxide has many beneficial properties beyond just the value of enhanced fluidity; it is cost-effective, green, and non-flammable. The combination of SFE and SFC in an on-line analytical instrument brings the advantages of the individual techniques together with the advantages of on-line coupling – reduced sample handling, increased throughput, improved recoveries, and reduced exposure of analytes to the external environment. Over the past decade, the Shimadzu Nexera UC on-line supercritical fluid extraction – supercritical fluid chromatography – tandem mass spectrometry (SFE-SFC-MS) instrument has been shown to provide exceptional analytical performance for trace detection in environmental, biological, forensic, and food analysis. When coupled with emerging advanced optimization routines, there should be virtually no small molecule present in any solid sample that the instrument cannot analyze. This talk will provide an overview of the SFE-SFC-MS instrument and its use across a wide range of application areas [1 – 7].

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# Enantioselective Ion Pairing Systems, my 50 Years of Contributions

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## Introduction

Looking back to the 1970s, the direct separation of enantiomers using liquid chromatography systems was a highly challenging task tackled only by a small group of colleagues. This period of time marks my devotion to this topic and the start of an intellectual race to identify and to formulate enantioselective molecular recognition principles and to develop dedicated "Chiral Selectors, SOs" and related "Chiral Stationary Phases, CSPs" to master the difficult problems of resolving the enantiomers of structurally very diverse chiral compounds. The driver for this dynamically growing field where mainly the Life Sciences, including Medicinal Chemistry and Pharmaceutical Chemistry, on the one hand, and, on the other side, companies which introduced the academically developed enantioselective so-called "Chiral Columns" to the market.

## Experimental

Enantioselective molecular recognition can be described by a three-point intermolecular interaction scenario based on a tetrader model whereby the fourth corner point is dedicated to a spatial descriptor. This model can be more generalized by multi-site interaction events between an SO unit and the enantiomers of the "Chiral Selectands, SAs", the analytes, leading to enantioselective systems. The discrimination of the SA enantiomers is based on the intermediate formation of two diastereomerically behaving (SO-(R)-SA) and (SO-(S)-SA) associates, which differ in physicochemical properties. The multimodal SO-SA bindings are to be described by simultaneously occurring interactions as of: hydrogen bonding, and/or pi-pi, and/or electrostatic, and/or hydrophobic character of the SO and SA molecular sites within the frame of a three-dimensionally oriented space domain.

## Results

In the above described context, I developed in the 70s the first chiral "ion pairing" SO for the use in RP-HPLC. It had the structure of a hydrophobic chiral triamine agent using $Zn^{2+}$ as Central ion leading to a type of chelating agent, which was positively charged. Besides the enantioselective chelating (D)- and (L)- N-dansyl-amino acids, it involved strong electrostatic interactions to neutralize the complex. It was the start of my constant search for novel direct and indirect enantio- and diastereoselective systems involving inter- or intramolecular electrostatic interactions of the SOs and the SAs as a vector.

In the 1980s, we introduced and patented the analytical and preparative indirect resolution of chiral drugs as of an aminoethanol structure (e.g., beta blockers) involving tartaric acid derivatives thereof. Preparatively, this concept is still used today for the synthesis of e.g., (S)-Timolol. Having these drugs in enantiomerically pure form in hand, we carried out diverse pharmacodynamic and pharmacokinetic studies with clinical partners.

In 1990/1991, we introduced the genuine chiral anion exchanger type columns for LC based on

Quinine (QN) and Quinidine (QD) derivatives, which got widely used and are still on the market. In parallel, we also developed chiral cation exchangers, and consequently, we came up with the idea to fuse both SO components into novel ampholytic SO compounds. Thus, we generated the so-called zwitterionic CSPs and ZWIC columns, which were introduced in 2008. Concept-wise, it turned out highly inspiring as it allows the resolution of also highly polar anionic, cationic, and ampholytic analytes with one chiral ZWIX type ion-exchange column.
Thus, the zwitterionic CSPs and chiral columns can be used in a multimode way and proved their suitability for diverse enantioselective applications, including highly sensitive and selective amino acid analysis employing HPLC-MS/MS and two-dimensional HPLC/HPLC systems for clinical studies.

**Conclusion**

Overall, the impact of the diverse enantioselective separation concepts in introducing small and polymer type chiral selectors (SOs) systems developed by many colleagues is highly relevant and indispensable in practically all fields of Science where “Chirality” plays a central role.

**Acknowledgements**

An intrinsic part of my lifelong “success” story is the working together with highly talented and motivated co-workers during my academic life, but also of the industry partner who produced these “chiral columns” and introduced them to the market. These collegial partnerships last over decades, for which I am truly thankful.

# The future of multimodal chiral stationary phases

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## Introduction

The growing demand for enantioselective pharmaceutical analysis has prompted the development of novel chiral stationary phases (CSPs) tailored for high-performance liquid chromatography (HPLC). The crucial unit of the CSP is a chiral selector (SO) responsible for distinguishing the enantiomers of an analyzed compound. There are several established groups of chiral selectors, whose chiral recognition mechanism, *i.e.*, the way they interact with various chiral analytes through non-bonding intermolecular interactions, has already been well characterized. Depending on the nature of the selector, the formation of transient diastereomeric complexes between this chiral selector and the enantiomers of analytes is facilitated by various intermolecular interactions, hydrogen bonds, π–π interactions, dipole-dipole interactions, ionic interactions, van der Waals forces, among others.[1] Long-range electrostatic (ionic) interactions are usually considered to be the primary attraction force with limited contribution to enantioselectivity. Enantioselectivity is instead primarily driven by oriented short-range interactions. Each type of selector exhibits a distinct set of interactions characteristic of its class. Recently, renewed attention is paid to mixed-mode stationary phases, which combine several interaction principles typical for different selector classes.[2] This combination of interaction is typically used to tune reversed-phase chromatography via the introduction of a functional group into the structure of the alkyl chain or as an additional unit on the silica surface. The latter principle can be used to develop stationary phases combining achiral and chiral separation function.[3] Does this imply that such a stationary phase is multimodal? And if the stationary phase is multimodal (applicable in different chromatographic modes), is it mixed-mode by nature? The aim of this contribution is to sort out the current terminology using various examples from laboratory practice and literature.

## Experimental

Chromatographic experiments in different chromatographic modes were performed using prototype multimodal columns 150×4 mm id, 3 μm, evaluated in HPLC using ECOM HPLC (ECOM, Czech Republic), Shimadzu Nexera LC-40 line (Shimadzu, Japan) at a constant flow rate of 0.75 mL $min^{-1}$ and temperature 25 °C.

## Results

The most prominent multimodal CSPs today are polysaccharide-based sorbents. For nearly all polysaccharide-based CSPs, examples of chiral resolution in alkane-alcohol (normal phase - NP), polar organic (PO), and reversed phase (RP) mode, as well as supercritical fluid chromatography (SFC) can be found.[4,5] It should be noted that polysaccharides are not considered to be mixed-mode CSPs. On the other hand, macrocyclic antibiotics can be considered as mixed-mode CSPs due to the presence of a broad variety of functional groups

combining intermolecular interactions, which can be found in polysaccharides with ionic interactions imposed by ionizable amino and carboxylic functions. Similar to polysaccharides, chirobiotic phases also perform well in different chromatographic modes – NP, PO, SFC – hence provide a multimodal elution regime. This indicates that multimodality of a CSP can be introduced by combining different interaction principles of two or more selector classes (mixing interaction modes) into a single selector, while maintaining the parent interaction forces active. Recently, we have combined a donor-acceptor selector scaffold with an ion-exchange fragment.[6] The resulting mixed-mode CSP proved to be also multimodal, providing chiral resolution of nonpolar and polar analytes in NP, PO, and SFC conditions. Interestingly, chiral resolution of some analytes has also been achieved under polar organic-water (HILIC-like) conditions. Moreover, the new CSP can be easily synthesized in both enantiomeric forms and thus enables a predictable reversal of elution order. This makes it highly advantageous for the determination of enantiomeric impurities as well as the preparative resolution of enantiomers.

**Conclusion**

To conclude, not every mixed-mode stationary phase is multimodal, and not every multimodal stationary phase must be of mixed-mode character. Nevertheless, multimodality can be introduced by careful selector design combining various fragments that work within the target CSP in a concerted manner. The main benefit of such CSPs is their predictable behavior, including the possibility of the reversal of elution order.

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# Analytical Challenges in the Development of Voclosporin HPLC methods: Application of High-Temperature Liquid Chromatography in pharmaceutical analysis

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**Abstract**

Voclosporin is a cyclic peptide calcineurin inhibitor used for the treatment of lupus nephritis, characterized by a complex synthetic route, polymorphism, and the formation of numerous structurally related impurities and geometric isomers. The development of robust and selective analytical methods is therefore a critical component of its process development and quality control strategy. This contribution summarizes the key analytical challenges encountered during the development of voclosporin and its late-stage intermediates, with particular emphasis on high temperature liquid chromatography (HT HPLC).

Conventional reversed phase HPLC methods often suffered from limited selectivity, broad peak shapes, and long analysis times when applied to voclosporin and cyclosporine derived compounds. HT HPLC was systematically investigated as a tool to overcome these limitations. Practical aspects of HT HPLC implementation, including mobile phase preheating at the column inlet and efficient cooling at the detector inlet to prevent thermal mismatch and mobile phase boiling, are discussed.

A major analytical challenge was the separation of E/Z geometric isomers formed during olefination steps. Extensive method development was carried out using reversed phase as well as normal phase chromatography. Porous graphitic carbon columns (Hypercarb) operated at high temperature provided markedly enhanced selectivity and enabled baseline separation of E/Z isomers of voclosporin and acetyl voclosporin under isocratic conditions. Stationary phase, column temperature and specific composition of the mobile phase providing isomers separation is discussed.

An equally significant challenge was the development of stability indicating analytical methods for the determination of related substances. The role of elevated temperature is discussed as a factor providing sufficient efficiency for all components while simultaneously affecting the selectivity of separation for certain compounds. Another parameter influencing separation selectivity is the use of tert butyl methyl ether as a modifier in the mobile phases. An additional critical aspect is the selection of an appropriate stationary phase capable of delivering the required selectivity while maintaining adequate thermal stability, thereby ensuring suitability for routine QC applications.

# Next-Gen Zwitterionic HILIC: Particle Morphology Matters

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**Abstract**

Zwitterionic stationary phases are among the most powerful HILIC options for retaining and resolving highly polar analytes. By combining closely spaced positive and negative charges within an overall neutral surface, these phases generate pronounced dipole-driven interactions that translate into strong, selective retention, improved control of polar selectivity, and frequently cleaner peak shapes for difficult sample matrices.

Recent progress has focused on transferring grafted zwitterionic chemistries from fully porous particles (FPP) to superficially porous particles (SPP) to further optimize performance. Although FPP offer high surface area and strong analyte–stationary phase interactions, they can impose practical limitations, including higher backpressure, longer cycle times, and greater vulnerability to contamination-related clogging, especially in sub–2 µm columns. Sub–3 µm SPP, by contrast, can achieve comparable efficiency and resolution while enabling lower pressure operation, faster separations, and enhanced bed robustness.

This oral presentation highlights advances in zwitterion-grafted SPP HILIC columns for HPLC and LC–MS workflows and examines the role of particle morphology in shaping chromatographic outcomes. Head-to-head comparisons of SPP and FPP formats are presented, including data on equilibration behavior, separation efficiency, sample volume and mass loadability, and injection repeatability/stability. Practical aspects of column inertness are also addressed through selected application examples.

# Quantification of Polyethylene in Compostable Plastic Formulations by Analytical Pyrolysis-based Methods with Split Modulation

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## Introduction

We developed two methods based on double-shot analytical pyrolysis-GC-MS (DSPy-GC-MS) and evolved gas analysis by mass spectrometry (EGA-MS) to quantify polyethylene (PE) in compostable plastic materials [1]. PE is often added to biodegradable plastics to improve mechanical properties and reduce production costs. Non-biodegradable organic components in biodegradable formulations must not exceed 1% by weight according to currently available regulations. However, no standardized procedure to measure such low amounts of polymer is available yet. As a result, materials labelled as compostable but containing excessive amounts of PE might end up in composting facilities, leading to incomplete composting processes and generating plastic-containing fertilizers.

## Experimental

The method was developed and validated using custom-made reference materials consisting of common biodegradable plastic formulations with known amounts of PE (0-5%). Experiments were performed with a microfurnace pyrolyzer (Frontier Laboratories) coupled with a GC-MS system (Agilent).

## Results

Preliminary EGA-MS experiments showed that the biodegradable polymer matrix undergoes thermal degradation at a significantly lower temperature than PE. This allowed us to develop a DSPy-GC-MS method consisting of two consecutive runs. In the first run, the biodegradable matrix is pyrolyzed while leaving PE intact. In the second run, PE is pyrolyzed using a higher temperature and quantified using the chromatographic areas of its pyrolysis products. Split modulation between the two runs is required to increase the instrumental sensitivity towards PE, while avoiding signal saturation due to the biodegradable bulk. The method showed negligible matrix interference, good linearity, and a limit of quantification for PE of 0.3%, well suited to assess the conformity of real-case compostable plastic materials.

While providing excellent performances, the double-shot method requires rather long analysis times (75 min). EGA-MS was therefore tested as an alternative, faster method to quantify PE. For this method, mid-run split switch was used to improve the sensitivity towards PE (**Figure 1**). This method also showed suitable performances, albeit with slightly lower sensitivity and a limit of quantification for PE of 0.9%.

## Conclusion

This is the first study reporting a quantitative method based on mass spectrometry to quantify PE in biodegradable plastic formulations. This is also the first study reporting the use of EGA-MS for quantitative analysis, and the use of mid-run split modulation to increase sensitivity.
Both developed methods can reliably quantify PE in compostable plastic materials when present in amounts of 1% or lower, providing a method to assess the conformity of compostable plastic materials. The EGA-MS method is faster, while the Py-GC-MS method can be used if a higher sensitivity is required.


## Acknowledgements

The National Italian Consortium for the Recycling of Biodegradable and Compostable Plastic (Biorepack) and Novamont S.p.A. are acknowledged for the collaboration on this topic and for providing the reference and unknown samples. The Frontier Laboratories Research Fund 2025 is acknowledged for financial support.


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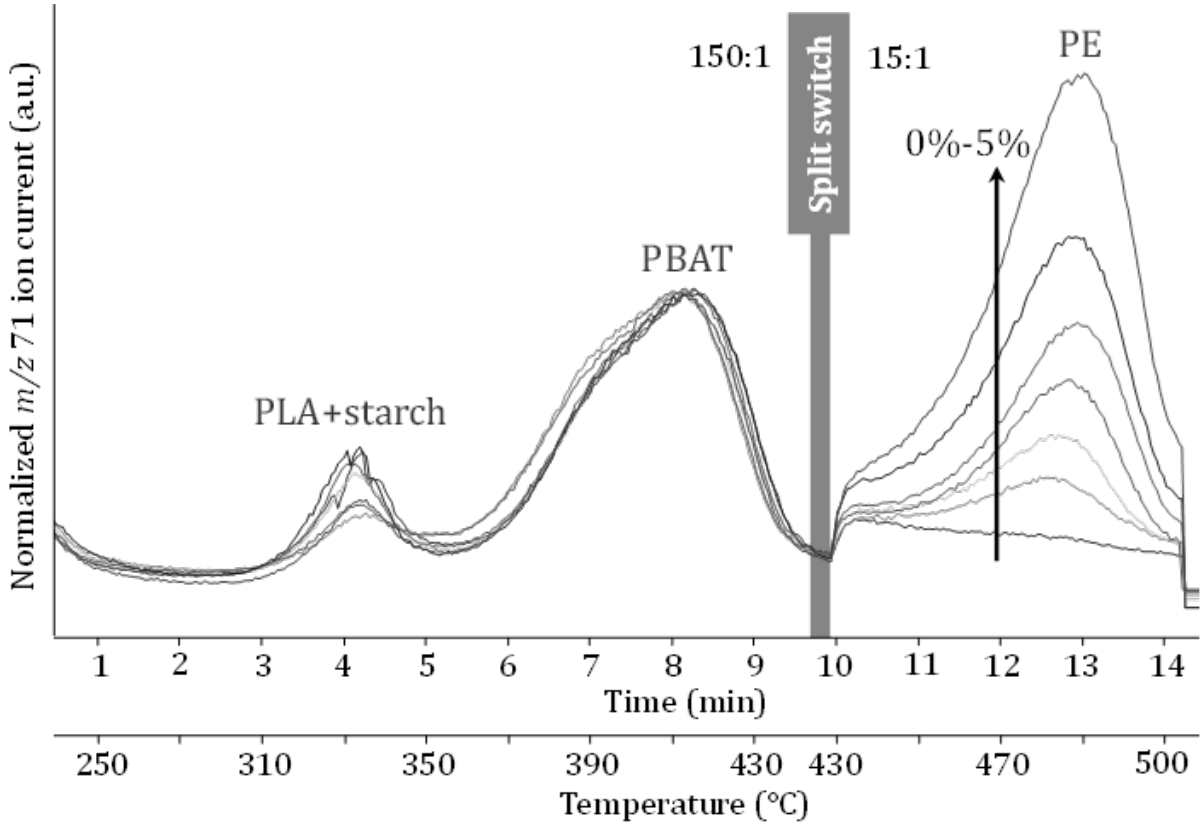
**Figure 1.** EGA-MS profiles of reference biodegradable plastic materials with known amounts of PE, showing the linearity of response of the method. The mid-run split switch point is also indicated.



# Untargeted Analysis of a Late Classic Period Maya Femur by Comprehensive Two-Dimensional Gas Chromatography (GC×GC)

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## Introduction

Red-painted human remains recovered from caves in the Petexbatun region of Guatemala suggest that pigments and body treatments formed part of Late Classic Maya mortuary practice[1]. In this study, we investigated the organic composition of red pigment on a painted human femur from Cueva de Sangre, Dos Pilas, Guatemala, to identify binders and additives associated with ritual treatment. Volatile compounds were characterized by solid-phase microextraction (SPME) coupled with gas chromatography with time-of-flight mass spectrometry (GC-TOFMS), while volatile and semivolatile compounds were analyzed by thermal desorption (TD) coupled with GC×GC-TOFMS.

## Experimental

Superficial pigment was collected as three subsamples from different locations on the femur. Samples were analyzed directly by SPME-GC-TOFMS for lighter volatile compounds and by TD-GC×GC-TOFMS for broader untargeted profiling of volatile and semivolatile constituents.

## Results

GC×GC-TOFMS analysis of the three subsamples yielded several thousand raw chromatographic peaks. After removal of unassigned peaks, blank contaminants, duplicates, column bleed, silicon-containing artifacts, and clearly modern synthetic compounds, 726 plausible ancient organic compounds remained for interpretation. The retained profile included 11 diketopiperazines consistent with proteinaceous material, 19 cholesterol-related compounds representing extensive sterol degradation, diterpenes and sesquiterpenoids consistent with a plant resin contribution, and lipid-derived species including oleic acid, trans-13-octadecenoic acid, and glycidyl palmitate. Hopane biomarkers further indicated substantial microbial diagenesis during long-term burial.

## Conclusion

The compound profile is consistent with a complex organic binding medium containing proteinaceous material, copal-like resin, and a minor lipid component associated with the red pigment on the bone surface. These findings support deliberate preparation of the pigment mixture and demonstrate the value of untargeted GC×GC for archaeological residue analysis.

## Acknowledgements

We thank Dr. Michele Bleuze for providing the sample.

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# Theoretical Decomposition and Practical Mitigation of Matrix effects in quantitative LC–MS calibration

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## Introduction

Matrix effects are a persistent source of bias in quantitative LC–MS workflows based on linear calibration and therefore complicate both method validation and systematic method optimization. In routine practice, they are most often evaluated using standard addition method based on two empirical quantitative descriptors: the matrix-matched response ratio [1] and the calibration-slope ratio [2]. However, the formal mathematical relationship between these descriptors has not been clearly established. As a result, important features of calibration behavior, especially those related to intercept and slope contributions, may remain obscured, leading to incomplete interpretation of matrix effects and potentially suboptimal analytical decisions [3].

## Experimental

In this study, we develop a unified theoretical and computational framework that decomposes matrix effects into two components corresponding to the intercept and slope terms of the calibration model. This formulation explicitly links conventional matrix-effect metrics to the underlying regression structure, thereby clarifying their interpretation and limitations. The proposed decomposition enables separate quantification of concentration-independent and dependent terms, providing a more detailed diagnosis of matrix-induced perturbations.

## Results

The practical utility of the framework is demonstrated using two chemically distinct matrices (human serum and a plant extract) where it enhances the interpretability method validation. By resolving matrix effects into separable components, the approach permits a systematic evaluation of analytical interventions with respect to their specific impact on calibration parameters, thus supporting more rational method development and optimization. The optimization process is facilitated by statistically based design of experiment.

## Conclusion

The proposed framework provides a generalizable strategy for the rigorous characterization of matrix effects in quantitative LC–MS and for guiding their mitigation. It is readily transferable across a broad range of targeted omics applications, including metabolomics, proteomics, and lipidomics, where accurate quantification in complex biological matrices remains a central analytical challenge.

**Acknowledgements**
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# Feed Injection-Enabled 2D-LC/MS for Enhanced Analyses of Lipophilic Drug Formulations

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## Introduction

Strong sample solvents and lipidic excipients hamper reversed-phase (RP) liquid chromatography (LC) analysis of lipid-based pharmaceutical formulations by causing solvent mismatch, resulting in peak distortion and restricting the applicable injection volume, which ultimately reduces the sensitivity to detect impurities.

## Experimental

In this work, we evaluated feed injection (FI) under gradient conditions for the analysis of sesame oil-based formulations dissolved in ethyl acetate (EtOAc) and integrated FI with two-dimensional LC coupled to mass spectrometry (2D-LC/MS) for impurity profiling.
Findings were compared to conventional flow-through injection (FTI) and sandwich injection, across injection volumes of 1–40 µL.

## Results

FI at low feed flow fractions (≤5% of mobile phase flow rate) provided ≥1:20 online dilution, sustaining column head focusing, and preserving peak shape and linearity across the full injection range.
In contrast, higher feed flow fractions (≥20% of the mobile phase flow rate, ≤1:5 dilution) resulted in severe fronting peaks and non-linear ultraviolet (UV) detector response.
Finally, the 2D transfers resolved matrix- and Active Pharmaceutical Ingredient (API)-related impurities without laborious offline reconstitution or dilution.

## Conclusion

This work extends the application field of FI from analyte diluents to complex lipid matrices, offering a robust, scalable workflow for pharmaceutical formulation analysis.

**Modern approach for analytical and preparative liquid chromatography by ECOM manufacturer**

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**Abstract**

The presentation will introduce modern trends and innovations in analytical and preparative liquid chromatography, with a focus on modular and automated solutions for research and production laboratories. Advanced system configurations combining column and solvent selectors, pH monitoring, and conductivity detection will be presented, emphasizing flexibility, efficiency, and easy integration into laboratory workflows. The presentation will also feature the new GMP-ready preparative system PrepTower, equipped with advanced process and detection functions, which has successfully demonstrated its performance in direct comparison with competing preparative platforms. A significant part of the presentation will be devoted to modern detection technologies, particularly the use of LED light sources in UV/VIS detectors, as well as practical possibilities for coupling preparative systems with UV/VIS and mass spectrometric detection, including intelligent dual-signal-controlled fraction collection.

# The Role of Analytical Chemistry in Solving Contemporary Challenges in Biomethane Plants

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**Abstract**

The increasing role of biomethane plants in the energy transition has brought not only technological opportunities but also complex analytical challenges that require precise monitoring and process control. Analytical chemistry plays a crucial role at multiple stages of biomethane production, from feedstock characterization and process optimization to gas quality control and environmental safety.

One of the key challenges in biomethane plants is the presence of hydrogen sulfide ($H_2S$), a toxic and corrosive compound that negatively affects process efficiency, infrastructure durability, and compliance with gas quality standards. Effective mitigation of $H_2S$ requires not only suitable removal media but also robust analytical tools to understand, monitor, and optimize the capture process.

This work highlights the indispensable role of analytical chemists in addressing such challenges by combining material selection with advanced analytical strategies. Special attention is given to the development and evaluation of appropriate media for $H_2S$ capture based on deep eutectic solvents (DESs), which are well known in analytical chemistry for their tunable properties, low volatility, and potential sustainability. The successful application of DESs in this context relies on accurate qualitative and quantitative analysis of sulfur species and on the optimization of operational parameters.

Without extraction and chromatographic techniques, including gas analysis and speciation studies, reliable assessment and optimization of $H_2S$ removal processes would not be possible. This contribution demonstrates that analytical chemistry is not merely a supporting discipline but a fundamental component of modern biomethane plant operation, enabling data-driven decisions, improved process stability, and enhanced environmental performance.

# Centrifugation-assisted micro-extraction using functionalized glass beads for rapid HPLC analysis

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**Abstract**

A novel, simplified microextraction technique we call the centrifugation-assisted extraction method, which uses C18-functionalized glass beads as the sorbent, has been developed. The procedure begins with extracting the analyte from a small volume of sample onto the functionalized glass beads. Then, an inert high-density fluorinated liquid is added, and the contents of the tube are centrifuged. This liquid layer acts as a physical barrier between the supernatant, which contains matrix interferences, and the functionalized glass beads, which retain the analytes. The supernatant and inert liquid can easily be removed, and the captured analyte can be eluted from the beads with a solvent for analysis by high-performance liquid chromatography using a diode-array detector. This procedure is demonstrated by extracting retinol from a small volume of human serum and has been validated according to the ICH M10 guideline by the European Medicines Agency. Our method is a rapid, cost-effective, environmentally friendly, and robust alternative to other techniques. It is well-suited for routine clinical and analytical laboratories because it significantly reduces solvent usage and simplifies the workflow to a single tube.

# Pipette tip micro solid phase extraction: a simple and efficient microextraction technique

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## Introduction

Sample preparation remains a crucial bottleneck in analytical chemistry, accounting for approximately more than half of the total analysis time [1]. While traditional techniques like liquid-liquid extraction and solid-phase extraction (SPE) are widely accepted, they often suffer from high consumption of toxic organic solvents, large sample requirements, and complicated multi-step procedures. To address these drawbacks, miniaturized techniques such as pipette tip micro solid-phase extraction (PT-µSPE) have emerged [2]. In this presentation, by focusing on our research, we will highlight different features of PT-µSPE as a promising miniaturized SPE variant that aligns with the principles of green chemistry and offers a highly economic and versatile approach for trace analysis.

## Experimental

The PT-µSPE technique is characterized by its compact and portable design. In this method, a standard pipette tip serves as the SPE cartridge, where a few (typically 2–7) mg of an adsorbent are "sandwiched" between two pieces of deactivated cotton or filters. The extraction is performed through repetitive aspirating and dispersing cycles, allowing for rapid mass transfer and achievement of equilibrium within minutes (Figure 1) [3]. The flexibility of this setup allows for the use of various advanced adsorbents. While inorganic materials (silica, CNTs, graphene) provide stability, organic adsorbents like molecularly imprinted polymers (MIPs) offer superior selectivity for target identification. Recently, high-surface area materials such as metal-organic frameworks have been integrated to overcome the limitations of chemical stability and repeatability under harsh conditions [4].

## Results

Our findings showed that the application of PT-µSPE, coupled with detection methods such as AAS, HPLC, spectrophotometer, and GC-MS, has remarkable efficiency across diverse matrices (Table 1). The technique has proven successful in detecting trace analytes, including heavy metals, antibiotics, plant hormones, dyes, and phenoxy herbicides, in complex samples such as seawater, foodstuffs, and body liquids. Compared to SPE, PT-µSPE requires significantly less sample volume and adsorbent, making it particularly conducive to biological analysis. The non-exhaustive nature of this miniaturized approach, combined with the reduction of extraction time to a few minutes, underscores its economic feasibility and high-throughput potential in modern laboratories.

## Conclusion

PT-µSPE represents an important advancement in sample preparation. By miniaturizing the classical SPE process into a pipette tip format, it addresses the issues of sample carryover and

excessive solvent waste. The integration of highly selective materials like MIPs and stable hybrid frameworks like MOFs positions PT-µSPE as a robust and versatile tool for the sensitive analysis of trace components in complex environmental and biological systems.

**Acknowledgements**
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**Figure 1.** Schematic illustration of the PT-µSPE procedure.

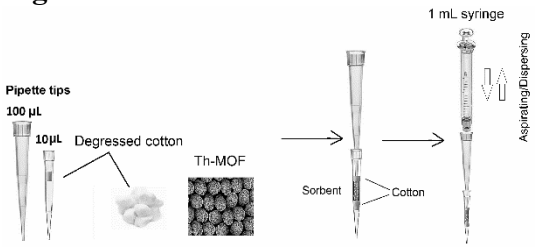


**Table 1.** Our recent research on the application of PT-µSPE

| Sample | Analyte | Adsorbent | Instrument | Enrichment Factor | LOD (µg/L) |
|---|---|---|---|---|---|
| Sea water, wastewaters | mitoxantrone | MIP, methacrylic acid | spectrophotometry | 49 | 0.2 |
| human plasma | malondialdehyde | graphene | spectrofluorometer | 50 | 0.3 |
| urine and pharmaceutical samples | mefenamic acid | zinc sulfide modified carbon nanotubes | HPLC | 42 | 50 |
| fish | mercury | porphyrinic zirconium metal-organic framework | cold vapor AAS | 120 | 0.02 |
| water and soil | diazinon | modified multi-walled carbon nanotubes | HPLC | 100 | 0.03 |
| juice and drinking water | Bisphenol A | cobalt metal organic framework | HPLC | 83 | 0.07 |
| seawater | methyl red | molecularly imprinted polymer | spectrophotometry | 32 | 0.5 |
| seawater | Rhodamine B and Malachite green | graphene oxide/zinc oxide nanocomposite | spectrophotometry | 20 and 30 | 1.0 and 1.2 |
| seawater | methyl orange and acid red | graphite based magnetic $NiFe_2O_4$ decorated exfoliated | spectrophotometry | 40 and 27 | 0.9 and 1.0 |
| cactus fruit, multivitamin syrup, vitamin B1 tablet and human plasma | Vitamin B1 | carbon nanotube | spectrofluorometer | 91 | 0.16 |
| seawater | malachite green, rhodamine B, methyl orange and acid red 18 | Co metal-organic framework | HPLC | 26, 31 and 28 | 0.09–0.38 |
| seawaters | malachite green | carbon nanotubes | spectrophotometry | 32.3 | 0.8 |
| seawaters | malachite green, rhodamine B, methyl orange and acid red 18 | MIP | HPLC | 107, 87, 43 and 32 | 0.083, 0.10, 0.12 and 0.17 |
| saliva, urine and wastewater | nicotine | tantalum metal organic framework | HPLC | 233 | 0.7 |
| human blood plasma and tablet | ciprofloxacin | molecularly imprinted polymer | HPLC | 48 | 1.5 |
| bottled water | Bisphenol A | thorium–metal organic framework | HPLC | 99 | 1 |
| pills | nalidixic acid and acetaminophen | molecularly imprinted polymer | spectrophotometry | 39 and 23 | 0.2 and 0.3 |
| shampoo | methyl and propyl parabens | Chromium-based metal organic framework | spectrophotometry | 340 | 0.24 |
| foodstuff | profenofos | molecularly imprinted polymer | HPLC | 31 | 0.33 |
| aqueous samples | Levofloxacin | molecularly imprinted polymer | spectrophotometry | 78 | 0.1 |

# LC-MS/MS salicylate profiling in *Arabidopsis thaliana* plants under infection with pathogens of different lifestyles

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## Introduction

Salicylates are precursors and metabolites of salicylic acid. Salicylic acid (SA) is a key phytohormone involved in plant immune responses. Its accumulation during defense reactions is influenced by biosynthesis, transport, and release from conjugated metabolites. Many aspects of SA metabolism under different conditions remain insufficiently understood.

## Experimental

A comprehensive liquid chromatography tandem mass spectrometry method for targeted and semi-targeted analysis of salicylates, their precursors, and metabolites, with a focus on the identification of new SA-related compounds, has been developed. The method combines reverse phase chromatography with optimized sample preparation, including selective extraction, hydrophilic-lipophilic balanced solid phase extraction, and analyte preconcentration. The method was applied to *Arabidopsis thaliana* plants infected with microorganisms exhibiting different lifestyles (e.g., hemibiotrophic bacterium *Pseudomonas syringae* and the necrotrophic fungi *Botrytis cinerea* and *Sclerotinia sclerotiorum*).

## Results

The results reveal differences in SA and SA-related metabolic profiles associated with distinct infection strategies.

## Conclusion

This approach provides a useful tool for studying SA metabolism and supports the discovery of previously unknown SA-related compounds involved in plant defence.

## Acknowledgements

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# ChromLine: Performance & Innovation

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**Abstract**

Chromservis s.r.o. is a Czech manufacturer of chromatographic columns and analytical consumables with over 35 years on the market, distributing to customers worldwide. This presentation introduces ChromLine — Chromservis's own product brand unifying its portfolio of HPLC columns, GC columns, sample prep platforms, and ready-to-use analytical kits developed in collaboration with university and industrial R&D partners across Europe. Alongside the portfolio overview, selected recent product launches and innovations are highlighted.

The talk also addresses Chromservis's approach to application support and custom method development, reflecting the company's role as an R&D partner rather than a conventional supplier.

# Rational approaches to MALDI-MS matrix design

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## Introduction

The use of small organic molecules as a matrix for MALDI-MS is one of the most common approaches in this analytical method. Historical, due to the lack of synthesis resources analytical chemists working in this field relied of selecting these compounds from their chemical storage cabinet based on crude rules like absorption or solubility. This empirical approach resulted in the discovery in most of the commercially available MALDI-MS matrices. Interestingly, the selection of the right combination of MALDI-Matrix and group of analytes is key to the success of this method. This triggered the need of a more rational approach for the MALDI-MS matrix design. The straightforward idea from an organic chemist's perspective is the rational design of compound libraries with physical properties matching the requirements from both analytical method and analyte group. The final goal of this approach is the identification of the major structural and physical properties of these matrices which are key for the design of MALDI matrices. To test this idea a library of potential MALDI-MS matrices was synthesized and evaluated to identify "structure-performance relationships" between matrices and brain lipids like sulfatides.

## Experimental

Synthesis of the compound library was done by Knoevenagel condensation of aromatic aldehydes and α-cyanoacetic acid derivatives leading to compounds with different substituents at the aromatic rings and different geometries. Compounds were purified by re-crystallization and characterized by NMR-methods (500 MHz). Beside this, a scaffold hopping approach was executed to substitute benzene rings by heterocycles to identify new matrix scaffolds. The design of the chemical library was done based on designing a virtual library using Lipinski rules and Tanimoto similarity scores. The physical properties pKa, logP, number of H-bond donors, and number of H-bond acceptors were calculated on Instant JChem version 15.1.19.0 (ChemAxon, Budapest, Hungary). Matrices were evaluated by MALDI-MS measurements using an UltrafleXtreme (355 nm Smartbeam II laser) system and using various analyte extracts like lipids, peptides and protein mixture. For the statistical analysis of the evaluation of a lipid brain extract ((Avanti Polar Lipids Inc, Alabaster, USA) a principal component analysis (PCR) was done.

## Results

The chemical synthesis led to a library of 59 compounds which were evaluated using sulfatides as constituents of a lipid brain extract in the negative ion mode [1]. The analysis of the mean S/N ratio of the sulfatide analytes signals showed that amide derivatives of the phenyl cinnamic acids are general better matrices for this analyte group in the negative ion mode. A second outcome was that cinnamic acid derivatives with a para phenyl substitution pattern show in

many cases a much better performance than ortho or meta substituted phenyl derivatives. In an additional step a multivariate statistical analysis established relevant features like pKa, clogP or hydrogen bonding as additional features which influence the performance of this class of MALDI-matrices for lipid detection. Further evaluations of these matrices with other analyte classes like peptides and proteins show that a correlation between the physical properties of the matrices and the analytes is a promising approach for the rational development MALDI matrices. Beside this, a scaffold hopping approach led to a novel group of MALDI-MS matrices with a pyrrol scaffold and altered properties and analyte group selectivity.

**Conclusion**

The analysis of the screening results shows the feasibility of a rational design approach and the usage of synthesis strategies origination from medicinal chemistry. This strategy could finally lead to a set of structural features and physical properties which can be used for the virtual screening of large compound libraries to identify MALDI matrices which are tailored for a certain analyte group.

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# Multiomics in laboratory medicine: from high-dimensional data to clinically actionable information

David Friedecký[1], Radana Brumarová[1], Eliška Ivanovová[1], Hana Janečková[1], Jakub Rozhon[1], Martina Kadláčková[1], Martina Horejšová[1], Antonín Bednář[1], Richard Masař[1], Aleš Kvasnička[1], Barbora Piskláková[1], Jaroslava Friedecká[1], René Lenobel[2], Ondřej Novák[2]

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## Introduction

Laboratory medicine is undergoing a transition from single-analyte testing towards integrated interpretation of complex biological data. Genomics, transcriptomics, proteomics, metabolomics, and lipidomics provide complementary information on molecular mechanisms of disease, treatment response, and individual patient variability. However, the clinical implementation of multiomics remains challenging due to preanalytical variability, analytical complexity, data integration, standardization, and interpretation in a clinically meaningful context. The aim of this contribution is to summarize the current role and future potential of multiomics approaches in laboratory medicine, with emphasis on their translation into routine diagnostics and personalized patient care.

## Experimental

Multiomics workflows combine high-throughput analytical platforms, including next-generation sequencing, mass spectrometry-based proteomics, metabolomics, and lipidomics, and advanced bioinformatic tools. Key methodological steps include standardized sample collection and storage, quality control, data preprocessing, feature annotation, statistical modeling, and biological interpretation. In a clinical laboratory setting, special attention must be paid to traceability, reproducibility, reference materials, harmonization of reporting, and integration with established biochemical and clinical parameters.

## Results

The integration of multiple omics layers enables a more comprehensive description of disease phenotypes than isolated molecular measurements. In oncology, multiomics contributes to molecular tumor classification, biomarker discovery, and therapy selection. In inherited metabolic disorders, metabolomics and genomics together improve diagnostic yield and facilitate interpretation of variants of uncertain significance. In cardiometabolic, inflammatory, and neurodegenerative diseases, combined molecular profiling may reveal early pathophysiological changes and support patient stratification. Despite these opportunities, several barriers remain, including limited availability of validated clinical algorithms, high data dimensionality, inter-laboratory variability, and the need for multidisciplinary interpretation involving laboratory specialists, clinicians, and bioinformaticians.

## Conclusion

Multiomics has the potential to significantly expand the diagnostic and interpretative role of laboratory medicine. Its successful clinical implementation will depend not only on analytical performance but also on standardization, validation, robust data integration, and clear communication of clinically actionable results. Laboratory medicine is well-positioned to coordinate this translation, linking advanced molecular technologies with patient-centered diagnostics.

## Acknowledgements

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# Back to the Old Masters of Lacquerware: Classification of Asian Lacquers Using Atmospheric Solids Analysis Probe High-Resolution Tandem Mass Spectrometry

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## Introduction

Asian lacquers are materials produced by the polymerization of sap from trees *T. vernicifluum*, *T. succedaneum* and *G. usitata*. The resulting material is a glossy, tough and durable. The history of the usage of these materials lasts thousands of years and lacquers represent an important part of Asian cultures. A common analytical task is the determination of the type of lacquer used. The method of first choice is pyrolysis coupled with gas chromatography and mass spectrometric detection (py-GC/MS) [1,2]. By using this method, the interpretation of the data is relatively challenging, and times of analysis are long. [2] Other methods used are time-of-flight secondary ion mass spectrometry (ToF-SIMS) [3], high performance liquid chromatography (HPLC) [4] and Fourier transform infrared spectroscopy (FTIR) [5]. A study has been presented on the differentiation of *T. vernicifluum* lacquers from different geographic regions; however, it includes only a single sample per region [3]. Such a limited dataset cannot adequately assess the significance of inter-regional differences relative to intra-local variability, nor determine whether the geographic origin of *T. vernicifluum* can be reliably distinguished. This communication deals with the utilization of atmospheric pressure solids analysis probe high resolution tandem mass spectrometry (ASAP-HRTMS) for classification of Asian lacquers.

## Experimental

Twenty-seven lacquer samples from 17$^{th}$ to 20$^{th}$ century were analyzed. Study was performed using Waters Cyclic IMS high resolution tandem mass spectrometer with integrated cyclic ion mobility cell and atmospheric solids analysis probe (ASAP) used as an ion source. Samples were inserted into a standard melting point capillary through a 1 mm hole radially drilled approximately 5 millimeters from the capillary bottom end. A standard solution of 2',4',6'-trihydroxyacetophenone monohydrate (THAP) in acetone (1 mg/ml) was used for lock mass correction. The best signal of characteristic lacquer components was obtained at the following setup: desolvation temperature 600 °C, corona current 2.0 μA, sample cone voltage 30 V.

## Results

Based on characteristic MS and MS/MS spectra, samples in which at least one type of Asian lacquer was identified were subjected to Principal component analysis (PCA). Selected signals of detected phenol and benzenediol derivatives were used for the construction of data matrix. In the resulting Score plots, the samples were reliably separated into three clusters corresponding to individual lacquer types, i.e. urushi, laccol and thitsi [6]. Moreover, in the cluster of *T. vernicifluum*, results indicated further separation into three subclusters that is subject of a present research.

## Conclusion

ASAP-HRTMS in combination with PCA appeared to be a reliable technique for the classification of Asian lacquers. The method is fast and does not require any sample pretreatment. Presently the method is tested by another set of lacquer samples with the aim of establishing it as a standard tool for distinguishing Asian lacquers.

## Acknowledgements

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# Using Charge Detection Mass Spectrometry with an Electrostatic Linear Ion Trap for the Analysis of Viral Vectors and Large Protein Complexes

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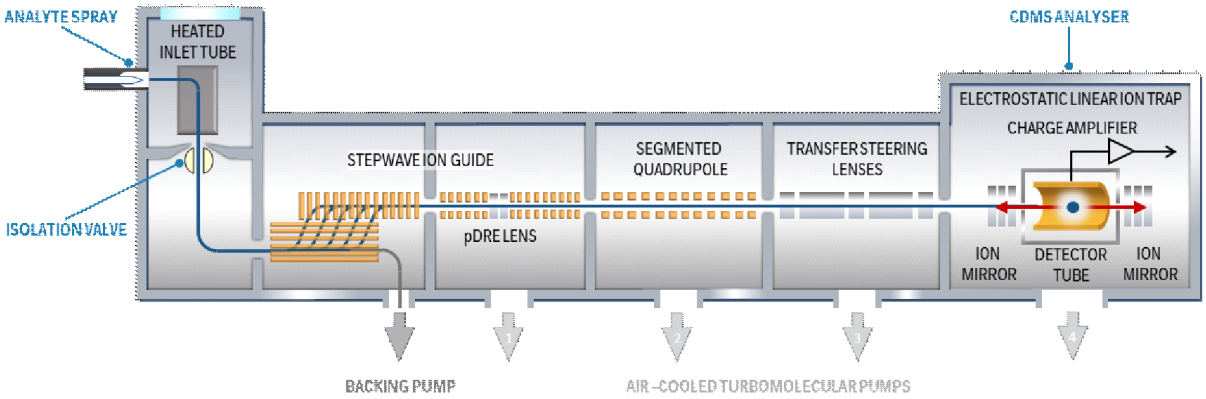
## Introduction

Determining the mass and understanding the heterogeneity of large and complex biomolecules has long been a barrier to advancement in the biological analytical chemistry. Conventional techniques fail to resolve the information needed to reconstruct, in detail, the structure, especially for megadalton-sized molecular assemblies, so it can take months or even years of tedious, expensive research to understand the basics of such biomolecular structures. With the breakthrough of the charge detection mass spectrometric techniques (CDMS), it is now possible to obtain a precise measurement of these challenging highly heterogeneous molecules, including viral vectors, virus-like particles (VLPs), RNA, and protein complexes, that were previously out of reach for mass characterization [1,2,3].

## Results

This oral presentation introduces the technique of CDMS and offers a deeper overview into its recent developments and examples of applications. The talk deals with the development of this young discipline, which is an alternative to conventional mass spectrometry by a measurement of both the m/z and charge (z) of individual ions, allowing for direct determination of accurate intact masses. By performing individual ion measurements, CDMS is particularly useful for over megadalton sized biomolecules, where determination of individual charge states is not anymore possible. The simultaneous measuremens of m/z and z for each of ions is performed by trapping these between two electrosttic mirrors in the electrostatic linear ion trap (ELIT, figure 1). Switching between trapping and transfer potentials causes ions to oscillate through a detector tube, including a periodic signal that is captured by a charge-sensitive amplifier. Fast Fourier transform (FFT) analysis of the time-domain signal is performed in real time with the charge of the ion determined from the amplitude of the fundamental frequency and m/z from the oscillation frequency.

**Figure 1.** Schematic of a prototype benchtop nanoESI-CDMS ELIT instrument.



## Conclusion

The conference audience receives a comprehensive overview of current developments of the CMDS technology. More development in this area is being reported both from academic and industry research groups making this approach one of fast developing hyphenated analytical techniques.

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# Pioneering 4D Multiomics and Functional Proteomics/Matbolomics 2.0

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**Abstract**
Discover how Bruker is advancing ion mobility mass spectrometry to help scientists see more, know more, and decide with confidence. Explore what's new across our portfolio for proteomics, metabolomics, pharma, imaging, and applied markets after ASMS 2026.

# Capillary Electrophoresis - Mass Spectrometry for the Characterization of Proteoforms on the Intact Level

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**Abstract**

Electromigrative techniques are powerful tools for the separation of intact proteins and their proteoforms. However, CE-MS is still restricted by the sensitivity and ease-of-use of the interface in conjunction with low injection volumes limiting its application for biological samples. Various solutions will be presented here overcoming these shortcomings.

Initially, the power of CE-MS for the characterization of proteoforms will be presented applying the *nanoCEasy* interface [1]. Efficient separation of proteins and proteoforms depends strongly on the applied capillary coating. Very recently we developed efficient coatings enabling finetuning the EOF [2] and, thus, increase the separation efficiency for proteins of certain mobility. Results on the application for protein separation from biological samples will be presented.

Furthermore, a CZE-MS method for a detailed characterization of mAb charge variants is presented showing similar high separation efficiencies as the widespread non-MS-compatible separation method based on ε-amino caproic acid [3]. A static HPMC-based capillary coating in combination with an ammonium acetate-based electrolyte at physiological pH enable a powerful separation of mAb charge variants and allow subsequent hyphenation to MS. Depending on the composition of the sheath liquid, subsequent MS analysis can be performed under denaturing as well as under non-denaturing conditions, showing slightly better quality for the denatured charge-deconvoluted spectra.

nanoLC-CE-MS is a promising tool for targeted protein and proteoform analysis in biological samples. Initially, a heart-cut nanoLC-CE-MS was setup and the performance regarding improved sensitivity as well as separation of proteoforms was demonstrated [4]. Due to the increased loadability, the nanoLC-CZE-MS setup exhibits a strongly improved increased concentration sensitivity compared to CZE-MS. The combination of high sensitivity and orthogonal selectivity enables the detailed characterisation of intact proteoforms at physiologically relevant concentrations. A novel selective comprehensive online nanoLC-CE-MS configuration will be presented and discussed in the context of targeted proteoform analysis in biological samples using proteoforms of histone as an example [5].

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# Analysis and investigation of acid-base and electromigration properties of peptides by capillary zone electrophoresis and isotachophoresis

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## Introduction

Peptides are extremely important biomolecules exhibiting various biological activities, e.g. antimicrobial, antitumor, antidiabetic, and antiobesitic effects [1, 2]. Recently discovered anorexigenic neuropeptides (prolactin-releasing peptide (PrRP), cocaine- and amphetamine-regulated transcript peptide (CARTP), and ghrelin (Ghr)) are explored as potential new drugs regulating in brain a food intake. However, they are not able to cross the hemato-encephalitic barrier when they are applied peripherally. For that reason, they are modified by the attached fatty acids [3]. Before testing their biological activities, their chemical purity must be checked and their physico-chemical properties must be determined. Hence, the aim of this work was to employ capillary zone electrophoresis (CZE) and isotachophoresis (CITP) [4] for analysis of above synthetic (lipo)peptides and for study of their acid-base and electromigration properties.

## Experimental

CZE and CITP analyses were carried out in Agilent CE 7100 analyzer (Waldbronn, Germany) equipped with UV-vis spectrophotometric detector set at 200 nm and contactless conductivity detector. The analyses were run in the hydroxypropyl cellulose or polyacrylamide coated fused silica capillaries with outer polyimide coating. Their ID/OD was 50/375 μm for CZE and 100/375 μm for CITP, and their total/effective lengths were 450/365 mm for CZE and 600/450/515 mm for CITP. Peptides were dissolved in 50 μL of water or BGE at concentration 1 μg/μL and were introduced into the capillary hydrodynamically by pressure 1 000–3 500 Pa for 10–20 s, the injected volume was 4.4–30.5 nL.

## Results

Purity degree of the above neuropeptides and their lipidized derivatives with covalently attached octanoic, myristic or palmitic fatty acids was determined by CZE. The (lipo)peptides were analyzed by CZE as cations in acidic and neutral background electrolytes (BGEs) composed of formic, acetic and phosphoric acids and MES, at pH 2.1–7.0, and as anions in alkaline BGEs constituted of sodium-Tricine and sodium-CAPS buffers, pH 8.5–11.0. Examples of CZE analyses of PrRP in two acidic, one neutral and three alkaline BGEs, are presented in Figure 1A. Mostly, a high purity degree of RP-HPLC purified (lipo)peptides was found.

Acid-base and electromigration properties of (lipo)peptides were investigated by both CZE and CITP methods. Effective mobilities of (lipo)peptides at 23 particular pH values were determined by CZE performed in BGEs composed of various buffers within a wide pH range (1.50–12.3) at constant ionic strength of 25 mM and constant temperature of 25°C. From the measured pH

dependence of the effective mobilities of peptides shown in Figure 1B, the isoelectric point of (lipo)peptides, the mixed acidity constants of their ionogenic groups and the actual ionic mobilities of their particular ionic forms were obtained by nonlinear regression analysis using the procedure described in [5]. The mixed acidity constants (related to 25 mM ionic strength) were recalculated to thermodynamic acidity constants (related to zero ionic strength) using the Debye-Hückel theory [5]. The thermodynamic acidity constant (pKa) of acidic groups (alpha-carboxyl and carboxyl groups of aspartic and glutamic acids) were in the range of 2.02–4.96. The pKa values of ionogenic groups of basic amino acids (histidine, lysine, and arginine) and N-terminal amino group span the interval 5.55–11.26.

Effective charge of (lipo)peptides was estimated by CITP using a cationic electrolyte system. The leading electrolyte was composed of 10 mM ammonium cation as the leading ion and 40 mM acetate as the buffering counterion, pH 4.0. The terminating electrolyte comprised 40 mM acetic acid, pH 2.9, providing hydroxonium cation as the terminating ion. The determination of effective charge is based on the linear relation between the CITP zone length of the analyte and its effective charge and injected sample amount [6]. The effective charge of strongly basic (lipo)peptides was found significantly (36–50 %) lower than the theoretical charge calculated from the acidity constants due to the counterion condensation.

## Conclusion

CZE and CITP proved to be powerful methods for analysis of (lipo)peptides and for study of their acid-base and electromigration properties. In particular, the purity degree, acidity constants (pKa), effective and ionic mobilities, isoelectric points, and effective charges of (lipo)peptides were determined.


## Acknowledgement

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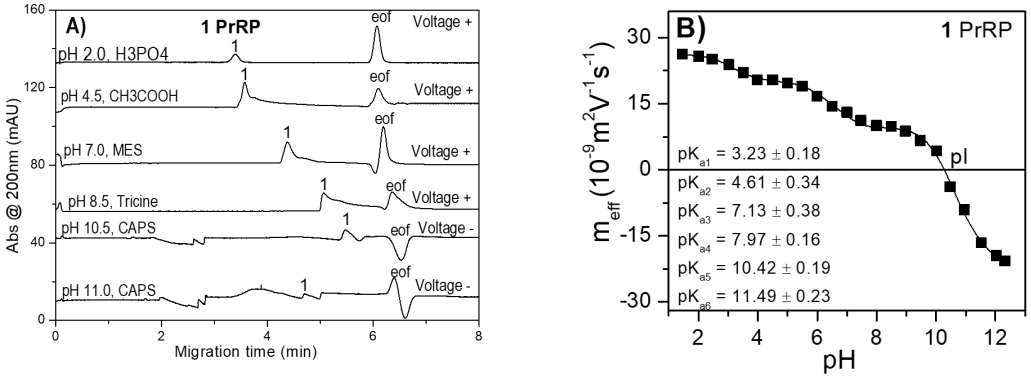
**Figure 1.** A) CZE analysis of prolactin-releasing peptide (**1** PrRP) in acidic, neutral and alkaline BGEs. B) Dependence of the effective mobility $m_{eff}$ of PrRP on pH of background electrolyte.



# From Tissue to Single Cells: Targeted Hormonomics in Root Protoplasts

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## Introduction

Plant hormones are small bioactive molecules that regulate physiological processes in plants. They act at very low concentrations, typically $10^{-12}$ to $10^{-15}$ moles per gram of fresh weight. Information on the spatio-temporal occurrence of phytohormones, their precursors and metabolites, improves our understanding of plant growth, organ development and responses to environmental stresses. Ultra-high performance liquid chromatography coupled with tandem mass spectrometry (UHPLC-MS/MS) has become the state-of-the-art technology for targeted determination of the phytohormone metabolome in plant tissues. However, current plant physiology is increasingly focused on smaller spatial scales, creating the need for methods capable of detecting phytohormones at the cellular level.

## Experimental

Fluorescence-activated cell sorting (FACS) coupled with UHPLC-MS/MS was employed to profile phytohormone metabolites in a single cell population of *Arabidopsis* root protoplasts. Sample preparation was identified as a critical step due to matrix effects influencing ionisation efficiency and mass spectrometric response. A novel sample preparation method based on dispersive solid phase microextraction (DSPE) was developed.

## Results

Key parameters, including number of protoplasts, sorbent capacity and solvent composition, were optimised using a statistically designed experiment to maximise the signal-to-noise ratio. The purification step significantly reduced matrix effects and improved analytical performance. Importantly, phytohormonal metabolites were successfully detected in the purified fraction of fluorescently labelled protoplasts. These results demonstrate the feasibility of analysing phytohormones at the level of a single cell population.

## Conclusion

DSPE represents a promising approach for sample preparation prior to targeted UHPLC-MS/MS analysis of phytohormones at the cellular level. The developed methodology enables sensitive and reliable profiling of plant hormone metabolites in highly limited biological material, contributing to advanced studies of plant physiology.

## Acknowledgements

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# Three-Level Microfluidic Architecture as a Framework for Modular Analytical Instrumentation

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## Introduction

Microfluidics enables precise fluid manipulation at the microlitre-to-nanolitre scale, with well-established applications in analytical chemistry spanning sample preparation, cell manipulation, electrophoretic and chromatographic separations, and direct analyte delivery to mass spectrometer ion source. These applications typically rely on single-device setups offering little beyond a basic mechanical attachment interface, while multi-device integration with electronic control remains largely absent from existing systems. We previously introduced a "three-level microfluidic architecture" [1] in which a microfluidic device (MFD) is integrated into a standardised-footprint module with fluidic and electrical connectors. Four such modules stacked together form a self-contained microfluidic unit capable of MFD control and on-device data acquisition. Here, we report the development of a platform for assembling multi-unit microfluidic setups, which accommodates up to three microfluidic units and provides a mechanical mounting interface, power supply, and USB 3.0 communication for PC-based control software.

## Experimental

Overall design of the platform was made in SolidWorks 2025; mechanical components were CNC-milled by OpeTech s.r.o. (Czech Republic). Electronic schematics and PCB layouts were developed in KiCad 9, with PCBs manufactured by AISLER Germany GmbH. Control software was implemented in Python 3.9 with a PyQt6 graphical interface. For functional validation using an application relevant to modern analytical chemistry, a full-glass double-emulsion MFD for production of UV-crosslinked microcapsules was designed in SolidWorks 2025 and fabricated by selective laser-induced etching and laser welding (LightFab GmbH, Germany).

## Results

The prototype of the microfluidic platform was successfully assembled. Mechanical components were manufactured to the designed specifications, and all components were integrated according to original design. The assembled platform is undergoing characterisation to verify stable electronics and firmware operation. Initial tests indicate that the modular stacking mechanism provides sufficient mechanical rigidity and repeatable module positioning, both of which are critical for applications involving optical microscopy. Functional validation with the microcapsule generator microfluidic module is planned as the next step, with the aim of demonstrating end-to-end operation of the complete system.

## Conclusion

A functional prototype of the platform was successfully assembled. Initial characterisation confirms stable electronics and firmware operation. The attachment mechanism provides sufficient rigidity and repeatable unit positioning, both critical for optical microscopy applications. End-to-end functional validation using the microcapsule generator module, in which the platform will control droplet formation and UV crosslinking, to produce microcapsules suitable for use in analytical chemistry workflows as miniature sample vials in analytical workflows, is currently in progress.


## Acknowledgements

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# Optimizing Gradient Conditions in Comprehensive 2D Liquid Chromatography: A Comparison of Chromatographic Response Functions

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**Abstract**

Numerical optimization of gradient elution profiles in liquid chromatography is typically based on building of retention model using isocratic or gradient retention data followed by mapping of targeted criteria, most often resolution of critical pairs of compounds. Previously we have shown that for both, reversed-phase liquid chromatography [1] and hydrophilic interaction liquid chromatography [2] separations, the approach of retention modelling/resolution mapping can be successfully used for achieving optimal separation conditions. In our recent work, we have developed the procedure for optimization of gradient profile and tandem mass spectrometric detection (MS/MS) in multiple-reaction monitoring mode simultaneously using so called gradient score approach, which utilizes several complementary criteria describing both, chromatographic separation power, time-window overlap in MS/MS and effective use of gradient time [3]. In present work, we have compared gradient score obtained for various gradient profiles in HILICxRP two-dimensional separations with "traditional" chromatographic response functions based on resolution.

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# Open-source capillary electrophoresis – democratizing separation science: Design principles and practical implementations from our laboratory

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## Introduction

Open-source hardware and software are becoming increasingly important in analytical chemistry because they enable researchers to construct low-cost, customizable, and fully transparent instrumentation. Capillary electrophoresis (CE) is particularly suitable for open-source development due to its relatively simple instrumental design, low operational costs, and modular architecture [1]. In recent years, numerous open-source concepts have emerged in analytical chemistry; however, comprehensive and practically usable open-source CE systems remain scarce. In our laboratory, we have focused on the development of modular, open-source CE instrumentation and software that can be freely reproduced and modified by the scientific community. In this lecture, we will summarize the design principles behind open-source CE instrumentation and present several practical implementations developed in our laboratory, including 3D-printed CE autosamplers [2], open-source data acquisition systems [3], temperature-controlled sampling devices [4], and open-source electropherogram processing software [5].

## Experimental

The presented instrumentation was developed using commercially available electronic components, Arduino microcontrollers, 3D-printed mechanical parts, and open-source programming environments. The CE systems consisted of modular high-voltage power supplies, capacitively coupled contactless conductivity detection ($C^4D$), laser-induced fluorescence detection, and custom-built data acquisition systems. Instrument enclosures and mechanical parts were designed using Tinker CAD software and fabricated by fused deposition modeling (FDM) 3D printing. Control software and graphical user interfaces were written in Arduino IDE and Python environments. The developed systems were tested in representative CE applications including inorganic ion analysis, biological thiol determination, and exhaled breath condensate analysis. Electropherograms were evaluated using the newly developed CAPRI open-source integration software.

## Results

The lecture will overview the concept of open-source CE and discuss the availability of open-source components for CE instrumentation, including high-voltage power supplies, detectors, data acquisition systems, and software tools. We will discuss the advantages and limitations of this approach. Examples of practical open-source devices developed in our laboratory will follow. These will include first a fully automated, 3D-printed autosampler for CE. The

autosampler is operated by an Arduino Mega microcontroller and allows programmable hydrodynamic injection of up to 14 samples, capillary flushing, high-voltage control, and automated data acquisition. The system demonstrated excellent injection repeatability with peak area RSD values below 2.7% and enabled fully autonomous operation of modular CE instruments. Second, as an indispensable part of any separation system, inexpensive Arduino-based high-resolution data acquisition (DAQ) modules will be presented. They are utilizing analog-to-digital converter modules with 16–24 bit resolution. The developed DAQ systems demonstrated performance comparable to commercial high-end 24-bit converters while reducing the cost of CE data acquisition electronics to below 50 USD. Their applicability was demonstrated in CE systems with contactless conductivity detection ($C^4D$) for inorganic ion analysis. Third, an Arduino-based temperature-controlled sampler for collection of exhaled breath condensate (EBC), capable of maintaining stable collection temperatures within ±1 °C using thermoelectric cooling and feedback regulation was also developed. The device was successfully applied in point-of-care diagnostics and CE-$C^4D$ analysis of inorganic ions and organic acids. Finally, the lecture will introduce CAPRI (CApillary Electrophoresis Peak Recognition and Integration), an open-source Python-based software developed for automated and manual processing of electrophoretic and chromatographic data. CAPRI incorporates automated, advanced baseline correction, peak recognition, manual and automatic integration, calibration modules, and graphical visualization tools. The software was benchmarked against commercial CE data systems and demonstrated comparable or improved integration performance, particularly for complex electropherograms with low signal-to-noise ratios.

**Conclusions**

The open-source paradigm enables significant democratization of separation science by allowing researchers to construct, modify, and share analytical instrumentation and software at a fraction of the cost of commercial systems. The presented examples demonstrate that open-source CE instrumentation can achieve analytical performance comparable to commercial instruments while maintaining flexibility, transparency, and low cost. We believe that the continued development and dissemination of open-source CE hardware and software will stimulate innovation, improve accessibility of analytical instrumentation, and support wider adoption of capillary electrophoresis in research and education.

**Acknowledgements**

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# Analysis of Biomedical, Environmental and Pharmaceutical Samples by Microchip Electrophoresis

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## Introduction

The increasing analytical demands in biomedical research, environmental monitoring, and pharmaceutical quality control call for methods that are rapid, efficient, and aligned with green analytical chemistry. Microchip electrophoresis (MCE) meets these requirements through high-speed separations, minimal reagent consumption, and straightforward integration with automated workflows. This contribution highlights recent advances in applying MCE to the analysis of complex biological, environmental, and pharmaceutical samples.

## Experimental

Analyses were performed using an in-house developed MCE platform comprising an electrolyte unit with peristaltic micropumps and membrane driving electrodes, and an electronic unit providing stabilized current control. The poly(methyl methacrylate) microchip featured two separation channels equipped with conductivity detectors (Figure 1). Additional miniaturized detection modules including a surface-enhanced Raman spectrometer, a photometric detector, and an ion mobility spectrometer coupled via a thermal-spray interface enabled flexible, multimodal detection tailored to diverse sample types.

## Results

The study demonstrates the capability of MCE, particularly in coupled-channel configurations, to address analytical challenges associated with biomedical, environmental, and pharmaceutical matrices. The combination of isotachophoresis (ITP) for preconcentration with zone electrophoresis (ZE) for high-resolution separation significantly enhances sensitivity for trace analytes. The following applications are presented, among others: (1) analysis of metabolic organic acids and neurological biomarkers in cerebrospinal fluid and urine, (2) determination of nitrite and nitrate in cerebrospinal fluid following microsolid-phase extraction, (3) quantification of carminic acid in food and pharmaceutical products using integrated photometric detection, (4) detection of synthetic dyes in pharmaceuticals via ITP–SERS, and (5) ZE–IMS and ITP–IMS determination of carboxylic acids in food, environmental, and biological samples. Across these applications, the developed microanalytical methods provided high repeatability, accuracy, and low detection limits, confirming the suitability of MCE for trace-level analysis in complex matrices.

## Conclusion

Microchip electrophoresis with coupled channels represents a versatile and powerful analytical platform capable of integrating sample pretreatment, multidimensional separations, and diverse

detection techniques. Its performance in biomedical, environmental, and pharmaceutical analyses underscores its potential as a multifunctional tool for modern microanalytical applications.

**Acknowledgements**
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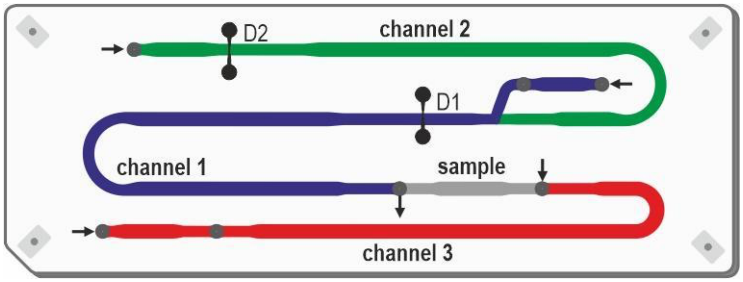
**Figure 1**. A schematic of the microchip with coupled separation channels and integrated conductivity sensors.



# UV-LED coupling for high-sensitivity glycan analysis in a commercial capillary electrophoresis system

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## Introduction

Laser-induced fluorescence (LIF) detection is the gold standard for high-sensitivity analysis in capillary electrophoresis (CE) [1]. LIF allows for signal-to-noise ratios several orders of magnitude higher than UV-vis absorbance detection. This sensitivity is crucial for fields like glycobiology, where analytes are often present in trace quantities. However, a significant limitation of commercial CE/LIF instruments is their lack of flexibility regarding excitation wavelengths (most are equipped with a fixed 488 nm laser).

The development of new fluorescent labels, such as the recently synthesized UV-excitable fluorophore 6-[4-(4-methylpiperazin-1-yl)-phenyl]pyridine-3-carbohydrazide (DMPPP hydrazide), requires excitation sources in the near-UV range (340 nm). While external lasers can be coupled to these instruments, they are often expensive and bulky. Light-emitting diodes (LEDs) offer a low-cost, compact, and power-efficient alternative. However, the highly divergent nature of LED light makes efficient coupling to the narrow apertures of CE systems a significant technical challenge [2]. This study presents and evaluates different strategies for coupling a 340 nm LED to a commercial Sciex P/ACE MDQ Plus system to enable high-sensitivity analysis of labeled glycans [3].

## Experimental

A 340 nm UV-LED (0.8 mW) was used as the excitation source. Three distinct coupling designs were developed and compared: (1) Indirect LED Coupling: The LED was housed in a 3D-printed adapter and coupled via a 3 mm ball lens to a standard optical fiber patch cable, which was then connected to the instrument's SMA port. (2) Direct LED Coupling with Ball Lens: To minimize light loss from the optical cable, the LED was placed in an aluminum housing with a 3 mm fused silica ball lens and mounted directly onto the instrument's SMA fiber port. (3) Direct LED Coupling with Two Plano-Convex Lenses: For maximum efficiency, a more complex setup was designed using two N-BK7 plano-convex lenses (12.5 mm diameter, 19.9 mm focal length). The first lens collimated the LED light, and the second focused it into the SMA port. This system was mounted on an XYZ linear stage for precise alignment.

Maltooligosaccharide standards (DP4–6) and *N*-linked glycans released from glycoproteins (ribonuclease B and ovalbumin) via PNGase F were labeled with DMPPP hydrazide at 50 °C for 18 hours. The excitation and emission maxima of the label were confirmed to be 325 nm and 470 nm, respectively.

Separations were performed in 50 μm I.D. fused silica capillaries (40 cm total length) coated

with linear polyacrylamide. The background electrolyte consisted of 1 M formic acid in a 1:1 (v/v) mixture of water and methanol. Samples were injected at 34.47 mbar for 15 seconds and the CE analyses were performed with a separation voltage of 30 kV.

**Results**

The performance of the three designs was evaluated based on the fluorescence signal of labeled maltooligosaccharides. The "Indirect" approach yielded a very weak signal, likely due to reflection and dispersion losses within the optical fiber. Switching to the "Direct Ball Lens" design resulted in a 10.7-fold increase in peak height and a 4.8-fold increase in the signal-to-noise (S/N) ratio. The "Direct Two-Lens" configuration provided the best performance, yielding a 31.2-fold increase in fluorescence signal and an 8.9-fold increase in S/N ratio compared to the indirect setup. This design effectively managed the divergence of the LED, delivering a much higher density of excitation light to the capillary detection window. Using the optimized two-lens configuration, the system achieved limits of detection between 99 and 105 nmol/L for the labeled maltooligosaccharides. These values are highly competitive with other LED-based systems and demonstrate the suitability of this setup for trace analysis.

The utility of the modified instrument was demonstrated through the analysis of DMPPP-labeled *N*-linked glycans from complex glycoproteins. The system successfully resolved the characteristic glycan patterns of ribonuclease B and ovalbumin. Remarkably, high-quality electropherograms were obtained from minute sample quantities: just 7.49 ng of ribonuclease B and 28.8 ng of ovalbumin.

**Conclusion**

The study demonstrates that a low-cost UV-LED can serve as an effective excitation source for commercial CE/LIF instruments when coupled using optimized direct-illumination optics. By bypassing standard optical fiber cables and using a two-lens focusing system on an XYZ stage, fluorescence signals can be increased by over 30-fold. This modification significantly enhances the versatility of existing laboratory equipment, enabling the use of novel UV-excitable fluorophores for high-sensitivity glycomic research without the need for expensive, specialized laser systems.

**Acknowledgements**

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# POSTERS

# Comparative TWIMS–MS Analysis of Binary and Ternary Lipocalin–Enterobactin Complexes

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## Introduction

Iron sequestration is a key mechanism of host innate immunity, in which lipocalin proteins play an essential role by binding bacterial siderophores such as enterobactin (Ent). Travelling wave ion mobility–mass spectrometry (TWIMS–MS) is a powerful separation science technique that enables the characterization of biomolecular conformations and noncovalent protein–ligand interactions through gas-phase ion mobility measurements and collision cross section (CCS) determination. In this study, TWIMS–MS was employed to investigate the binding interactions of Lipocalin 1 (Lcn1) with Ent and to compare its ion mobility behaviour with the well-characterized Lipocalin 2 (Lcn2)–Ent system.

## Experimental

The non-covalent complexes Lcn1 and Lcn2 with desferric and ferric Ent were characterized by electrospray ionization quadrupole time-of-flight mass spectrometry (ESI-Q-TOF-MS) (Synapt G2Si, Waters) using home-made gold-plated ESI emitters. The samples were prepared in 20µM concentration of protein and ligand using 150 mM ammonium acetate pH 7.4. The TWIMS–MS, enabled separation of ions based on their gas-phase mobility. The CCS values were determined for observed charge states using established calibration procedures.

## Results

The Lcn1 and Lcn2 were observed to bind Ent, forming stable complexes. The CCS values determined based on TWIMS–MS experiments for the binary Lcn1–Ent complex were calculated as 1655 ± 08 Å² ($7^+$) and 1656 ± 23 Å² ($8^+$), while for ternary complex of Lcn-1-ferriic-Ent the CCS values were measured as 1675 ± 13 Å² ($8^+$) and 1702 ± 25 Å² ($9^+$). For binary Lcn2–Ent complex, CCS values of 1802 ± 11 Å² ($8^+$) and 1824 ± 23 Å² ($9^+$) were obtained. We observed the partial interaction of Lcn1–Ent complex while the Lcn2 showed complete intercalation of Ent. The comparative ion mobility analysis revealed differences in drift time distributions and conformational behaviour between Lcn1 and Lcn2 complexes, indicating variations in siderophore recognition and metal-dependent binding interactions.

## Conclusion

TWIMS–MS provides a robust platform for resolving and comparing structurally related protein–siderophore complexes. The observed differences in CCS values and ion mobility profiles highlight subtle structural and binding variations between Lcn1 and Lcn2, contributing to a deeper understanding of siderophore recognition and metal-mediated host–pathogen interactions.


## Acknowledgement

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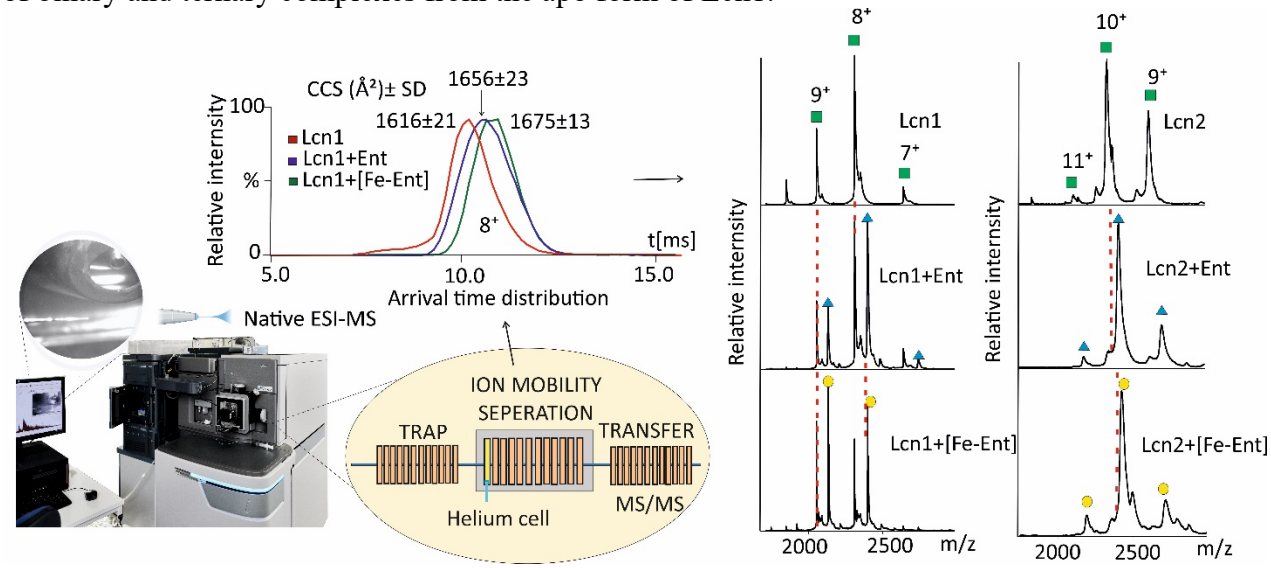
**Figure 1.** Native ESI-MS spectra of Lcn1 and Lcn2 binding with Ent. The ion mobility separation of binary and ternary complexes from the apo form of Lcn1.



# Comprehensive Evaluation of Melanin from Black Barley: Isolation, HPLC-based Quantification, and a Feeding Experiment in Rats

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## Introduction

Cereals represent a significant source of bioactive compounds, including pigments such as anthocyanins, carotenoids, and melanins, which may exhibit antioxidant effects and contribute to the modulation of metabolic and inflammatory processes. Among these, melanin has recently attracted attention due to its potential roles in plant defence, oxidative stress mitigation, and photoprotection. In this study, melanin was successfully extracted and characterized from the pigmented barley (*Hordeum vulgare* subsp. *distichon* (L.) Körn. convar. *nudum* (L.) A. Trof. var. *nudimelanocrithum*) which was specifically bred to enhance pigment content and nutritional quality. For quantitative analysis, a robust high-performance liquid chromatography (HPLC) method was developed and validated using a YMC Triart DIOL HILIC column.

While the influence of some of plant bioactive compounds on cytochrome P450 enzymes (CYP), key enzymes involved in drug metabolism, has been described, the potential interaction of melanins with these processes remains unclear. To evaluate CYP activity in a feeding experiment with laboratory rats, we used established protocols with subsequent HPLC detection.

## Experimental

An extraction protocol involving sequential alkaline and acidic hydrolysis was developed for the isolation of melanin from the plant matrix. The extracted melanin exhibited solubility only under alkaline conditions (pH > 8) and high stability against extreme pH values and thermal stress. For its quantitative analysis, a HPLC method using a YMC Triart DIOL HILIC column was developed and validated. This analytical approach was followed by a fourteen-week feeding experiment conducted on male Wistar albino rats divided into four groups (n = 10). The groups were differentiated according to the barley variety included in the diet: the conventional variety Caesar and the melanin-rich variety Nudimelanocrithon. Each group was further divided into a control variant (CT) and a high-fat diet variant (HFD), with barley constituting 60% of the feed ration. A microsomal fraction was isolated from the liver to determine the activity of selected CYP enzymes involved in human drug metabolism, which were analysed using established HPLC methods.

**Results**
The method developed for the determination of isolated melanin demonstrated linearity within the concentration range of 0.0391–0.6250 mg/mL ($R^2$ = 0.9989), with relative errors not exceeding ±15%. Accuracy, evaluated based on recovery, ranged from 91.9% to 105.7%, and precision (%RSD) was below 5% for all validated concentrations. Among the evaluated enzymes, CYP1A1/2, for example, exhibited significantly lower activity as a result of both obesity and administration of black barley.

**Conclusion**
The analytical method described here allowed us to get reliable results of quantitation of content of melanin in biological samples. The method developed was a part of a comprehensive approach to the extraction, reliable quantification, characterization, and biological evaluation of plant-derived melanin in cereal crops, supporting its potential applications in the food, cosmetic, and pharmaceutical industries.
For these applications, it has been important to show that the barley with a high melanin content may modulate CYP enzyme activity without significantly affecting the overall antioxidant capacity of the organism. These findings are important for assessing the safety and functional potential of melanin-enriched products.

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# HPLC-UV determination of quercetin and its glycosides in onion: effect of biotic stress

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## Introduction

Onion (*Allium cepa* L.) is one of the most widely cultivated vegetables worldwide, with production increasing significantly in recent years [1]. It is valued not only for its sensory properties but also for its biologically active compounds, particularly flavonoids such as quercetin and its derivatives, which account for up to 80–95% of total flavonols in onions [2].

Despite its benefits, large-scale onion production generates substantial amounts of waste, including peels, outer scales, roots, and substandard bulbs [3]. This waste is unsuitable for feed or fertilizer, partly due to the risk of spreading pathogens such as *Sclerotium cepivorum* [4]. However, onion waste represents a valuable source of bioactive compounds, including flavonoids, fiber, and fructans, with potential applications in the food, pharmaceutical, and cosmetic industries. Most current research remains at the laboratory scale and requires further development for industrial implementation [5].

In this context, deep eutectic solvents (DES) have emerged as a promising alternative to conventional organic solvents due to their low cost, simple preparation, tunable properties, and biodegradability [6]. DES are typically composed of a hydrogen bond acceptor (HBA), such as a quaternary ammonium salt, and a hydrogen bond donor (HBD), such as a sugar, whose interactions reduce the melting point and form a liquid eutectic mixture. Natural deep eutectic solvents (NADES) are particularly attractive because of their ability to dissolve poorly water-soluble compounds and their biocompatibility, making them suitable for green extraction processes [7]. Studies have demonstrated their higher efficiency in extracting phenols and flavonoids compared to conventional solvents [8].

However, onion cultivation is also affected by various pests and diseases, including the onion leaf miner (*Phytomyza gymnostoma*), onion weevil (*Ceutorhynchus suturalis*), and viral infections such as Onion yellow dwarf virus or Leek yellow stripe virus. These factors can cause plant damage, yield losses, and may also influence the content and composition of flavonoids, either directly or through the use of chemical treatments.

## Experimental

The onion varieties Stuttgartská, Sturon and Všetana were selected for the analysis. Plants in the experimental field were naturally exposed to pests and viral infection. When signs of attack were visible, the plot was treated by azoxystrobin and deltamethrin (June 14$^{th}$, 2022), effectively removing all contamination. 0.05 g of homogenized sample (OSW or onion) was extracted with 10 ml of NADES Betaine - Lactic acid. Betaine – lactic acid NADES (1:2) was prepared by mixing 19.7 g of betaine (Be) with 35.6 g of 0.85% lactic acid (LA) and 44.7 g of $H_2O$. Extractions were carried out in a laboratory sonic tank at 42 kHz and laboratory temperature for 30 minutes. The extracts were centrifuged and major flavonoids (quercetin 3,4'-diglucoside, spiraeoside and quercetin) and protocatechuic acid were identified and quantified by high-performance liquid chromatography with UV detection.

## Results

**Table 1.** The content of quercetin, quercetin glucoside and quercetin diglucoside [mg/kg]

| **Všetana** | QDG | QG | Q |
|---|---|---|---|
| Healthy | 5504.26 | 27111.67 | 24131.69 |
| Virus | 6643.47 | 25599.28 | 24808.88 |
| Pests | 6617.38 | 30115.10 | 19096.51 |

| **Stuttgartská** | QDG | QG | Q |
|---|---|---|---|
| Healthy | 5785.95 | 25338.81 | 17250.58 |
| Virus | 5741.55 | 23475.15 | 16407.26 |
| Pests | 6993.71 | 26632.88 | 17194.66 |

| **Sturon** | QDG | QG | Q |
|---|---|---|---|
| Healthy | 5141.98 | 21096.76 | 16614.49 |
| Virus | 5816.07 | 26490.83 | 21294.76 |
| Pests | 5675.51 | 22272.85 | 19214.84 |

| **Karmen** | QDG | QG | Q |
|---|---|---|---|
| Healthy | 5165.01 | 17294.98 | 17573.94 |
| Virus | 5372.14 | 17582.24 | 18077.13 |
| Pests | 4406.64 | 18253.08 | 21453.49 |

## Conclusion

Plant protection treatment shifted the analyte ratio strongly to quercetin glucoside in all samples, including plants not showing any signs of biotic stress. Under normal conditions, quercetin is the most contained analyte, details are given in [9]. The results further vary according to biotic stress factors, further increasing the amount of quercetin diglucoside, except for the red variety Karmen, where production of quercetin was stimulated by pests.

## Acknowledgements

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# LC-UV analysis of salbutamol sulfate in an *in-vitro* model of human respiratory tract

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## Introduction

Salbutamol, a short-acting brochodilatator, is commonly prescribed for various respiratory diseases, asthma and exercise-introduced bronchospasm. [1] It acts as an agonist at $\beta_2$ adrenergic receptors, which are located on the smooth muscle of the airways. [2] Salbutamol is currently available in various dosage forms such as inhalation dosage forms, syrups and extended-release tablets. [3] Inhaled dosage forms are the most popular, effective and gentle form of administration for these pulmonary diseases.[4] The main advantage of inhalation route of administration is that the drug is delivered directly to the respiratory tract, which is acompained by other benefits, such as: a low dose of the applied drug (in micrograms), rapid onset of action, elimination of side effects and the effect of the first pass through the liver. [5] Inhalation technique errors are often a major problem in administering inhaled medications. [6]

The aim of this work was to determine the amount of salbutamol sulfate drug in an inhaled dosage form retained in individual parts of a pharmacopoeial apparatus, which represent a human pulmonary tract. We have designed an HPLC method for the determination of salbutamol sulfate in samples obtained after using a pMDI inhaler (Ventolin® Inhaler N).

## Experimental

The nebulization test was performed using a pharmacopoeial apparatus Impactor Glass Twin Impinger (Copley Scientific Limited, Colwick, Nottingham, UK), which represent an in-vitro model of human respiratory tract. Physiologic inspiration was simulated by a vacuum pump LCP5 (Copley Scientific Limited, Colwick, Nottingham, UK) with an air flow rate 60 ± 0,5 L/min controled by a DFM4 flow meter (Copley Scientific Limited, Colwick, Nottingham, UK). Quantitative analysis samples taken from the inhalaer and three parts of the apparatus was performed using RP- HPLC method.

The HPLC method was performed on a Dionex UltiMate 3000 Series UHPLC System (Thermo Fisher Scientific, Waltham, MA, USA). Chromatographic data were acquired using Chromeleon® version 7 software (Thermo Fisher Scientific, Waltham, MA, USA). Symmetry C18 column (4.6 mm i.d x 250 mm, 5µm particle size ) (Waters Corporation, Milford, MA, USA) was used as stationary phase. The obtained data were processed in Microsoft Excel. Mobil phase consisted of methanol and aqueous solution of ortho-phosphoric acid (7.3 mM) in the ratio of 70:30 (V/V) with pH 3,4. The elution was isocratic with a flow rate 1 mL/min. The injection volume was 20 µl. The column temperature was maintained at 40°C. The wavelength of detection was set to 229 nm.

## Results

The HPLC method optimization process involves investigation of varying mobile phase compositions and different detection wavelengths on the separation and detection procedure of the method.

The mobile phase optimization process was carried out in two steps. In the first step, a different methanol/water ratio was tested. In the second step, an aqueous solution of ortho-phosphoric acid was used. This aqueous solution was mixed with methanol in different ratios. The addition of ortho-phosphoric acid improved separation efficiency (number of theoretical plates), peak symmetry and peak resolution in comparison with pure water. In comparison of all analyzed parameters, the mobile phase consisting of methanol and aqueous solution of ortho-phosphoric acid in the ratio of 70:30 was selected. Optimization of the detection wavelength involved measuring of UV spectra of salbutamol sulfate standard and a salbutamol solution obtained from a pharmacopoeia device. The detection wavelength of 229 nm was chosen based on the absorption spectra. Finally, the method was applied to determine the content of salbutamol sulfate in each compartment of the device after nebulization procedure.

## Conclusion

The study presents that the largest amount of salbutamol sulfate drug is delivered to the lower impingement chamber, which represent lower respiratory tract and lungs (the main site of action). The obtained results create a good basis for study of traditional and innovative drugs administered by inhalation from diffferent types of inhalers. The developed methods could also be used in clinical practice to verify the correctness of inhalation technique.

## Acknowledgements

This work was supported by the Slovak Research and Development Agency under Contract no. APVV-23-0508.

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# A new screening method for the determination of phytosterols in vegetable oils

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## Introduction

Vegetable oils are a valuable source of nutrients and essential fats in human nutrition. Phytosterols (plant sterols) are naturally occurring compounds present in various vegetable oils, with their concentration and composition depending on the type of oil. They exhibit a number of beneficial biological effects, including anti-inflammatory and anticancer properties. Despite their relatively low content in plant foods, they have significant health-promoting potential. Therefore, qualitative and quantitative analysis of phytosterols in vegetable oils is essential. According to the literature, more than 200 different phytosterols have been identified in various plant species to date. Analysis of phytosterols in vegetable oils requires careful sample pretreatment. Sterols are most often determined by chromatographic methods after isolation of the so-called unsaponifiable fraction of the oil. The main phytosterols typically determined include β-sitosterol, campesterol, stigmasterol, and brassicasterol. However, previously published methods involve relatively complex and time-consuming saponification and subsequent purification steps prior to analysis.

The aim of this work was to develop and test a new analytical method based on gas chromatography separation with MS detection for the screening analysis of essential phytosterols in vegetable oils without the need for saponification and sample purification.

## Experimental

All analyzed samples were purchased from local food stores in Slovakia.

Two sample preparation procedures were used for the analysis. The first procedure consisted of dissolving 20 mg of the oil sample in 1 ml of chloroform. The second procedure consisted of dissolving 20 mg of the sample in 1 ml of a mixture of acetonitrile and hexamethyldisilazane (1:2, v/v), with the addition of 2 µl of trifluoroacetic acid as a derivatization catalyst, followed by 15 min derivatization at 50°C in a thermoshaker. Sample preparation was performed in 2 ml vials, which were directly used for measurement.

Analyses were performed on an Agilent GC-MS 6890N-5973 system (Agilent Technologies, Palo Alto, CA, USA). A DB-5MS capillary column (5% phenyl-methylpolysiloxane, 30 m x 0.25 mm internal diameter, 0.25 µm film thickness) from Agilent Technologies was used. The optimized temperature program was: initial temperature 150 °C (1 min), ramped to 320 °C at a rate of 15 °C/min. The injector was heated to 300 °C and operated in split mode (split ratio 1:10). Helium was used as the carrier gas at constant flow rate of 1,9 ml/min. Mass spectrometric was performed using electron ionization (70 eV) in full scan mode (m/z range 50–500). Compounds were identified by comparing their mass spectra with the NIST 05 library, literature data, and linear retention indices.

**Results**

The developed method was used to determine phytosterols in vegetable oil samples (rapeseed, sunflower, corn, palm, and linseed oil) prepared using both procedures. Both approaches are suitable for screening analysis of sterols. Procedures 2 (with derivatization) resulted in improved chromatographic performance, including narrower peaks (reduced tailing) and a higher response on the MS detector due to the formation of the trimethylsilyl derivatives.

The phytosterol content in the analyzed samples was within one order of magnitude, which is consistent with published data. The method showed good repeatability (RSD of determined content < 5%), low detection limits, and short analysis time (up to 15 minutes, Fig. 1). The solvent consumption is only 1 ml per sample, making the method environmentally acceptable according to the principles of green analytical chemistry.

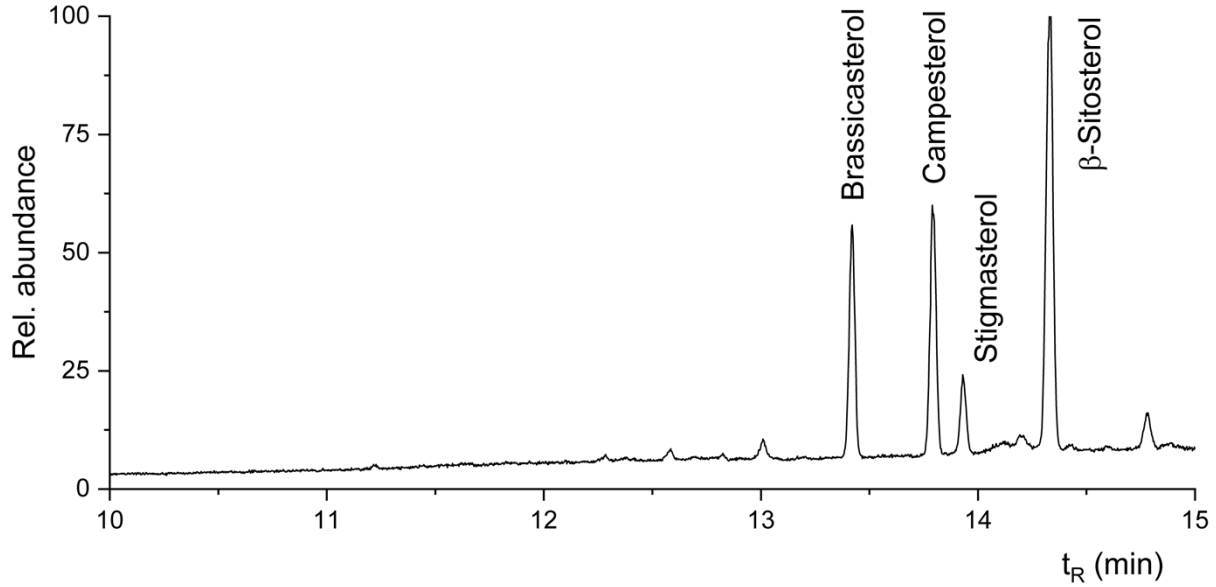
**Conclusion**

A simple and rapid method for the screening determination of phytosterols in vegetable oils was developed and tested. The method does not require time-consuming saponification or sample purification and is based on direct dissolution of the oil samples (with or without online derivatization) followed by GC-MS analysis. Both tested sample preparation procedures are applicable; however, derivatization (procedure 2) provides better chromatographic performance and higher sensitivity. The proposed method is fast (analysis < 15 min), reproducible (RSD < 5%), with low solvent consumption (1 ml per sample) and complies with the principles of green analytical chemistry. It is suitable for rapid screening of the content of major phytosterols in vegetable oils.

**Acknowledgements**

This work was supported by the Slovak Research and Development Agency under contract numbers APVV-21-0323 and APVV-23-0234

**Figure 1.** GC-MS-SIM chromatogram of rapseed oil prepared by procedure 2.



# The application of molecular CD spectroscopy for the chiral analysis of amino acids and oligopeptides

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## Introduction

Molecular spectroscopy using circular dichroism (CD) phenomenon is widely used analytical method for physico-chemical or structural study and mainly for analysis of optical active substances [1]. Its main advantage is the ability to distinguish among individual enantiomers, which is not possible by molecular absorption or fluorescence spectroscopy [1].

Amino acids (AAs) contain a carboxyl group and an amino group in their molecular structure and this configuration allows amino acids to act as bidentate ligands for metal ion complexation. In addition, if these atoms are also present in the side chain, they can also bind metal ions as tri- or tetradentate ligands). The formation of ternary metal complexes of the type $[M(AA)(L)]^{2+}$ is possible in case, when L is another bidentate ligand, e.g. 1,10-phenanthroline (*phen*), 2,2'bipyridine (*bipy*), ethylenediamine (*en*). In the case of Cu(II) and Pd(II) ions, it is advantageous to study the square-planar complexes which exhibit high stability. The same types of ternary complexes are formed with amino acids present in peptides [2], [3]. In this work, the physicochemical and spectral properties of ternary complexes of selected amino acids were studied using molecular CD, absorption, and fluorescence spectroscopy for their potential qualitative and quantitative analysis.

## Experimental

The spectroscopic measurements were performed on the following instruments: a Jasco J-810 CD spectropolarimeter (Jasco, Japan) and a Lumina fluorescence spectrometer (Thermo Scientific, USA). The measured molecular spectra were smoothed using the Savitzky-Golay filter. All chemicals used (tryptophan (Trp), histidine (His), methionine (Met), glutathione (GSH), $CuSO_4$, $PdCl_2$, diamines (DA): phen, bipy, en) were of the highest purity. The solutions of $[M(phen)]^{2+}$ a $[M(bipy)]^{2+}$ complexes were mixed with AAs or oligopeptides in slightly basic environment (pH 8.0) and at room temperature ($t$ = 25 °C) and their absorption, fluorescence and CD spectra were recorded. These spectra were analyzed and used for calculation of stability constants of ternary complexes and quantitative analysis.

## Results

Tryptophan was chosen as a model amino acid for studying the formation of ternary $[Cu(DA)(AA)]^{2+}$ complexes because it exhibits suitable spectroscopic properties. The interaction of the $[Cu(DA)]^{+}$ complex with D- and L forms of Trp in aqueous solution was studied using molecular CD and absorption spectroscopy in UV region. The formation of ternary metal complexes of 1:1 stoichiometry having log $K > 5$ was proved:

$$[Cu(DA)]^{2+} + Trp \Leftrightarrow [Cu(DA)(Trp)]^{2+}$$

This value ensures that the ternary chiral complex is formed quantitatively in excess of one of the components (Trp, Cu(II) complex). Since Trp exhibits the highest native luminescence of all

natural amino acids, it was tested whether the ternary complex $[Cu(DA)(Trp)]^{2+}$ would exhibit any luminescence. The Cu(II) ion present in its ternary complex exhibits a quenching effect. The value log $K \sim 5$ calculated on the base of the luminescence data, which is consistent with previous results. In addition, the molecular CD spectra measured for Cu(II) ternary complexes in the UV and VIS region can be utilized for the analytical determination of both L- and D- chiral forms Trp under optimal experimental conditions (pH ~ 8.0, $c_{Cu(DA)}$ / $c_{AA} > 2.5$) whereas molecular absorption and luminescence spectroscopy methods can be used to determine the total Trp content. Analogously, the interaction of the $[Cu(DA)]^{2+}$ complex with other amino acids (His, Met, GSH) was studied, and the stability constants for ternary complexes were calculated from the spectroscopic data. On the contrary, the Pd(II) ion forms more stable metal complex species than Cu(II) ion in their ternary complexes, likely due to the increased affinity of the Pd(II) ion for the donor sulfur and nitrogen atoms present in Met and GSH.

## Conclusion

In this study, the formation of ternary Cu(II) and Pd(II) complexes with selected amino acids was investigated by molecular circular dichroism (CD), absorption, and luminescence spectroscopy. It was demonstrated that the metal ternary complexes of 1:1 stoichiometry are formed, for which physico-chemical parameters were calculated. These results were used to optimize experimental conditions for the quantitative analysis of optical isomers of selected amino acids. This work may find application in the quantitative analysis of chiral amino acids in their multicomponent mixtures, particularly when combining the use of various metal complexes and experimental techniques of molecular spectroscopy.

## Acknowledgements

The study was supported by Masaryk University (project MUNI/A/1682/2025) and by EU COST (NECTAR, LUCES).

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# A novel HPLC method with fluorescence detection for the determination of ozanimod in mouse plasma and its application in a pharmacokinetic study

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## Introduction

Ozanimod is a sphingosine-1-phosphate receptor modulator approved for the treatment of multiple sclerosis and ulcerative colitis, whose hepatic metabolism is predominantly mediated by cytochromes P450 (CYP) [1]. Despite its increasing clinical use, no validated analytical method for the determination of ozanimod in plasma had been published prior to this work, representing a significant gap for both pharmacokinetic research and drug monitoring. The ketogenic diet (KD) – a high-fat, carbohydrate-restricted nutritional intervention that shifts the body's primary energy source from glucose to ketone bodies – is increasingly considered as an adjunct strategy in the management of multiple sclerosis, partly due to the neuroprotective effects of β-hydroxybutyrate [2]. The concurrent use of ozanimod and KD in multiple sclerosis patients therefore represents a clinically relevant scenario in which diet-induced modulation of hepatic CYP enzymes could potentially alter drug pharmacokinetics. However, the impact of the KD on CYP-mediated drug metabolism in vivo remains poorly understood and has not previously been investigated for ozanimod.

## Experimental

A fluorescence-based HPLC method was developed for the quantification of ozanimod in mouse plasma [3]. Sample preparation involved protein precipitation using acetonitrile with 0.1% HCl, centrifugation (12,000 g, 10 min), evaporation under nitrogen at 40 °C, and reconstitution in 100 µL of mobile phase; nabumetone served as the internal standard. Chromatographic separation was performed on a Chromolith HighResolution RP-18e monolithic column (100 × 4.6 mm; Merck) with an isocratic mobile phase consisting of 16 mmol/L sodium acetate solution (pH 4.7) and acetonitrile (1.7/1; v/v). Ozanimod and nabumetone were detected by fluorescent detection (with 278 nm excitation and 383 nm emission) at 8 and 18 min, respectively. The method was validated according to FDA guidelines. Using this validated method, the pharmacokinetic profile of ozanimod was subsequently determined in mice fed either a control or ketogenic diet for 28 days. Hepatic CYP mRNA expression levels and enzyme activities were measured in parallel.

## Results

Method validation demonstrated excellent linearity over the range 5–500 ng/mL ($R^2$ = 0.9996), with LOQ of 10 ng/mL, inter-day precision (RSD) below 6.6%, and mean recovery from spiked plasma samples of 75–89%. The method was subsequently applied to a pharmacokinetic study: KD-fed animals showed a 17% increase in the area under the plasma concentration–time curve (AUC) of ozanimod compared to controls. These pharmacokinetic changes were accompanied by significant alterations in hepatic CYP enzyme activities: CYP1A activity increased by 41% and CYP3A activity decreased by 47%, consistent with changes observed at the mRNA level.

## Conclusion

A new validated HPLC-fluorescence method was developed and applied to assess the pharmacokinetic profile of ozanimod in mice under dietary modulation. The ketogenic diet resulted in a moderate increase in systemic ozanimod exposure, accompanied by changes in hepatic CYP enzyme activities and mRNA expression. These findings suggest that the KD has the potential to influence CYP-mediated drug metabolism and may be worth considering in patients combining this dietary regimen with pharmacotherapy.

## Acknowledgements

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# Fractionation and characterization of humic acids by a combination of stepwise and linear gradient elution in RP-HPLC

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## Introduction

Humic substances (HSs) are ubiquitous natural materials occurring in huge amounts in soils, sediments and waters as a product of the chemical and biological transformation of animal and plant residues [1]. HSs are also complex mixture of amorphous, yellow to black colored, hydrophilic, polyelectrolyte-like, polydisperse macromolecules and probably not their two molecules are identical. With respect to their supramolecular structure, there is still a lot of controversy about their nature. Reversed-phase high performance liquid chromatography (RP-HPLC) as prevailing chromatographic method has achieved in the field of analysis and characterization of HSs only very limited application. In most cases authors used conventional organic solvents (methanol, acetonitril) typical for RP-HPLC separation of small molecules together with weakly pH buffered aqueous part of mobile phase. However, these mobile phases did result in moderate satisfaction with HS samples [2,3].

In this work, RP-HPLC using combination of step-wises and linear gradient of dimethylformamide (DMF) in buffered aqueous mobile phase and a wide-pore (30 nm) octadecylsilica column was evaluated for purpose of characterization of RP-HPLC fractionated humic acids [4,5].

## Experimental

Fractionation and characterization of HSs was carried out by the HPLC system consisting of pump provided by a quarternary low-pressure gradient, autosampler L-720, LiChrospher ODS WP 300 RP-18 (250 x 4; 4 x 4) mm column and guar column, column oven, diode-array detector and fluorescence detector.

Stepwise gradient program was set from 0.0 to 3.6 min isocratic 0% (v/v) B (99% DMF / 1% phosphate buffer; c = 5mM, pH = 3.00) in A (1% DMF / 99% phosphate buffer; c = 5mM, pH = 3.00), and from 3.7 min, every 4 min there was isocratic step added increasing content of B in A by 10% (v/v) up to the last step increased by 9% (v/v) ending in 99% (v/v) B in A, maintained till 55.0 min isocratic 99% (v/v) B in A, from 55.1 min to 60.0 min linear decrease from 99% (v/v) B in A to 0% (v/v) B in A and between runs 10 min re-equilibration was maintained [5,6].

Linear gradient program was set from 0.0 to 5.0 min isocratic 0% (v/v) B in A, from 5.1 to 35.0 min linear increase from 0% (v/v) B in A to 95% (v/v) B in A,from 35.1 to 40.0 min isocratic 95% (v/v) B in A, from 40.1 to 45.0 min linear decrease from 95% (v/v) B in A to 0% (v/v) B in A and between runs 10 min re-equilibration was maintained[4].

Two types of HAs were analysed in this work, the first one was commercially available standard of HSs from Sigma - Aldrich company (**Aldrich**) and the second one was extracted from soil of Dunajská Streda locality, Slovakia (**DS J**) and isolated by modified IHSS fractionation scheme [6].

## Results

In the first step the step-wises RP-HPLC method was used to obtain elution profiles and for fractionation (collecting all 11 fractions) of both samples. In the second step the step-wise gradient RP-HPLC method was applied for characterization of each fraction, which means re-injection all

collected fractions and also used linear gradient RP-HPLC method for re-injection these fractions to prove that each fraction stays the same even through re-injection, independently on type of used gradient (Fig. 1). The gradient method is predetermined for efficient combination with the other methods of HL characterization, preferably separation methods. Individual fractions obtained by the RP-HPLC method can be analyzed by separation methods acting on different and independent separation principles or mechanisms as e.g. size-exclusion chromatography (SEC), immobilized-metal affinity chromatography (IMAC) etc.

**Conclusion**

Obtained data indicate, that this mode of application of RP-HPLC system could be employed as a separation system for more detailed characterization of such complicated natural macromolecules as analysed HAs and obtain so more information about their attributes.

**Acknowledgements**

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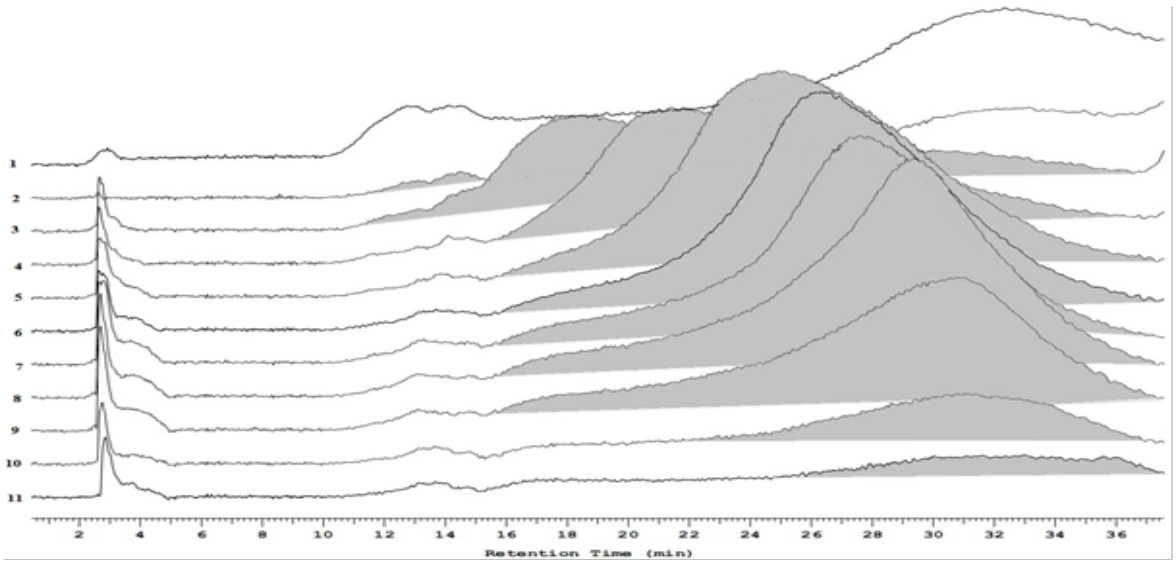
**Figure 1.** Background corrected RP-HPLC profiles of all collected fractions of DS J sample using linear gradient. FLD (ex. 470 nm, em. 530 nm).



# Online SPE-HPLC-MS/MS determination of urinary DINCH plasticizer metabolites for exposure assessment in Slovak adult population

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## Introduction

Plasticizer DINCH (1,2-cyclohexane dicarboxylic acid diisononyl ester; Hexamoll® DINCH) was introduced in 2002 and gradually replace phthalates used as plasticizers in production of plastic products, especially those coming into close contact with humans, mainly food packaging, toys and medicinal devices due to its more favorable toxicological profile [1]. However, in the case of high levels of exposure, hormonal changes, effects on kidney and liver function and on lipid metabolism were described. As biomarkers of exposure to DINCH, mainly metabolites hydroxy- (OH-MINCH) and carboxy- (cx-MINCH) monoesters are used [2,3]. The aim of this study was to determine the exposure level to DINCH plasticizer in the Slovak adult population based on the analysis of oxidized metabolites in urine using a combination of online SPE with an isotope dilution liquid chromatography and tandem mass spectrometry.

## Experimental

Urine samples were collected from 300 adults aged 20-60 years from three regions of Slovakia. Oxidized metabolites were analyzed after their deconjugation from glucuronide conjugates. 210 microliters of sample were injected into a C18 SPE column (20 mm × 4.6 mm, 3.5 μm) at isocratic elution with water : acetonitrile (90 :10) containing 0.05% acetic acid for 5 min, then the flow was switched to the analytical column. Triple quadrupole mass spectrometer with a heated electrospray ionization source and operated in negative mode was used for measurements. Monitored mass transition quantifiers for cx-MINCH, D8-cx-MINCH, OH-MINCH, and D8-OH-MINCH were 327.2→173.1, 335.2→173.1, 313.2→153.1, and 321.2→161.1, respectively. Concentrations were calculated from matrix linear calibration curves in the range of 0.12 to 60 μg/L.

## Results

The method was approved within the HBM4EU proficiency test scheme with Z-score $|Z| \leq 0.9$; the requirement for quality approval was the interval of $|Z| \leq 2$. Intraday and interday precisions were better than 4 % and 8 %, with limits of quantitation of 0.1 μg/L for cx-MINCH and 0.14 μg/L for OH-MINCH. Presented study provides very first results on exposure of Slovak adult population to DINCH plasticizer. The detection frequency of at least one metabolite was 100 %. For adults we found median values of DINCH metabolites lower than in children, but similar to published values for older adolescents [3]. No interregional differences were found within Slovakia. The recommended safe limit for the sum of OH-MINCH and cx-MINCH metabolites of 4.5 mg/L was not exceeded. The maximum concentration determined in examined urine samples reached 48 μg/L.

## Conclusion

It can be concluded that each participant was in contact with products containing DINCH. However, the level of exposure of the Slovak adult population to the plasticizer DINCH was found to be low and based on currently available scientific knowledge, no adverse impact on health is expected, and no actions are currently necessary. However, due to the rapidly increasing production and use of this substance, it can be assumed that the exposure of the population to DINCH will increase in the near future. In accordance with the precautionary principle, regular biomonitoring is therefore required.

## Acknowledgements

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# rDUVLAESCI-MS/MSI: a novel approach for direct analysis of biological surfaces and high-speed mass spectrometry imaging (MSI)

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## Introduction

Ambient mass spectrometry has significantly enhanced the insight into the molecular composition of complex biological samples under native conditions, requiring minimal sample preparation [1]. Advancements in laser-based designs, including UV and deep UV lasers, enabled non-destructive, low-fragmentation analysis of various solid samples and dried spots/layers while significantly improving lateral resolution [2] as well as acquisition speed for mass spectrometry imaging (MSI). The presented work introduces a novel, fully automated Remote Deep-Ultraviolet Laser Ablation coupled with Electrospray Ionization−Atmospheric Pressure Chemical Ionization (rDUVLAESCI) ionization source, which can be easily used for either spot analysis or MSI of a wide range of organic molecules with varying polarities and molecular weights.

## Experimental

The rDUVLAESCI benefits from the coupling of a 193 nm Analyte G2 laser ablation unit (Photon Machines) with a hybrid Q-TOF mass spectrometer Synapt G2S (Waters), enabling the simultaneous acquisition of complementary ESI and ESCI mass spectra in a single analytical run. Careful optimization and synchronization of both instruments were essential for successful MSI measurements. Laser ablation parameters, including spot size, ablation speed, laser frequency, and scan time of the mass spectrometer, were optimized to ensure proper pixel per second synchronization during imaging experiments. Additional optimization involved selection of the "sheath liquid" for the ionization source, evaluation of UV laser fluence, and preliminary quantitative experiments investigating the relationship between signal intensity and rat brain tissue thickness (12, 20, and 30 μm).

## Results

The rDUVLAESCI-MSI enabled acquisition rate up to 25 pixels/s in a single ionization mode imaging ESI or ESCI (see **Figure 1)** and 12,5 pixels/s in simultaneous dual ionization mode imaging. High quality 2D distribution maps of analytes present in thin mouse brain tissue sections (12-30 μm thick) were successfully displayed with unmatched speed. Among the detected molecules, which are the majority substances in brain tissue, were cholesterol and its derivatives (dehydrocholesterol, oxocholesterol, and desmosterol), free fatty acids (palmitic acid, stearic acid), sphingosine, diacylglycerols (mainly dipalmitoyl glycerol), phospholipids, ceramides and adenine. The promising quantitative potential of rDUVLAESCI-MS has been shown on the linear responses for the brain tissue section with increasing thickness from 12 to 30 μm.

## Conclusion

The developed rDUVLAESCI-MS/MSI method represents a fast and versatile approach for direct analysis and MSI of biological surfaces under ambient conditions. The successful highspeed MSI of various lipid-related compounds and small biomolecules in mouse brain tissue demonstrates the ability of rDUVLAESCI-MSI for rapid and detailed molecular imaging of complex biological samples.

**Acknowledgements**
The research was funded by Internal Grant Agency, Palacký University Olomouc (IGA_PrF_2026_024).

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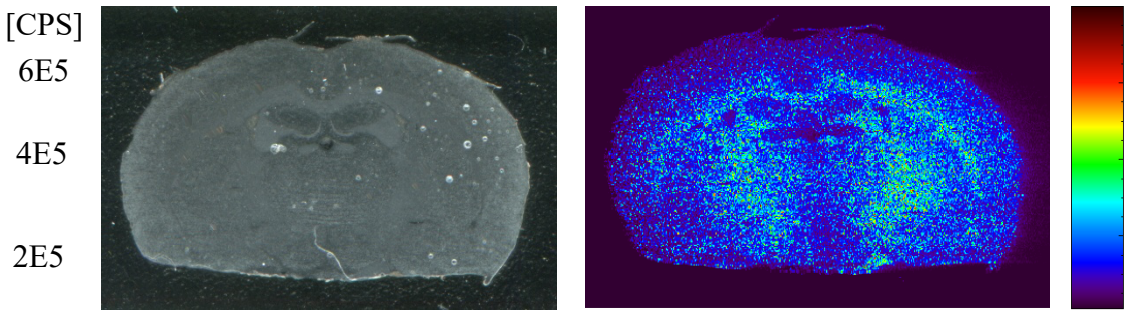
**Figure 1.** 25 pixels/s imaging of brain tissue in ESCI+ ionization mode



# Ion Mobility – Mass Spectrometry in Investigation of Nitrile-Imine Cross-Linking Reactions in Gas-Phase Peptide Ions

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## Introduction

Diaryltetrazole-peptide conjugates (XAAA-tet-K ) containing N-terminal amino acid residues – phenylalanine (F), tyrosine (Y), 3-nitro-tyrosine (3-$NO_2$-Y), tryptophan (W), serine (S) or threonine (T) – and a lysine linked to a 4-(2-phenyl 2H-tetrazol-5-yl)benzyl group at C-terminus were investigated. Protonated molecules $[M+H]^+$ of these peptides may undergo the loss of $N_2$ from the tetrazole ring (Fig. 1) via collision-induced dissociation (CID) or photodissociation (UVPD). Both precursor ions and fragment ions ($[M + H]^+$ and $[M + H – N_2]^+$) were analysed by cyclic ion mobility mass spectrometry. Experimental collision cross sections ($CCS_{exp}$) were compared with $CCS_{calc}$ values calculated by Mobcal (ion trajectory method) for structures obtained by density functional theory for low-Gibbs-energy conformers [1]. Ion mobility contribution to revealing gas-phase ion structures is presented [2,3].

## Experimental

Analyses were carried out on a SELECT SERIES Cyclic Ion Mobility Spectrometer (c-IMS) (Waters Corp., Wilmslow, U.K.) using direct infusion (5 µl/min) with an electrospray ion source in positive mode. Each sample was dissolved and diluted to a working concentration of 1 µg/ml in ACN/$H_2O$ (50/50, v/v) with 1% acetic acid and measured six times. Precursor ions were fragmented in the trap at 42 V and 35 V for conjugates with aromatic and non-aromatic amino acids, respectively. Poly-DL-alanine (2 µg/ml) in ACN/$H_2O$ (50/50, v/v) with 0.1% formic acid was used for CCS calibration.

## Results

In the mobilograms, the precursor ions $[M + H]^+$ provided one major symmetrical Gaussian peak accounting for a relative intensity of 98–99%, which was accompanied by none, one, or two minor peaks with relative intensities up to 2%. The $CCS_{exp}$ values matched with the $CCS_{calc}$ values for the lowest Gibbs energy structures, with a few exceptions. For $[YAAA\text{-}tet\text{-}K + H]^+$, the best fit ($CCS_{exp}$ = 271.6 Å$^2$) was obtained for the structure with a relative Gibbs energy of 5 kJ·mol$^{-1}$ ($CCS_{calc}$ = 272.2 Å$^2$), whereas the structure with the lowest Gibbs energy displayed a significantly higher $CCS_{calc}$ of 284.8 Å$^2$. Specifically, this ruled out a possible hydrogen bond between the tyrosine hydroxyl group and the tetrazole group. In contrast, $[TAAA\text{-}tet\text{-}K + H]^+$ was the only ion that exhibited a hydrogen bond between the amide N-H group and the tetrazole group. Notably, the peptides with aromatic amino acid residues showed no π-π stacking with the diaryltetrazole aromatic rings. The loss of $N_2$ yielded ions ($[M + H – N_2]^+$) with lower $CCS_{exp}$ values in comparison to the respective precursor ions. This was consistent with more compact structures formed as a result of cross-link cyclization after $N_2$ elimination.

## Conclusion

Multiple pass ion mobility separation with high resolving power provided useful information for both the precursor ions $[M + H]^+$ and the $[M + H - N_2]^+$ ions, contributing to the differentiation of structures with very similar energies. In some cases, structures with slightly higher Gibbs energy were preferred in the gas phase over the lowest Gibbs energy isomers. This highlights the importance of experimental CCS determination. Together with collision-induced dissociation, UV photodissociation and quantum chemistry calculations, ion mobility contributed to revealing the gas-phase ion structures.


## Acknowledgements

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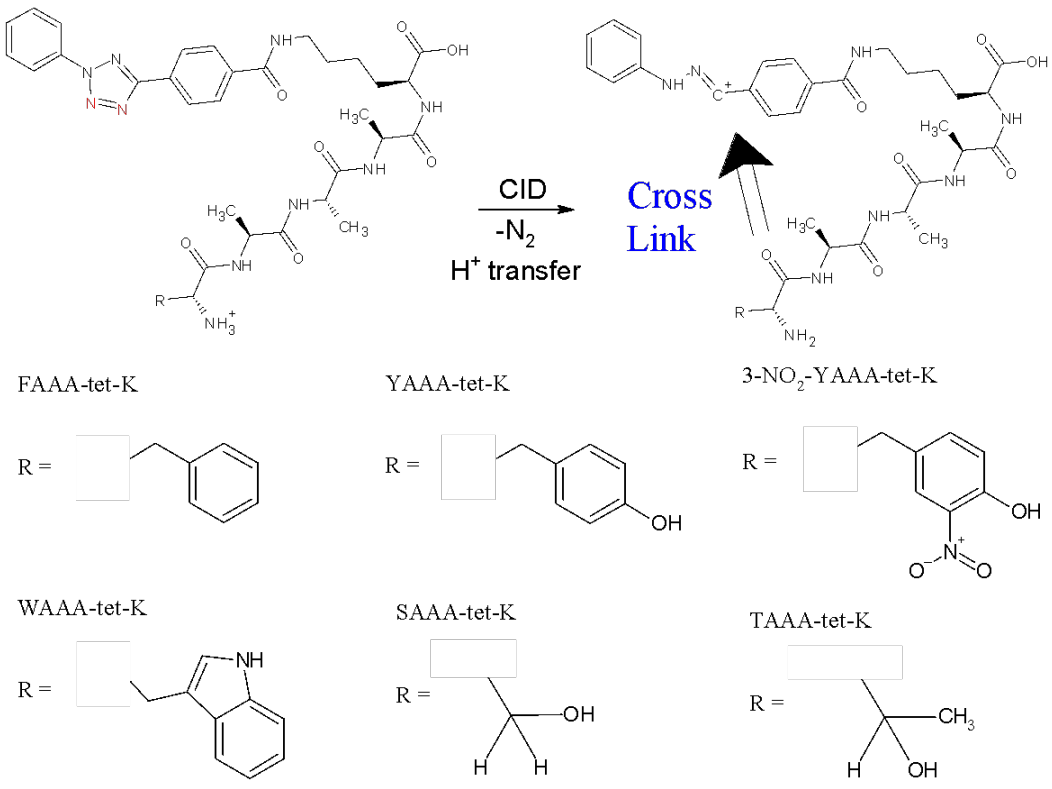
**Figure 1.** Structures of analyzed diaryltetrazole-peptide conjugates and loss of $N_2$ by CID



# Optimisation of monoclonal antibody analysis by CZE-MS and stability testing

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## Introduction

Therapeutic monoclonal antibodies (mAbs) require robust analytical methods to monitor structural heterogeneity and stability, as degradation and modifications may impact product quality and efficacy [1]. Stability testing in post-production handling of biopharmaceuticals gains popularity over last years. Quality control in post-manufacturing world is essential for administration of safe formulations, therapy cost-effectiveness and less drug product waste.

Furthermore, development of new analytical methods for improved mAb analysis is of high importance. Capillary zone electrophoresis–mass spectrometry (CZE-MS) offers a fast and efficient alternative for mAb analysis. In this study, a CZE-MS method is optimized using in-capillary pre-concentration and subsequently applied to the analysis of mAbs.

## Experimental

Samples for stability testing were prepared by simulating various post-production handling situations. In total, samples for 6 conditions at 4 time-points were prepared. MAb samples infliximab (IFX, standard) and rituximab (RTX, MabThera) were reduced at 37 °C for 1 hour by adding TCEP reducing agent at concentration 1 mM. Intact and reduced samples were diluted in ammonium acetate (AA) and ammonium bicarbonate (AB) and analysed by CZE using Agilent 7100 CE (Agilent, Santa Clara, US) system coupled to UV detection.

## Results

Method development focused on optimizing in-capillary pre-concentration using RTX. Testing various AA concentrations in combination with the 2 M acetic acid BGE revealed that 250 mM AA provided the optimal buffer for sample stacking, yielding the highest peak areas. An injection time of 250 s was identified as optimal for highest sensitivity. For dynamic pH junction, combination of 2 M acetic acid with 10 mM AB and 300 s injection time showed most optimal conditions. These parameters establish the foundation for current MSI optimization, with aim to analyse multiple sample segments in a single CZE-MS run.

Analytical methods are currently being applied to stability studies of therapeutic mAbs under simulated post-production conditions. Data acquisition is ongoing across multiple conditions and time points, with the aim of evaluating methods suitability for monitoring structural changes and/or degradation.

## Conclusion

An optimized CZE method with in-capillary pre-concentration was developed, providing improved sensitivity for mAb analysis. Stability testing is currently an ongoing work focused on use of multiple analytical techniques to assess effect of post-production handling on mAbs.

**Acknowledgements**
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# Water-based Randall extraction of bioactive compounds from plants

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## Introduction

In nature, bioactive compounds are synthesized within living organisms, such as plants. The complexity of these matrices makes extraction an essential step for the analysis of bioactive compounds. For such matrices, analytical method recovery is not as important as its selectivity. Method selectivity is dependent on selectivity of purification and selectivity of extraction, and those two parameters are highly dependent on the solvent. Nowadays, organic solvents, both novel and traditional, are predominantly used. Yet one solvent is often overlooked: water. Water is a selective, cheap and abundant solvent. Furthermore, properties of water and aqueous solutions can be easily modified. Acid-base equilibria in an aqueous medium can be leveraged to further increase selectivity for acidic and basic organic compounds. The polarity of water is strongly dependent on temperature and can be reduced to the level of certain organic solvents by heating, which would significantly increase solubility of mildly polar compounds. Certain properties can even be utilized to enhance purification process. Increase in ionic strength of solution will reduce solubility of organic compounds, which may increase efficiency of purification process. Moreover, purification of aqueous extracts is simplified by the immiscibility of water with many organic solvents, because liquid-liquid extractions are generally simple.

Randall extraction is a modification of a well-known Soxhlet extraction method. It can achieve full analyte recovery faster than conventional Soxhlet due to the use of boiling and rinsing steps. Extracts can then be concentrated during the recovery step for further analysis. Recovered solvent can be reused in future extractions thus lowering extraction cost and reducing solvent waste.

This study was focused on the use of water as an extraction solvent in Randall extraction. Boiling step of this extraction method allows extraction of mildly polar compounds with hot water. Dried flowers of chamomile (Matricaria chamomilla) were used as a sample for analysis. Procedure was applied for the determination of four bioactive compounds: chlorogenic acid, caffeic acid, quercetin and laricitrin. All these compounds possess properties that are beneficial to human health and can therefore be of interest to pharmaceutical and food industries. Water-based extraction was compared to the extraction using Acetone-Methanol mixture (AM).

## Experimental

For water-based extraction, one gram of sample was extracted with roughly 60 ml of water as described in Table 1. The extract was transferred to a 10 ml volumetric flask. Flask was then filled to the mark. Five milliliters of extract were centrifuged, and aliquot (2 ml) was taken from the supernatant. Aliquot was diluted with 2 ml of saturated NaCl solution and acidified with 100 µl of 1M $H_2SO_4$. Resulting solution was extracted with 1 ml of ethyl acetate (EtAc) and 1 ml of diethyl ether (DE). EtAc and DE extracts were combined and evaporated. Five hundred microliters of hexamethyldisilazane and 300 µl of pyridine were added to the residue, trifluoroacetic acid was added as a catalyst. Solution was analyzed by GC-MS after incubation (30 minutes) at laboratory temperature.

For AM extraction, one gram of sample was extracted with roughly 60 ml of AM (1:2) as described in Table 1. The extract was fully evaporated; 5 ml of acetone was added to the residue. Resulting

mixture was sonicated for 10 minutes. Solution was transferred to a 5 ml volumetric flask. Flask was then filled to the mark. Solution was centrifuged and aliquot (2 ml) was taken from supernatant. Aliquot was evaporated. Five hundred microliters of hexamethyldisilazane and 300 µl of pyridine were added to the residue, trifluoroacetic acid was added as a catalyst. Solution was analyzed by GC-MS after incubation (30 minutes) at laboratory temperature.

**Results**

Experiments demonstrated that aqueous and AM extracts contained significant amounts of different carbohydrates and other matrix compounds, which made chromatographic separation and analyte detection significantly harder. Simplistic yet efficient purification of aqueous extracts was made possible by unique properties of water. Purification was done by liquid-liquid extraction, which would be essentially impossible if AM was used as a solvent. Aqueous extract purification demonstrated higher analyte recovery than AM purification procedure.

During the purification of AM extract, the extract was exposed to elevated temperatures during the evaporation, which could lead to undesirable chemical reactions, such as Maillard reaction. These side reactions could lead to reduction in analyte recovery. In contrast, during the purification of the aqueous extract, the immiscibility of water with EtAc and DE allowed for a simple reextraction at laboratory temperature. The selectivity of this reextraction was further increased by shifting the medium to an acidic pH and recovery was increased by the addition of a saturated NaCl solution. While EtAc and DE extracts were evaporated as well, they contained significantly less carbohydrates, which could somewhat limit pyrolytic reactions. As a result, analyte peak areas were higher, with the peak areas of chlorogenic and caffeic acid being multiple times higher when compared to peak areas in purified AM extract.

**Conclusion**

In conclusion, Randall extraction with water is a viable extraction technique for the extraction of bioactive compounds from plants. Water can extract polar and mildly polar compounds with the same efficiency as organic solvents, AM in this case. Moreover, its properties allowed for simple and efficient purification procedure, while purification of AM extracts demonstrated lower analyte recovery. Looking forward, this method could be further enhanced by the introduction of advanced extraction techniques, such as microextraction.

**Acknowledgements**

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**Table 1.** Extraction parameters for Randall extraction with water and AM

| | AM extraction | | | Water extraction | | | |
|---|---|---|---|---|---|---|---|
| Step | Boiling | Rinsing | Recovery | Boiling | Rinsing | Recovery | Cooling |
| Time, min | 60 | 60 | 25 | 60 | 60 | 40 | 20 |
| Temperature, °C | 180 | 180 | 180 | 130 | 220 | 220 | - |

# Determination of CBD in Isolates, Drops, and Tinctures by a simple HPLC-UV method

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## Introduction

Cannabidiol (CBD) is a secondary metabolite belonging to a group of phytocannabinoids obtained from the plant *Cannabis sativa*. It is small, lipophilic non-psychoactive molecule, which has shown great therapeutic potential at molecular, cellular or organ level [1]. Most studies evaluating acute administration of CBD have concluded that it shows no potential for abuse. For this reasons, there are many formulations (ointments, creams, capsules, oil drops, tea, food etc.) that are sold through online platforms or retail stores whose effectiveness have not been verified [2]. However, it remains unclear whether the CBD content in these products corresponds to the amounts declared on their packaging.

## Experimental

Quantitative analysis of cannabidiol in various products was performed using high-performance liquid chromatography (HPLC). HPLC experiments were conducted using YOUNG IN Chromass (Anyang-si, Korea) chromatography system coupled with UV detector (λ=228 nm). Separation was performed on a 2.6 μm Kinetex® C18 column. Here, 20 mM ammonium formate with 0.1% formic acid (phase A) and acetonitrile (phase B) were used as mobile phase.

## Results

Working standard solution (100 μg/mL) was prepared by diluting the 1 mg/mL stock standard in methanol. Six calibration solutions (1, 5, 10, 20, 50, 100 μg/mL) were prepared by diluting the working standard solution in methanol to a final volume of 1 mL each, from which a six-point calibration curve was constructed. A solution with a concentration of 100 μg/mL was prepared from the isolate, which exhibited a high degree of purity and confirmed an elution time identical to that of the CBD standard. The drops, containing 10% cannabidiol, were labelled as containing 4 milligrams of CBD per drop. The drop was diluted with methanol in a 1:20 ratio and further prepared for analytical evaluation by diluting it in a 1:100 ratio. The total CBD content in the drops (after recalculation) was 937.10 ± 15.81 mg (9.37%), which is close to the value stated by the manufacturer. The tincture, prepared from an extract of hemp seeds and leaves, was diluted in a 1:40 ratio. The total CBD content in the tincture (after recalculation) was 2.84 ± 0.14 mg.

## Conclusions

The study demonstrates the use of high-performance liquid chromatography as an analytical tool for commercially available preparations. The tested formulations confirmed their purity and composition, which was also verified by the manufacturer. The presented results provide a suitable starting point for further studies focused on quality control of such commercial products.

**Acknowledgements**
This work was supported by the project VEGA 1/0302/24 and FaF/1/2026. Also, this work was supported by Konopná farma Liptov s.r.o., which provided us with its products for testing.

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# Electrochemical oxidation opens the aromatic ring of veratrole

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## Introduction

Veratrole (VER) is a naturally occurring catechol derivative. Its structural moiety can be found in plant metabolites and also in synthetic drugs. Generally, the oxidation of catechol and its derivatives in plants leads to the formation of *o*-quinone [1]. However, strong chemical or enzymatic oxidation can lead to the aromatic ring cleavage and the creation of muconic acid [2,3]. The possibility of electrochemical oxidative cleavage of catechol and its derivatives was studied in this work using VER as a model compound.

## Experimental

Electrolysis of veratrole (0.75 mmol $L^{-1}$) in Britton-Robinson buffer (BRB) at pH 9 with 50 % (v/v) of ACN was performed using the Autolab PGSTAT204 (Metrohm Autolab, The Netherlands) in three-electrode configuration: a large surface boron-doped diamond electrode (BDDE provided by Slovak Diamond Group, STU FEI, Slovakia), a carbon fibre brush electrode (CFBE) or a platinum net was used as a working electrode, Ag|AgCl (1M-KCl) was used as a reference electrode and an auxiliary platinum electrode was in separated cathode compartment filled with BRB at pH 9. Electrolysis was performed for either 1 or 3 hours. Cyclic voltammetry was performed in the same configuration as electrolysis using corresponding working electrodes (BDDE M-BDD-3 with disc diameter 3.0 mm from BioLogic, France; glassy carbon BASi MF-2012 electrode, GCE, with disc diameter 3.0 mm; or a platinum BASi MF-2013 with disc diameter 1.6 mm, both from Bioanalytical Systems, USA).

Samples after electrolysis were acidified and extracted with ethyl acetate. These extracts were analyzed using GC/MS either directly or after derivatization by silylation. GC/MS analysis was performed on the Agilent 7890A with 5975C inert MSD (Agilent Technologies, CA, USA) equipped with 30 m × 0.25 mm × 0.25 µm DB-5ms capillary column (Agilent Technologies, CA, USA). The temperature program settings were: 50 °C – 2 min – 10 °C/min – 300 °C – 15 min.

## Results

Cyclic voltammograms (CV) of VER recorded with BDDE showed one main four-electron oxidation peak at 1.33 V and one shoulder peak at 1.60 V, followed by two reduction peaks at -0.36 V and -0.68 V in the reverse scan and a new anodic peak at 0.86 V in the second anodic scan. This behavior implies, that electrochemical oxidation of VER produced reducible products, which were then re-oxidized in the second anodic scan. Comparison of CVs of VER and catechol confirmed that the peak at 0.86 V corresponds to the oxidation of catechol, while the reduction peak at -0.36 V appears at the same potential as reduction peak of *o*-benzoquinone (oxidation product of catechol). This observation suggests that catechol could be one of the products of VER anodic oxidation. On GCE, only one oxidation peak at 1.33 V was observed in the first scan, while in the reverse scan there was a small reduction peak (-0.25 V) forming redox couple with peak at -0.09 V in the second anodic scan. This pair of peaks differ from those of the catechol/benzoquinone pair suggesting that there are different products formed during VER oxidation on carbon electrode. On the Pt electrode, there was only one visible peak of VER oxidation at 1.35 V.

Controlled potential electrolysis of VER at potential of the limiting oxidation current followed by the GC/MS analysis brought new unexpected findings. After 1-hour electrolysis on BDDE, three peaks corresponding to dimethyl ester of muconic acid in different isomeric forms (*cis,cis-, cis,trans-* and *trans,trans-*) were identified beside a peak of dimethoxyphenol. The isomers of dimethyl muconate (dimethyl hexa-2,4-dienedioate, DMM) were more abundant than dimethoxyphenol. The analysis of silylated samples after VER electrolysis revealed two isomeric forms of methoxybenzene diol (most likely 2-methoxybenzene-1,4-diol and 3-methoxybenzene-1,2-diol) and also the presence of dimethoxyphenol and catechol. After 3-hour electrolysis these products were in higher abundance, except for the dimethoxyphenol, which had 8-times lower abundance. Based on these results, the electrochemical oxidation of VER on BDDE leads to the cleavage of the aromatic ring and the formation of DMM in different isomeric forms and dimethoxyphenol is probably an intermediate product of the VER electrolysis, which undergoes further transformation. The same products were found after electrolysis on CFBE and on platinum electrode, but with slightly different abundances.

## Conclusion

Electrolysis of VER followed by GC/MS analysis proved that the anodic oxidation induces the cleavage of the aromatic ring into a muconic acid dimethyl esters in a mixture of three geometric isomers. Catechol, dimethoxyphenol and methoxybenzene diol (two isomers) were identified as other electrolysis products. The same products were found using all three electrode materials (BDDE, CFBE and Pt) but in different quantitative ratio suggesting fine variations in the reaction mechanism on different electrode surfaces.

## Acknowledgements

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# A Robust Droplet Microfluidic Platform for High-Throughput Enzyme Kinetics and Inhibitor Screening Studies

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## Introduction

Characterization of enzyme kinetics and inhibitor screening represent pivotal steps in drug discovery and biotechnological development. Conventional bulk-phase assay formats, however, suffer from considerable drawbacks including excessive reagent consumption, limited experimental throughput, and poor control over reaction microenvironments. Droplet microfluidics offers a compelling alternative by encapsulating individual reactions within monodisperse, nanoliter-scale aqueous compartments suspended in an immiscible oil phase, whereby each droplet functions as an independent, isolated microreactor [1,2].

## Experimental

A droplet microfluidic platform was developed on a PDMS chip integrating four inlets, a serpentine passive mixing unit, and a 42-loop incubation channel, with fluorescence readout provided by laser-induced excitation and photomultiplier tube detection. The system employs on-line concentration modulation through real-time adjustment of individual syringe pump flow rates, enabling continuous and simultaneous variation of substrate, inhibitor, and enzyme concentrations within a single experimental run, eliminating the need for laborious off-line serial dilution protocols. Platform performance was validated with respect to droplet generation reproducibility, thermal stability across the range 21–43 °C, and equivalence between on-line and off-line dilution approaches.

## Results

The platform was employed to investigate the kinetic behavior of *Escherichia coli* β-galactosidase using the fluorogenic substrate fluorescein-di-β-D-galactopyranoside. Michaelis-Menten parameters $K_M$ and $V_{lim}$ were extracted by non-linear regression and were in good agreement with literature reference values [3]. The effect of enzyme concentration on reaction rate was systematically examined and confirmed the expected linear dependence within the tested range. Kinetic measurements were performed at two physiologically relevant temperatures (22 °C and 37 °C) yielding comparable kinetic parameters at both conditions, consistent with the expected behavior of this enzyme. Inhibition profiling with three competitive inhibitors: isopropyl-β-D-thiogalactopyranoside, caffeine, and D-galactose —provided $K_i$ and $IC_{50}$ values in good correspondence with literature-predicted data, with total aqueous phase consumption in sub-milliliter range per complete inhibitor series [4,5].

## Conclusion

The presented droplet microfluidic platform with integrated on-line concentration control constitutes a robust and reagent-efficient tool for comprehensive enzyme kinetic characterization at two relevant temperatures, including inhibitor profiling and systematic investigation of the effect of enzyme concentration. The system demonstrates clear translational potential for high-throughput applications in drug discovery pipelines.

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# Retention and Separation of 3-mer Phosphorothioate Oligonucleotides and Their Diastereomers in RPLC

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## Introduction

With increasing development and characterization of therapeutic oligonucleotides (ONs), the demand of chromatographic methods for analysis of oligonucleotides´ impurities and metabolites grows [1]. Phosphorothioate (PS) oligonucleotides represent the first generation of therapeutic antisense oligonucleotides [2]. Phosphorothioate modification introduces a chiral center to the molecule which leads to the formation of diastereomers. The ability of chromatographic techniques to resolve PS oligonucleotide diastereomers can be utilized to investigate for example the stereoselective metabolism of therapeutic ONs containing one to four PS linkages, i.e. two to sixteen diastereomers [3].

## Experimental

All chromatographic measurements were performed on the Waters Acquity UPLC H-Class system (Waters, Milford, USA). The Empower 3 software was used for system control, data acquisition, and results processing. Following columns were tested: ACQUITY$^{TM}$ PREMIER Oligonucleotide BEH C18 and ACQUITY UPLC BEH Phenyl (dimensions 50 or 100 × 2.1 mm, particle size 1.7 μm; Waters). Serial column coupling was also evaluated. Short 3-mer ONs investigated were synthetized by Generi Biotech (Hradec Králové, Czech Republic). Mobile phases were composed of ammonium acetate at various concentrations and pH values and acetonitrile. The effect of separation temperature on retention and separation was also tested.

## Results

The best aqueous part of mobile phase to acetonitrile for diastereomeric separation was 25mM ammonium acetate, pH 6.8 for both tested stationary phases, i.e. C18 and phenyl-hexyl. The separation temperature had significant impact on the retention and separation of individual diastereomers/3-mer oligonucleotides. Usually, increased temperature 35 °C – 40 °C yielded the best results (for illustration see Figure 1). For some of the peaks we observed reversal of elution order with the change of ligand of the stationary phase. In the case of the mixed sample containing ONs with nucleobase adenine, serial column coupling of C18 and phenyl-hexyl columns yielded the best separation compared to the use of single columns.

## Conclusion

In this work the effect of various chromatographic parameters, i.e. stationary phase type, mobile phase composition, gradient steepness and separation temperature on retention and separation of short phosphorothioate oligonucleotides and their diastereomers was evaluated. The obtained results proved that reversed phase mode of liquid chromatography represents a powerful tool for short PS oligonucleotides´ diastereomeric separation.

**Acknowledgements**
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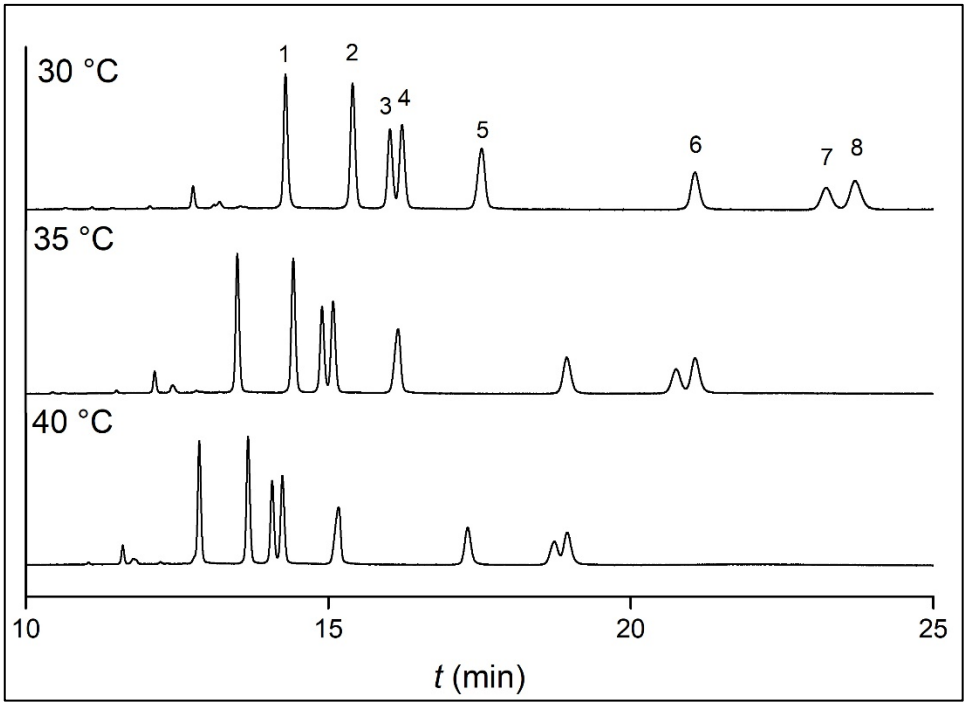
**Figure 1.** The effect of temperature on retention and separation of the mixed sample of 5'-T*T*T-3' (U2), 5'-T*TT-3' (U5) and 5'-TT*T-3' (U6) and their diastereomers. * denotes PS modification. Peaks: U2: 5, 6, 7, 8; U5: 2, 4; U6: 1, 3. ACQUITY UPLC BEH Phenyl column. Gradient elution.



# A configurable gradient score criterion for targeted LC-MS/MS optimatization via min-max normalization

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## Introduction

Designing robust targeted LC–MS/MS methods for large analyte panels is still a demanding task, particularly when chromatographic separation must be balanced with MS/MS acquisition constraints. This issue becomes especially relevant in the analysis of complex phenolic profiles, where many structurally related compounds may elute within a limited chromatographic window. Insufficient separation, excessive overlap of MRM acquisition windows, or unsuitable retention-time distribution can reduce method reliability and practical applicability. Therefore, objective tools are needed to compare candidate chromatographic conditions and support rational method development. In our previous work, the Gradient Score concept [1] was introduced as a single-number descriptor for evaluating gradient suitability. In this study, this concept is further developed into a configurable framework applicable to both gradient design and column selection.

## Experimental

Chromatographic experiments were performed using an Agilent 1260 Infinity II LC system coupled to a QTRAP 4500 MS/MS detector. The mobile phase consisted of water with 0.1% acetic acid as solvent A and acetonitrile as solvent B. Eight reversed-phase columns were evaluated: ACE C18, ACE C18-PFP, ACE C18-AR, Kinetex F5, Kinetex Biphenyl, YMC C18, Raptor C18, and Arion C18. A total of 68 phenolic standards were analyzed under targeted LC–MS/MS conditions. Initial experiments were based on conventional linear gradient profiles. Subsequently, nonlinear gradient profiles were designed and tested to improve chromatographic performance and MRM acquisition efficiency.

## Results

Column selection was optimized using the configurable Gradient Score based on three subcriteria: peak distribution index (PDI), penalized width index (PWI), and penalized selectivity index (PSI). Among the tested stationary phases, the Raptor C18 column was identified as the most suitable overall candidate. The ACE columns showed particularly strong performance in terms of separation efficiency, reflected by favorable PWI values. However, they provided less suitable peak distribution across the chromatogram and lower selectivity for critical peak pairs, as indicated by PDI and PSI. In contrast, the Kinetex columns appeared to be the least suitable for the final method. Although they provided good separation of critical pairs, several analytes showed broad, asymmetric, or tailing peaks. The Raptor C18 column was therefore selected for further gradient optimization. Linear gradient profiles were first evaluated to investigate the influence of the initial percentage of mobile phase B and gradient steepness on the individual GS subcriteria. Subsequently, nonlinear gradient profiles were optimized with the aim of achieving a more uniform distribution of analytes throughout the chromatogram. The optimal gradient profile was selected based on the GS calculated from detection window overlap (WO), gradient time (GT), and PSI. The results indicate that nonlinear gradients (Figure 1) generally provide improved values of the GS subcriteria.

## Conclusion

The configurable Gradient Score enabled objective optimization of both stationary phase and gradient profile. Raptor C18 provided the best overall balance of peak distribution, peak width, and critical-pair selectivity. Nonlinear gradient optimization further improved the GS-based evaluation by reducing detection-window overlap while maintaining suitable gradient time and selectivity.

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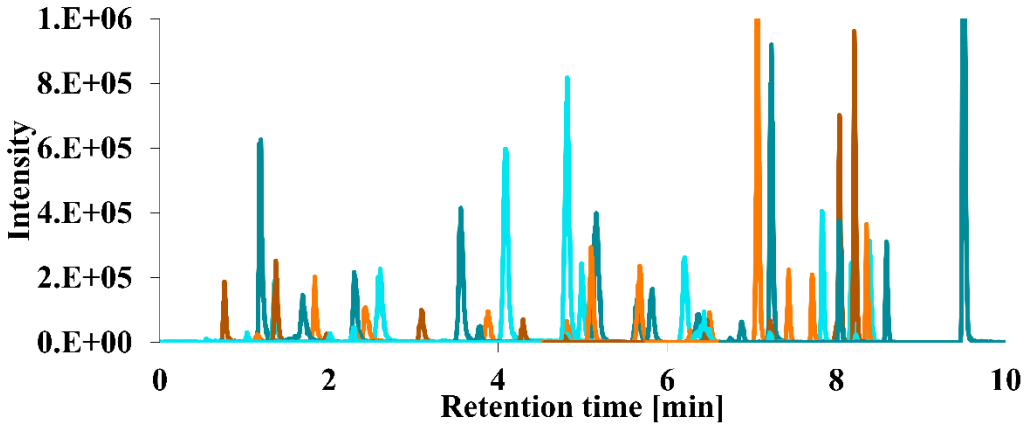
**Figure 1.** Chromatogram obtained using the optimized nonlinear gradient method



# Use of nano sheath liquid flow CE-ESI/MS for analysis of intact and pepsine digested monoclonal antibody trastuzumab

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## Introduction

Capillary electrophoresis (CE) with UV detection is a well-established method for the analysis of monoclonal antibodies (mAbs), particularly in their native state [1]. The CE methods hyphenated with mass spectrometry (MS) are being developed especially for more detailed characterisation of the mAbs charge variants [2]. Here, the CE-MS methods are applied for the characterisation of trastuzumab and its pepsin digestion products. Near-native CE-MS is used to monitor the extent of digestion and identify the digestion products, CE-MS in an acidic background electrolyte (BGE) and positively coated fused silica capillary enables detailed characterisation of the resulting fragments. Large volume sample stacking (LVSS) is employed for the analysis of the generated peptides. The aim of the work was to develop CE-MS methods for optimisation pepsin digestion conditions for best yield of the F(ab´)$_2$ fragment.

## Experimental

All measurements were performed using an in-house-built portable CE system equipped with an automatic carousel and two high-voltage power supplies (Spellman), coupled to an Orbitrap Exploris 240 mass spectrometer (ThermoFisher Scientific) via a nano–sheath-liquid CE–ESI/MS interface [3]. Fused silica capillaries (25/150 μm ID/OD) with permanent coatings (neutral hydroxypropylmethylcellulose, HPMC, or positively charged PAMAPTAC [4]) were used. MS data deconvolution and peptide identification were carried out using Biopharma Finder 5.3.

## Results

*Near-native CE–ESI/MS*

Measurements were performed in BGE composed of ammonium acetate at pH 5.75 and an ionic strength of 20 mM in an HPMC-coated capillary ($L_{tot}$ = 45 cm) with minimal electroosmotic flow mobility ($\mu_{EOF} \approx 0.1\times10^{-9}m^2V^{-1}s^{-1}$). The sheath liquid consisted of 0.5 M acetic acid in 30% v/v IPA. Under these conditions, intact trastuzumab was separated into two major zones along with several less abundant charge variants.

After pepsin digestion, removal of the Fc fragment led to sharpening of the main peaks and increased electrophoretic mobility. The F(ab′)$_2$ fragments were clearly separated from the two intact antibody forms (see Fig. 1), enabling effective monitoring of digestion progress. After 2 hours, residual intact trastuzumab forms were still detectable, whereas complete cleavage of the parent mAb was achieved after 4 hours.

*CE–ESI/MS in acidic BGE*

Near-native measurements indicated that $M_r$ the two main F(ab′)$_2$ digestion products differed by approximately 2 Da. Such a small mass difference is insufficient to explain their separation, suggesting a difference in their charge and thus a variability in sequence.

CE performed in 50 mM formic acid with 20% v/v acetonitrile (used as both BGE and sheath liquid) in a 4% PAMAPTAC-coated capillary enabled separation of reduced trastuzumab light chain (LC) and heavy chain (HC) fragments after pepsin digestion. The HC appeared as a single

dominant peak, whereas the LC was separated into two peaks differing by approximately 1 Da.

*LVSS–CE–ESI/MS*
LVSS-CE–ESI/MS was performed in a capillary ($L_{tot}$ = 40 cm, 25/150 µm ID/OD) with a covalently bonded cationic 2% PAMAPTAC coating for peptide mapping. The sheath liquid had the same composition as the BGE ($NH_4FA$, pH 2.75, I = 10 mM, 4% ACN), and the sample zone (1 mg/mL mAb pepsin digest in 4% ACN) occupied 23% of the total capillary length.
The method enabled identification of peptides generated by pepsin digestion and was used to assess the extent of proteolysis. Analysis of the undigested antibody yielded only a limited number of peptides. After 2 h of digestion, a significant increase in peptides derived from the Fc region was observed, while peptides from the Fab region remained scarce. Prolonged digestion further increased the intensity of Fc-derived and pepsin-related peptides, with only minor changes in Fab-derived peptide signals. These results indicate preferential and extensive digestion of the Fc region, whereas the F(ab′)$_2$ fragment is considerably more resistant to pepsin cleavage.

## Conclusions
The combination of near-native and acidic CE–ESI/MS with LVSS–CE–ESI/MS enabled characterisation of trastuzumab and its pepsin digestion products at multiple structural levels. Near-native conditions preserved charge heterogeneity of the intact antibody and allowed clear monitoring of Fc removal and F(ab′)$_2$ formation. Acidic separations further resolved subtle microheterogeneity within F(ab′)$_2$ and light chain fragments that could not be explained by mass differences alone, indicating charge- and sequence-related variability.
Peptide mapping by LVSS–CE–ESI/MS confirmed a non-uniform digestion pattern, with preferential and extensive cleavage of the Fc region, while F(ab‘)$_2$ region showed markedly higher resistance to pepsin.

## Acknowledgements
This work was supported by the Czech Academy of Sciences, project no. RVO 61388963.

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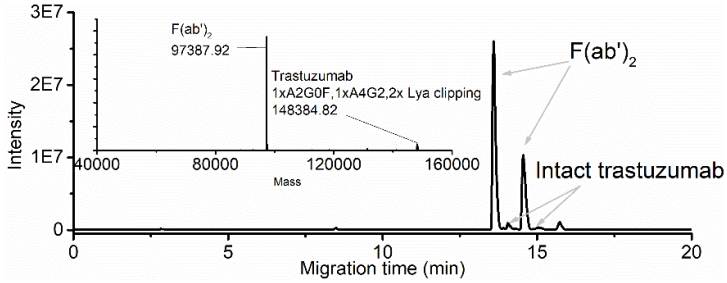
**Figure. 1** TIC electrophoregram of the pepsin digested trastuzumab under near-native conditions. The inset is deconvoluted MS spectrum of peaks in range of migration time 12-17 min.



# Characterization of Cefiderocol Stability and Degradation Products Using Modern Analytical Methods

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## Introduction

Cefiderocol, a novel siderophore cephalosporin antibiotic, plays a significant role in the treatment of severe infections caused by multidrug-resistant Gram-negative pathogens. This synthetic β-lactam compound is characterized by its unique "Trojan horse" mechanism, enabling active transport into bacterial cells via iron uptake systems. As a result, cefiderocol effectively overcomes key resistance mechanisms such as reduced membrane permeability, efflux pump activity, and the production of a broad range of β-lactamases. Owing to these properties, it has become an important therapeutic option in the management of infections caused by pathogens such as *Pseudomonas aeruginosa* and *Acinetobacter baumannii* [1,2,3].

Despite its growing clinical importance, the chemical stability of cefiderocol remains insufficiently explored. Its molecular structure contains several functional groups susceptible to hydrolytic and oxidative degradation, which may lead to the formation of degradation products with potentially altered pharmacological properties. [1,3].

While high-performance liquid chromatography (HPLC) is widely used for monitoring drug stability and quantification, the identification of degradation products requires more advanced analytical approaches. Therefore, liquid chromatography coupled with mass spectrometry (LC–MS) represents a powerful complementary technique, enabling detailed characterization of degradation pathways and structural elucidation of formed products. The combination of these analytical methods provides a comprehensive tool for studying cefiderocol stability and its degradation behavior under various stress conditions. [4]

## Experimental

Chromatographic analyses were performed using a reverse-phase high-performance liquid chromatography (RP-HPLC) system (ChroZen, YOUNG IN Chromass, Anyang-si, Korea) coupled with a UV detector. The separation was carried out on a Sunfire C18 column. The mobile phase consisted of 0.1% formic acid in LC–MS grade water (A) and acetonitrile (B). The flow rate was set to 1.0 mL/min, and the column temperature was maintained at 40 °C. Sample injection was performed with an injection volume of 10 µL. Detection was achieved using UV spectrophotometry at a wavelength of 260 nm.

Subsequently, LC–MS analysis was employed for the identification and structural characterization of selected degradation products. The LC-MS experiments were performed on a Waters Acquity UPLC system (Waters, Milford, MA) coupled to the XEVO TQD (Waters) triple quadrupole mass spectrometer. Separation was performed using a Cortecs UPLC T3 column (2.1 mm x 100 mm, particle size: 1.6 mm) (Waters) with a VanGuard precolumn, using the gradient elution method. The column temperature was set at 40°C. The mobile phase consisted of 0.1% HFo in LC-MS water (solvent A) and acetonitrile (solvent B). Solvents A and B were run in a gradient regime.

The mobile phase was delivered at a flow rate 0.5 ml/min. The injection volume was 10 µL. All compounds were detected using positive electrospray
ionization mode (ESI+) and Scan, SIM, and Product ion mode.

**Results**

The applied stress conditions led to varying degrees of cefiderocol degradation depending on the nature of the stressor. The most pronounced degradation was observed under acidic and alkaline conditions, while moderate changes were detected under thermal stress.
HPLC analysis revealed the presence of multiple additional peaks corresponding to degradation products. LC–MS analysis enabled the detection of characteristic fragmentation patterns and facilitated the proposal of tentative structures for selected degradation products.

**Conclusion**

This study provides a comprehensive evaluation of cefiderocol stability under stress conditions and demonstrates the applicability of HPLC and LC–MS methods for monitoring its degradation behavior. The identification and structural characterization of degradation products contribute to a better understanding of cefiderocol stability and may support its quality control, storage optimization, and safe clinical use. Furthermore, the results highlight the importance of advanced analytical approaches in addressing current gaps in the characterization of cefiderocol degradation pathways.

**Acknowledgements**

This research was supported by the projects UK/1257/2026, and VEGA 1/0302/24

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# Multimodal cation exchange-type tyrosine-based chiral stationary phases: Synthesis and applications in high-performance liquid chromatography

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## Introduction

High-performance liquid chromatography (HPLC) using chiral stationary phases (CSPs) is among the most prevalent techniques for the separation of enantiomers. Among the most widespread CSPs, those containing ion-exchangers as chiral selectors (SOs) have emerged as powerful tools for the separation of polar and polarizable compounds. In addition to well-established commercial materials, such as Cinchona alkaloid-based chiral weak anion exchangers (WAX) and zwitterionic ion-exchange phases (ZWIX), chiral cation-exchange CSPs represent a valuable alternative. These materials have demonstrated broad applicability in the enantioseparation of racemic amines, encompassing a wide spectrum of pharmaceutical compounds [1].

The fundamental concept of the proposed work is to combine the key properties of the donor-acceptor and cation-exchange type SOs. The introduction of the DNB moiety enhances the π− π-interactions capacity of the chiral selector. While π-acidic (i.e., electron-deficient) analytes are expected to interact primarily with the electron-rich aromatic unit of the modified tyrosine motif, π-basic analytes should have high affinity to the electron-deficient DNB group. Moreover, the selectors include a sulfonic acid (i.e., strong cation exchanger, SCX-type, CSP I, II) or a carboxylic acid moiety (i.e., weak cation exchanger, WCX-type, CSP III) as the respective ion exchange sites. A key feature of the synthetic strategy was the efficient covalent immobilization of the chiral selectors onto the silica support via a copper(I)-catalyzed azide-alkyne cycloaddition reaction (i.e., *click-chemistry*).

## Experimental

The synthesis of the chiral selectors is described elsewhere [2]. The prepared selectors were immobilized on 3-azidopropyl-modified silica (3 μm, 120 Å) and slurry-packed into stainless steel chromatographic columns (150×3.0 mm, id) in Galochrom s.r.o. (Prague, Czech Republic). The chromatographic evaluation of CSPs was performed in various chromatographic modes (NP, PO, HILIC) using isocratic elution of selected analytes (β-blockers, proton pump inhibitors, antimalarics). The evaluation was performed on Agilent 1100 series (Agilent Technologies, Waldbronn, Germany) and ECOM HPLC (ECOM, Prague, Czech Republic) using a constant flow

rate of 0.75 mL $min^{-1}$ at room temperature. The detection wavelength was typically set to 254 nm.

**Results**

Systematic evaluation of the new CSPs employing HPLC demonstrated excellent chiral recognition across multiple classes of analytes and chromatographic modes. Using the polar organic mode, we document the applicability of the novel CSP in HPLC-MS, *e.g.*, for the chiral resolution and detection of omeprazole enantiomers in less than 5 minutes. Further noteworthy outcomes include the successful separation of other important chiral analytes, including β-blockers.

**Conclusion**

This work introduces improved tyrosine-based CSPs that effectively integrate donor-acceptor and cation-exchange functionalities, rendering them a versatile and powerful addition to the available toolkit for challenging chiral separations.

**Acknowledgements**

This work was supported by TACR, project no. TQ15000270.

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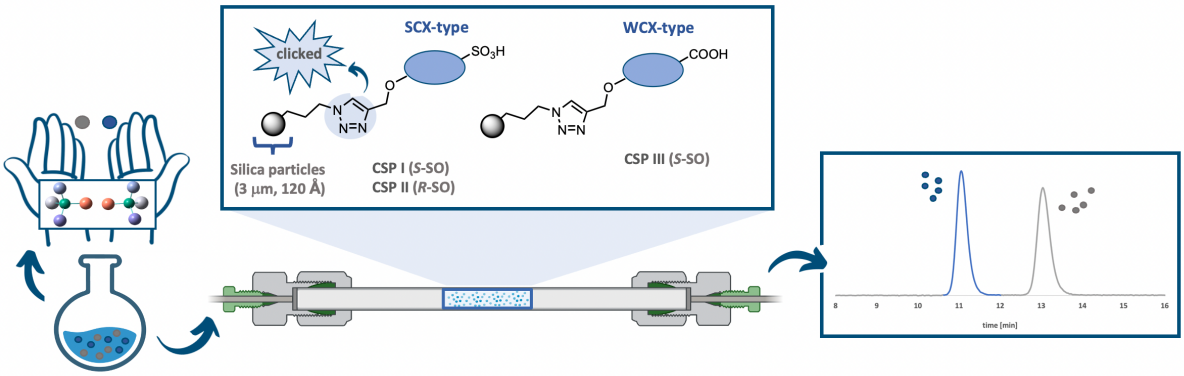
**Figure 1.** Graphical abstract



# Hexafluoroisopropanol-induced N-octyl Glucopyranoside Biphasic Coacervate System for Protein Fractionation and Enrichment

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## Introduction

Protein or peptide fractionation prior to LC-MS/MS analysis is commonly used to increase the number of identified proteins. Commonly used fractionation techniques for proteins and peptides include gel electrophoresis, solid phase extraction, size exclusion chromatography, and molecular weight cut-off filtration [1]. Coacervation has emerged as a promising strategy for selective extraction, enrichment, and fractionation of complex protein samples. Early studies demonstrated that perfluorinated alcohols can induce coacervate formation, enabling efficient protein extraction and highlighting their utility as alternative separation media [2,3]. In the present work, we investigate the formation of coacervate systems composed of hexafluoroisopropanol (HFIP) and nonionic surfactants for protein extraction and fractionation in the top-down LC-MS proteomic workflow.

## Experimental

Several nonionic surfactants (Brij C10, Brij L4, NOGP, PEG 400, Triton X-100, Tween 80, and Tween 85) were screened for their ability to form coacervates in aqueous solutions upon the addition of HFIP. All LC-MS experiments were performed on a Shimadzu LCMS-2020 quadrupole mass spectrometer (Shimadzu Scientific Instruments, Inc.; Columbia, MD, USA). A Viva C4 column (2.1 x 30 mm; 5 µm) equipped with an UltraShield UHPLC Precolumn Filter (all from Restek, Inc.; Bellefonte, PA, USA) was used as a stationary phase. Mobile phases consisted of water:acetonitrile mixture with the addition of difluoroacetic and formic acid. The injection volume was 5 µL, and the column oven temperature was 60 °C. MS was performed with electrospray ionization in the positive ionization mode with selected ion monitoring.

## Results

N-octyl glucopyranoside (NOGP) was chosen as the surfactant to pursue in this study because: (1) it consists of a single molecular specie; (2) it does not interfere with protein LC-MS analysis as it elutes before and is well separated from the first eluting protein, and (3) it is known to solubilize hydrophobic proteins, including membrane proteins. The results showed that the concentration ranges of HFIP within which two-phase systems exist are much narrower for NOGP systems than other systems previously studied, which consisted of anionic, cationic, and/ or zwitterionic surfactants. The coacervate volume tended to slightly decrease as more HFIP was added until the systems abruptly transitioned into a single-phase system at a critical HFIP concentration. The addition of a salt to a coacervate system results in a change in the relative volumes of the two phases, though the extent of this change is dependent upon both the identity of the salt and its concentration.

## Conclusion

In this work, HFIP-induced coacervate systems formed with nonionic surfactants were systematically evaluated as platforms for protein extraction and fractionation. Optimization of LC-MS conditions and sample preparation protocols enabled reliable quantification of proteins. These results support the use of nonionic coacervate systems as tunable tools for enhancing proteomic analyses.

## Acknowledgements

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# Influence of Solid Matrices on Signal Behaviour in SS-ETAAS

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## Introduction

Slurry sampling electrothermal atomic absorption spectrometry (SS-ETAAS) represents an attractive alternative to conventional wet digestion due to minimal sample preparation, reduced contamination risk and direct analysis of solid samples [1]. However, the analytical performance of SS-ETAAS is strongly influenced by matrix effects caused by the complexity and physicochemical properties of the analyzed materials [2]. Although slurry sampling has been successfully applied to various environmental, biological and food samples, a systematic comparison of matrix-induced signal suppression and enhancement across highly diverse solid matrices is still limited [3].

The presented work focuses on the influence of matrix effects on the determination of Pb and Fe in different solid samples using SS-ETAAS. Particular attention was devoted to the relationship between matrix complexity and atomization behaviour, optimization of slurry preparation and the role of chemical modifiers.

## Experimental

Nine different solid matrices of biological, food and environmental origin were analyzed, including human placenta, human hair, mushrooms, plums, walnuts, smooth flour, vegetable leaves, nutritional supplement and soil samples. Suspensions were prepared using optimized liquid media followed by ultrasonic homogenization and direct introduction into the graphite furnace.

The influence of particle size, sample amount, liquid medium composition, dilution factor and chemical modifiers on analytical performance was systematically investigated. Matrix effects were evaluated by comparing the absorbance of analytes in standard solutions and in the presence of solid sample matrices.

## Results

The obtained results demonstrated that matrix effects strongly depended on the character of the analyzed sample and significantly differed between Pb and Fe determination, as illustrated in Figure 1. Figure 1 presents a heatmap visualization of matrix effects observed during SS-ETAAS determination of Pb and Fe in various biological, food and environmental solid samples. Blue colour represents signal suppression, while red colour corresponds to signal enhancement relative to aqueous standards. While Pb signals remained relatively stable in most matrices, Fe exhibited pronounced signal suppression and enhancement effects.

The most significant suppression was observed for Fe in human hair samples, where the analytical signal decreased by approximately 52%. In contrast, signal enhancement was observed for walnuts and smooth flour samples, where Fe absorbance increased by approximately 49% and 21%, respectively. These observations clearly demonstrate that atomization behaviour in SS-ETAAS is highly matrix-dependent.

The study further showed that universal optimization conditions cannot be applied for all matrices.

The effectiveness of chemical modifiers strongly depended on both the analyte and sample composition. For example, $Pd(NO_3)_2$ improved Pb stabilization in placenta samples, while it negatively affected Fe determination under comparable conditions.
Despite the complexity of the investigated matrices, the optimized SS-ETAAS procedure provided satisfactory analytical performance with good linearity, low detection limits and repeatability below 5% RSD for most samples.

## Conclusion

This work demonstrates that matrix effects represent one of the key factors influencing slurry sampling ETAAS analysis of solid samples. The obtained results revealed substantial differences in analyte behaviour among biological, food and environmental matrices, especially for Fe determination. The study highlights the necessity of matrix-oriented optimization of slurry preparation, atomization conditions and modifier selection in order to achieve reliable analytical performance in SS-ETAAS.

## Acknowledgements

This work was supported by the Scientific Grant Agency of the Ministry of Education, Science, Research and Sport of the Slovak Republic (VEGA Grant No. 1/0727/25) and by the Slovak Research and Development Agency (APVV-24-0235), Slovak Republic.

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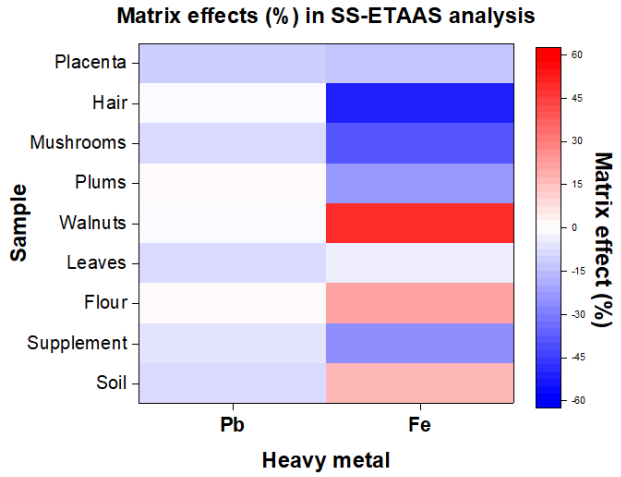
**Figure 1.** Heatmap visualization of matrix effects observed during SS-ETAAS determination of Pb and Fe in various solid matrices



# A Novel Approach to Stir Bar Sorptive Extraction with Natural Deep Eutectic Solvent

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## Introduction

The sample preparation is a crucial part of the analytical process and with growing emphasis on sustainable procedures [1]. This can be achieved through miniaturization, automation and replacing toxic chemicals with natural alternatives [1, 2] Among the increasingly utilized substances are natural deep eutectic solvents (NADES) which are binary or ternary mixtures composed of a hydrogen bond donor and a hydrogen bond acceptor in a defined molar ratio [3]. NADES have found wide application in extraction techniques, where they are replacing conventional chemicals. In combination with NADES several extraction techniques can be employed such as stir bar sorptive extraction (SBSE) [1]. SBSE is one of the microextraction techniques where the surface of a magnetic stir bar is covered with suitable medium such as NADES and is used for the extraction and preconcentration of analytes from the sample [4].

## Experimental

For the preparation of a hydrophobic natural deep eutectic solvent (HNADES), thymol and benzyl alcohol mixture in a 1:1 molar ratio was heated at 50 °C and stirred at 130 rpm for 15 minutes in a water bath. Subsequently, 50.0 mg of $Fe_3O_4$ magnetic nanoparticles were introduced into 2.0 mL of the HNADES, and the mixture was ultrasonicated (40 kHz, 10 min) until a uniform black ferromagnetic HNADES (HNADES-FF) was formed. This HNADES-FF was then applied as a coating material for the magnetic stir bar in SBSE procedure.

## Results

SBSE performed using the prepared HNADES-FF enabled the extractions of four *N*-nitrosamines: *N*-nitrosodiethylamine (NDEA), *N*-nitrosodiphenylamine (NDPhA), *N*-nitrosodibutylamine (NDBA) and *N*-nitrosopiperidine (NPIP) which were analyzed by HPLC-UV. Several parameters of the SBSE procedure required optimization. One of the key steps in the optimization process is the desorption of analytes from the surface of the glass magnetic stir bar covered with HNADES-FF. The selection of a suitable desorption solvent was identified as an important factor for achieving higher extraction recoveries. Following the SBSE procedure, where desorption was performed using 2.0 mL of acetonitrile, NDEA was still detected, suggesting insufficient desorption efficiency. This can also be seen in the HPLC chromatogram in Figure 1, which indicates a twofold enrichment of the sample in the desorption solvent. To improve this step, acetonitrile, ethanol and propan-2-ol were evaluated as the potential desorption solvents, selected based on their differing physicochemical properties that may enhance analyte desorption from the HNADES-FF coated glass magnetic stir bar.

In the case of acetonitrile as the desorption solvent, the extraction recoveries were 28% for NDEA, 20% for NPIP, 72% for NDPhA, and 55% of NDBA. These results suggest that acetonitrile is less suitable as a desorption solvent for the more polar analytes, such as NDEA and NPIP, while it is

more effective for the less polar analytes, particularly NDPhA and NDBA. The highest recovery for NDEA, close to 50%, was achieved with propan-2-ol as desorption solvent.

## Conclusion

The optimization and preparation of HNADES-FF enable its use in green analytical chemistry and microextraction techniques. Application of HNADES-FF in SBSE proved effective for the extraction of *N*-nitrosamines. Efficiency of extraction depended on process optimization, particularly the choice of desorption solvent. Acetonitril improved recoveries for NDPhA, NDBA and NPIP, while the highest recovery for NDEA was achieved with propan-2-ol. Further optimization of SBSE is required to enhance extraction recoveries.


## Acknowledgements

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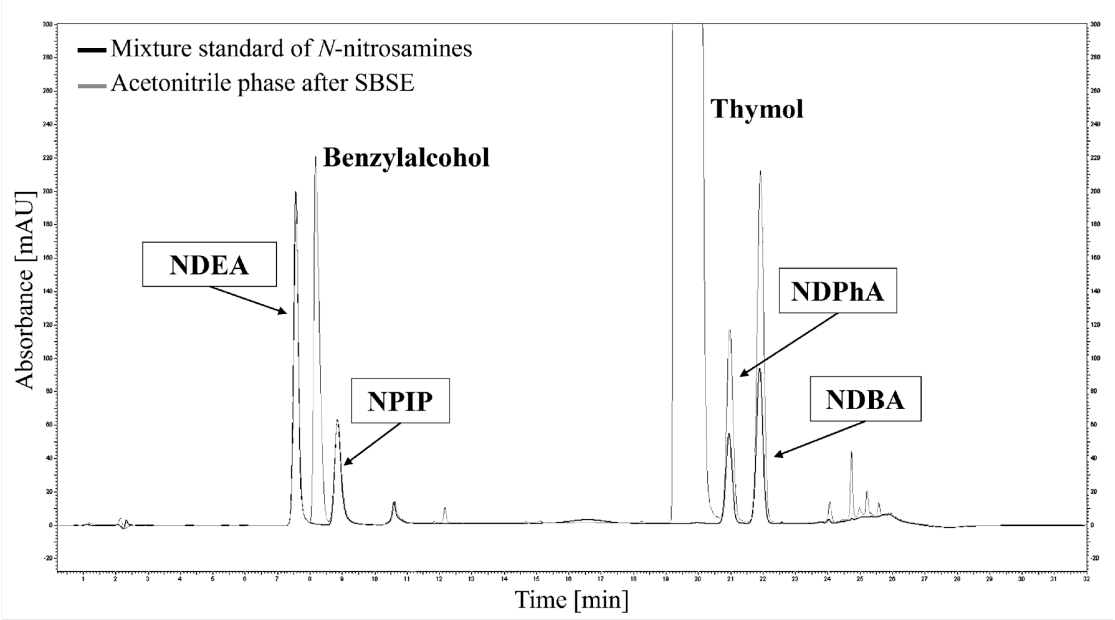
**Figure 1.** HPLC chromatogram of the separation of N-nitrosamine mixture standards after SBSE using acetonitrile as the desorption solvent



# Determination of Residual Urinary Cisplatin in Patients Suffering from Germ Cell Tumors by Triple Quadrupole LC-MS/MS

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## Introduction

Cisplatin (CisPt) represents a platinum-based chemotherapeutic widely used to treat various cancers, including germ cell tumors. The interindividual variability in renal excretion of CisPt remains clinically relevant for both efficacy and toxicity of chemotherapy. Reliable quantification of CisPt in urine requires selective and sensitive analytical methods capable of detecting residual levels in a complex biological matrix. One such method is HPLC-ESI-MS/MS analysis of CisPt following derivatization with diethyldithiocarbamate (DDTC) [1, 2].

## Experimental

The residual urinary cisplatin was derivatized with DDTC to form stable Pt-DDTC complexes. The palladium(II) acetate (PdAc), which formed Pd-DDTC complex, was used as an internal standard. Both complexes were separated using reversed-phase HPLC (Acquity UPLC BEH C18, 100 ×2.1 mm, 1.7µm) coupled with triple-quadrupole tandem mass spectrometry with electrospray ionization in positive mode (XEVO TQS, Waters) (Figure 1). The optimized HPLC-ESI-MS/MS method was validated in accordance with FDA guideline on the development of bioanalytical methods, covering linearity, selectivity, sensitivity, trueness, repeatability, intermediate precision, recovery, matrix effects, and robustness. The validated method was applied to 109 urine samples collected from male patients with germ cell tumours. The patients were undergoing cisplatin-based chemotherapy at the National Cancer Institute, Slovakia. The measured residual urinary cisplatin (uCisPt) levels were normalized to creatinine (uCisPt/Cr) and expressed as µg/mmol creatinine.

## Results

The validated HPLC-ESI-MS/MS method showed excellent analytical performance, with linearity over 0.25-75 µg/L (R=0.9995), LOD 0.13 µg/L, and LOQ 0.27 µg/L. Trueness repeatability and intermediate precision met FDA acceptance criteria, and robustness testing confirmed the stability of chromatographic and MS parameters (Table 1). The validated HPLC-MS/MS method allowed the determination of uCisPt/Cr in 109 patient samples with a mean and median of 136.3 and 131.3 µg/mmol, respectively. The uCisPt/Cr levels did not differ significantly according to histology, primary tumor site, treatment regimen, response, stage or IGCCCG risk group.

## Conclusion

The HPLC-ESI-MS/MS method for the quantification of residual uCisPt was successfully developed and validated. Its application to clinical samples confirmed its suitability for bioanalytical monitoring of platinum-based chemotherapeutics and for supporting pharmacokinetic or toxicological studies. The method provides a reliable tool for assessing

residual CisPt levels in patient urine and contributes to improved understanding of treatment-related variability. In the tested sample GCTs cohort, the uCisPt/Cr levels were not consistently associated with clinicopathological characteristics, disease-free survival and overall survival.

**Acknowledgements**
This work was supported by the Internal Grant Agency of Palacký University in Olomouc, IGA_PrF_2025_027 and IGA_PrF_2026_024.

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**Figure 1** MRM chromatograms for Pt-DDTC (blue) and Pd-DDTC (orange) in the real patient urine.

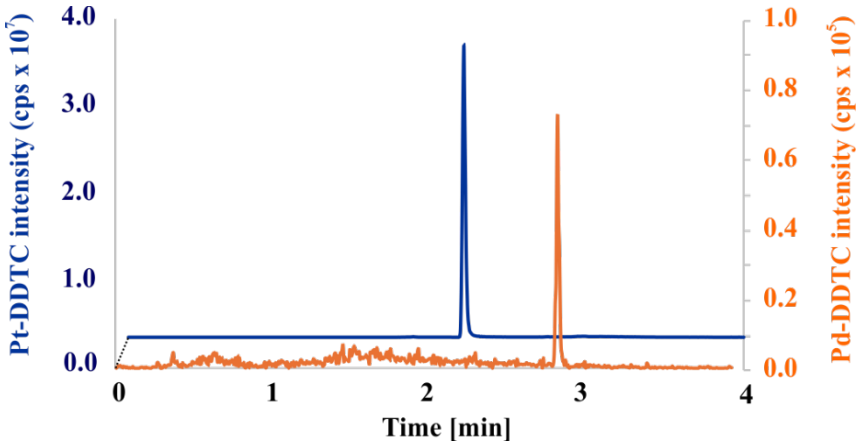


**Table 1** Summary of the validation results.

| **Validated Parameter** | **Result** |
|---|---|
| Calibration range [µg/L] | 0.25 – 75.0 |
| Linearity (correlation coefficient) | 0.9995 |
| Instrumental limit of detection (LOD) [µg/L] | 0.13 |
| Method limit of detection (LOD) [µg/mmoL] | 0.013 |
| Instrumental limit of quantification (LOQ) [µg/L] | 0.27 |
| Method limit of quantification (LOQ) [µg/mmoL] | 0.027 |
| Trueness (recovery, %) | LL: 107<br>ML: 107<br>HL: 108 |
| Repeatability (RSD, %) | 7 |
| Intermediate precision (RSD, %) | 16 |

LL = 0.25 µg/L, ML = 2.5 µg/L, HL =25 µg/L

# Arrival Time Distribution Profile Fitting for the Analysis of Isomeric Mixtures by Ion Mobility-Mass Spectrometry

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## Introduction

Ion mobility-mass spectrometry (IM-MS) is a powerful technique for the analysis of isomeric compounds. Modern high-resolution approaches, such as Trapped Ion Mobility Spectrometry (TIMS), Cyclic Ion Mobility Spectrometry (cIMS), and Structures for Lossless Ion Manipulations (SLIM), provide substantially improved resolving power. Nevertheless, the practical application of these techniques can still be limited by strongly overlapping arrival time distribution (ATD) profiles in mixtures [1]. In addition, peak broadening of individual ATD profiles at high resolving power may further complicate mixture separation. This effect can be eliminated by separating adducts of isomeric molecules with alkali metal cations [2]. On the other hand, broadening can be beneficial when it serves as a distinctive feature of individual isomers. In this work, we compare two approaches of determining the isomeric ratios of protonated molecules using characteristic ATD profiles of individual isomers. The first approach is based on Gaussian-function fitting [3], whereas the second employs multiple linear regression (MLR), implemented in commonly used data-analysis software such as OriginPro.

## Experimental

The Gaussian fitting procedure was developed on data obtained by Traveling-Wave Ion Mobility-Mass Spectrometry (TWIMS) with electrospay ionization source operated in negative mode on a Synapt G2-S ion mobility-mass spectrometer (Waters, Manchester, UK). Hyaluronan-derived oligosaccharides: ΔHA2AN, HA2AN, HA3NN, HA4AN, HA4NA, HA6AN, HA6NA, HA6ANdeac, and HA8AN were kindly provided by Contipro a.s. (Dolní Dobrouč, Czech Republic). The integer denotes the number of building blocks composed D-glucuronic acid (GlcA) and N-acetyl-D-glucosamine (GlcNAc). The symbols Δ and deac indicate a double bond between C4 and C5 of GlcA and deacetylation of one GlcNAc residue, respectively. Stock solutions were prepared at concentrations of 1 mg/mL in methanol:water (1:1, v/v) and subsequently diluted to 1 µg/mL (HA2 and HA3), 5 µg/mL (HA4 and HA6) or 10 µg/mL (HA8). Arrival time distribution (ATD) profiles were fitted using the Peak Analyzer Wizard in OriginPro 2015.

The MLR procedure was developed on data acquired on a Select Series Cyclic IMS equipped with an electrospray ionization source operated in positive mode (Waters Corporation, Wilmslow, UK). Three mixtures of isomers with a total concentration of 100 ng/mL in methanol/ water(50/50, v/v) were used: a) 3-MMC and buphedrone (*m/z* 178.13); b) 3-FMC and 4-FMC (*m/z* 182.10); c) BDB and methedrone (*m/z* 194.13). Data acquisition, processing, and evaluation were performed using Masslynx version 4.2 (Software Change Note 1016, Waters Corp., Wilmslow, UK) and statistics software OriginPro 2020 (OriginLab, Northampton, USA).

## Results

Arrival time distribution (ATD) profiles of hyaluronan-derived oligosaccharides were fitted using OriginPro 2015, specifically the Peak Analyzer Wizard function. This approach was especially useful for mobilograms exhibiting non-Gaussian peak shapes, including fronting or tailing due to the presence of multiple unresolved conformers. For simple systems, a single Gaussian function adequately described the ATD profile, whereas a superposition of a few Gaussian functions was used to fit more complex distributions. For example, HA4NA was described using two Gaussian functions and HA6AN by five. These analyte-specific ATD functions enabled the deconvolution of binary mixtures even when only a single broad peak was observed in mobilogram. The fitting procedure provided satisfactory results for different oligosaccharide ratios (e.g. for model mixture $_{\Delta}$HA2AN/HA2AN, coefficient of determination ($R^2$) was 0.9995). Moreover, the approach showed strong correlation with HPLC-based isomer ratio determination (e.g., for HA4ANred/HA4NAred, ratios 27.1:72.9 (HPLC), and 25.6:74.4 (ATD functions) were determined) [3].

Later on, the data processing was simplified by using MLR applied to entire ATD profiles. The isomeric ratio was expressed as $a_1$:$a_2$, where $y = a_0 + a_1x_1 + a_2x_2$. Mobilograms of individual isomers and their mixtures represent the independent variables ($x_1$, $x_2$) and the dependent variable (y), respectively. This approach simplified workflow while preserving full utilization of ATD signal shape information. For the 3-MMC/buphedrone pair, seven pass separation yielded $R^2$=0.9978, while lithium adduct separation followed by direct peak integration gave $R^2$=0.9998. The 3-FMC/4-FMC pair remained unresolved even after ten passes. However, MLR applied to $[M+H]^+$ data produced $R^2$ = 0.9961, while partially resolved lithium adducts measured after 25 passes yielded $R^2$ = 0.9949. For the BDB/methedrone pair, neither ion mobility separation nor adduct formation provided sufficient resolution. Therefore, ATD profiles of fragment ions were used for isomer discrimination, resulting in $R^2$ = 0.9822 [2].

## Conclusion

Both approaches rely on the repeatability of ATD profiles and enable the determination of the isomeric ratio in incompletely resolved isomeric mixtures by ion mobility-mass spectrometry. The Gaussian fitting strategy can be interpreted as an explicit deconvolution of individual component peak shapes, while the MLR approach treats the entire ATD profile as an empirical fingerprint, thereby simplifying data processing.

## Acknowledgements

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# Replacement of Acetonitrile by Greener Solvents in Reversed-Phase Liquid Chromatography of Intact Proteins

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## Introduction

The increasing awareness of environmental issues and the transition to green analytical chemistry (GAC) have gained popularity among academia and industry in recent years. One of the principles of GAC is the reduction and replacement of toxic solvents with more sustainable and environmentally friendly ones [1–3]. However, the retention of large molecules is extremely sensitive to the mobile phase composition. Thus, a very minor change in eluent strength can result in the complete release of the solute from a column thanks to the phenomenon known as an "on−off" elution mechanism [4]. Green organic solvents usually suffer from high UV cut-off wavelengths and high viscosity when mixed with water. In certain applications, these attributes may harm the sensitivity and separation efficiency of LC methods.

## Experimental

A standard mixture of various intact proteins with various isoelectric points and molar masses (up to 66 kDa) was separated by gradient elution on Dionex UltiMate 3000 Series UHPLC System using Restek Viva C4 (2,1 × 100 mm, 300 Å, 5 μm) and Waters XBridge Protein BEH C4 (2,1 × 100 mm, 300 Å, 3,5 μm) columns. Elution gradient of water–acetonitrile (eluent B1), water–ethanol (eluent B2), and water–isopropyl alcohol (eluent B3) was performed with 20 – 90 % of B1, 5 − 60 % of B2 / B3, respectively, within 15 − 40 min. 0.2% (v/v) formic acid was used as a mobile phase additive. The column temperature was 40 °C. The mobile phase was pumped at the flow rates of 0.2 − 0.4 mL/min.

## Results

The most common greener alternatives to acetonitrile (ACN) used as organic modifiers in LC are nowadays ethanol (EtOH) and isopropyl alcohol (IPA). EtOH displays rather the same characteristics in the separation of biomolecules with reversed-phase chromatography compared to ACN. Therefore, EtOH is the most suitable solvent to replace ACN in reversed-phase chromatography. Nevertheless, the elution gradients for each column needed to be adjusted and compared.

## Conclusion

The application of green analytical methods in the industry is limited, as the main challenge is to find a compromise between the environmental friendliness of the analytical method and the quality of its validation parameters. The future of green solvents in the analysis of peptides and proteins is promising; nevertheless, ongoing research to fully realize their potential and address current challenges is crucial.

**Acknowledgements**
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# A Highly Sensitive Headspace Gas Chromatographic Method Fully Optimized for Fast Routine Determination of Carboxyhemoglobin in Postmortem Blood

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## Introduction

Carbon monoxide (CO) poisoning is a major cause of fatal intoxication and a frequent subject of forensic investigation [1,2]. The determination of carboxyhemoglobin (COHb) in blood is crucial for confirming CO exposure. However, standard spectrophotometric methods [3,4] may provide unreliable or misleading results when analyzing postmortem samples affected by degradation processes such as putrefaction, thermal damage, or contamination.

To address these limitations, a gas chromatographic method was developed and validated for COHb determination in compromised postmortem blood. The method is based on automated headspace analysis after chemical release of CO using potassium ferricyanide and saponin. Separation is achieved using dual capillary columns, and detection is enhanced by converting CO to methane with a methanizer coupled to a flame ionization detector.

This approach enables accurate, sensitive, and reliable COHb quantification across a wide concentration range, even in degraded samples, making it suitable for routine forensic practice [5].

## Experimental

Postmortem whole blood samples were collected during autopsies. For gas chromatographic analysis, 100 mg of blood was transferred into a sealed headspace vial and flushed with nitrogen to create an inert atmosphere. The sample was then treated with an oxidizing solution of potassium hexacyanoferrate and saponin to release carbon monoxide from carboxyhemoglobin. After equilibration, samples were analyzed using automated headspace GC-FID equipped with dual capillary columns and a methanizer for sensitive detection of CO. COHb concentrations were calculated by comparing the CO signal of each sample with that of a fully CO-saturated reference blood sample. For method comparison, spectrophotometric analysis was also performed on diluted blood samples following chemical reduction and absorbance measurement at selected wavelengths. All analytical conditions of GC-FID method were optimized to ensure reliable performance, high sensitivity, and suitability for routine forensic application.

## Results

The developed GC-FID method was successfully optimized for routine forensic use, requiring only 100 mg of blood and achieving full CO saturation within 10 minutes. Equilibration at 50 °C for 10 minutes ensured efficient CO release and reproducible results.

Method validation confirmed excellent performance, with linearity across 0.1–100% COHb ($R^2$ = 0.9984). Precision ranged from 1.4–2.9% (intra-day) and 5.7–11% (inter-day), while accuracy remained within −4.7% to 6.2%. The method showed very high sensitivity (LOD < 0.01% COHb; LOQ = 0.1% COHb) and no significant interferences. Comparison with the standard spectrophotometric method in 35 postmortem samples showed strong agreement (R = 0.9939). However, GC-FID provided reliable results even in degraded samples where spectrophotometry failed due to decomposition-related interferences. Overall, the method demonstrated high robustness, sensitivity, and suitability for routine forensic toxicology, particularly in challenging postmortem cases.

## Conclusion

A gas chromatographic method for routine determination of COHb in postmortem blood was developed and validated as an alternative to the standard spectrophotometric approach. The method demonstrated excellent sensitivity, specificity, and accuracy, with LOD < 0.01% COHb and LOQ = 0.1% COHb. It proved reliable even in degraded samples, including putrefied and thermocoagulated blood. After optimization for routine forensic use, the method was successfully implemented and tested in real casework over a 4-year period, confirming its suitability for forensic toxicology practice.

## Acknowledgements

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# Development of a Python-Based Workflow for Design of Experiment in LC–MS Method Optimization of Plant Peptide Hormones

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## Introduction

Plant peptide hormones are small signaling molecules involved in plant growth, development and stress responses. Their analysis is analytically challenging due to the complexity of biological samples. LC–MS is commonly used for peptide analysis, but optimization of experimental conditions can require many experiments and extensive data processing.

Design of Experiments (DoE) offers a more systematic approach by evaluating several experimental factors simultaneously. Although Python is widely used for scientific data analysis, accessible tools focused on DoE and analytical workflows are still limited. The aim of this work is therefore the development of a Python package that combines experiment design, data evaluation and visualization into a single workflow.

## Experimental

The workflow was developed in Python using open-source scientific libraries. The package was designed to support experimental design generation, data import, statistical evaluation and graphical visualization of results.

The workflow was tested during LC–MS method development for the plant peptide hormone phytosulfokine-α (PSK-α). A reference standard of PSK-α was used to optimize MS response. Selected experimental parameters were evaluated using response surface methodology and regression models. Visualization tools such as Pareto plots and response surface heatmaps were implemented for interpretation of the results.

## Results

The developed workflow enabled automated generation and evaluation of DoE experiments in a single computational environment. Compared to conventional one-factor-at-a-time optimization, the DoE approach reduced the number of experiments while allowing evaluation of interactions between experimental factors.

Application of the workflow to PSK-α optimization showed successful improvement of MS response and enabled visualization of optimal experimental regions using contour plots and heatmaps. The developed package simplifies analytical method optimization and improves reproducibility by integrating experimental planning, statistical analysis and visualization into one workflow.

## Conclusion

The presented work demonstrates the potential of combining Design of Experiments with automated Python-based tools for LC–MS optimization of plant peptide hormones. The developed

workflow provides a simple and reproducible approach for analytical method development and can be extended to additional peptide hormones and experimental designs.

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# Mirror vs Native: Who Binds Stronger?

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## Introduction

Cyclodextrins (CDs) are cyclic oligosaccharides composed of D-glucose subunits linked via α-1,4-glycosidic bonds. Known for more than 130 years, they are typically produced by enzymatic conversion of starch and are widely applied in the food, pharmaceutical, and chemical industries, including targeted drug delivery and agriculture. In 2024, an international team led by Sir J. Fraser Stoddart published in Nature Synthesis an efficient method for the preparation of mirror-image L-cyclodextrins, opening new avenues for CD research and applications [1].

In pharmaceutical practice, the ability to control enantioselective separation is essential, as one enantiomer may be therapeutically active while the other may be inactive or, in the worst case, toxic. Previous studies on mirror-image CDs (L-CDs) [1–3] demonstrated the possibility of reversing enantiomer migration order. The ability to reverse migration order simply by switching between D- and L-CDs could substantially reduce method development time and improve method reliability compared to current approaches, which are often complex, empirical, and associated with uncertain outcomes.

In this work, we aimed to compare the interaction strengths between 6-methyltryptophan enantiomers and α-D- and α-L-CDs. Previous studies with mirror image CDs have only demonstrated that reversal of enantiomer migration order is possible. However, a detailed comparison of interaction strengths and the corresponding interaction constants would provide deeper insight into the mechanisms governing chiral recognition.

## Experimental

All experiments were performed using an Agilent 7100 capillary electrophoresis system equipped with a DAD detector. The analyses were carried out in an uncoated fused-silica capillary (Polymicro Technologies) with an internal diameter of 50 μm, a total length of 34 cm, and effective lengths of 25.5 cm or 8.5 cm (for short-end injection). The background electrolyte consisted of a 50 mM phosphate buffer adjusted to pH 2.2 with NaOH. α-D-cyclodextrin was purchased from Merck KGaA (Darmstadt, Germany). Cyclodextrins (α-D-CD and α-L-CD) were added to the background electrolyte at increasing concentrations prior to analysis. Binding constants were evaluated from shifts in migration times [4].

## Results

In 2025, Armstrong et al. [2] demonstrated that 6-methyltryptophan enantiomers migrate in reversed order when α-L-CD is used instead of native α-D-CD. The authors used a 10 mM sodium phosphate buffer at pH 2.2 together with a discontinuous electrolyte system containing a short plug of 10 mM CDs in order to minimize the consumption of the newly synthesized selector. A CD concentration of 10 mM was selected because it represented the solubility limit of the CD in the electrolyte.

In our work, we followed up on these results and investigated the interactions of 6-methyl-D-tryptophan and 6-methyl-L-tryptophan with both native and mirror-image cyclodextrins over a concentration range from 0 to 10 mM (ten concentration levels). Binding constants were subsequently estimated from mobility shifts using Scatchard analysis. The obtained data provided deeper insight into the mechanisms of chiral recognition and the differences in enantioselective interactions between native and mirror-image cyclodextrins.

## Conclusion

In our work, we confirmed that mirror-image cyclodextrins represent a new and promising class of chiral selectors with significant potential in enantioselective separations. Moreover, capillary electrophoresis proved to be a highly suitable technique for investigating these systems because of its minimal consumption of electrolytes and chiral selectors, enabling systematic studies even with scarce and expensive compounds such as L-cyclodextrins.

## Acknowledgements

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# Apparent pH of the Supercritical Carbon Dioxide-Based Fluids Used in Analytical Chemistry

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## Introduction

Supercritical carbon dioxide is an important fluid considered a green, sustainable solvent that may replace toxic, nonpolar organic solvents. The physicochemical properties of supercritical CO2 were thoroughly explored and described, including density, viscosity, dielectric properties, and miscibility with organic solvents. In current state-of-the-art analytical approaches, supercritical CO2 is typically mixed with polar organic modifiers and additives, which alter physicochemical properties. It was confirmed that CO2 can react with alcohol modifiers to form alkylcarbonic acid under supercritical fluid conditions, in the same manner as CO2 and water form carbonic acid. Such interaction creates an acidic environment with apparent pH $\leq$ 5.5, determined for CO2/methanol (MeOH) mixture [1,2]. Additionally, West et al. [2], described the apparent pH of CO2-MeOH solvents containing several additives, including acids, with an apparent pH close to 1, bases, and ammonium salts, with an apparent pH of 6-8. Nevertheless, the acidic-basic properties and apparent pH of different modifiers, including ethanol, isopropanol, acetonitrile (ACN), and MeOH/ACN mixtures, were not determined.Hence, the aim of the study was to systematically explore and determine the apparent pH of various $CO_2$/organic modifier/additive mixtures under various temperatures and pressures, used in supercritical fluid chromatography and supercritical fluid extraction.

## Experimental

Apparent pH was determined using spectral similarity analysis, exploiting systematic pH-dependent changes in the UV-VIS spectra of anthocyanins, i.e., pelargonidin, petunidin, cyanidin, and malvidin. Spectra acquired in $CO_2$-based fluids were compared with reference spectra measured in aqueous buffer solutions with determined pH in the range of 1 to 10. Reference spectra were defined as the averaged spectra at each pH, combining the measurements under various buffer/organic solvent ratios, temperatures, and pressures. Spectral similarity was quantified using the correlation coefficient. The resulting correlation-pH relationship was interpolated by spline fitting to assign the pH corresponding to maximum similarity. Only correlations > 0.9 were considered significant. Apparent pH was determined for individual anthocyanins, and the final value was calculated as their mean for the $CO_2$-based solvents.

Methanol, ethanol, isopropanol, acetonitrile, and methanol/acetonitrile in several ratios were tested as organic modifiers of $CO_2$. The concentration of the modifier ranged from 2 to 40 %. Additives included acids, i.e., formic, acetic, citric, trifluoracetic, and methanesulfonic acid, water, bases, i.e., ammonia in methanol, aqueous ammonia ($NH_4OH$), diethylamine, and triethylamine, and ammonium salts, i.e., ammonium formate, ammonium acetate, and ammonium fluoride, at various concentrations.

## Results

The acidic character of the $CO_2$-methanol mixture was determined with the apparent pH in the range 3 – 4.5, while for other tested modifiers, ethanol, isopropanol, and acetonitrile, the apparent pH corresponded to 4.5-5. Water as an additive resulted in an apparent pH decrease for $CO_2$/MeOH and $CO_2$/MeOH/ACN, while it remained unaffected for other organic modifiers.

Among the acidic additives, methanesulfonic acid and 0.5% formic acid produced a strong acidic environment, with apparent pH values near 1. When other acids, e.g., formic acid at lower concentrations, acidic acid, citric acid, were used, the apparent pH was strongly dependent on the modifier type and percentage. On the contrary, all tested basic additives and ammonia salts exhibited similar behavior within the study. The apparent pH stabilized at neutral conditions regardless of the tested conditions. A negligible effect of temperature (20-60 °C) and pressure (100–380 bar) on the apparent pH was observed.

## Conclusion

This study investigated the acidic-basic properties of $CO_2$-based solvents, typically used in analytical chemistry approaches. The correlations between UV-VIS spectra of anthocyanins in aqueous buffer solutions, their mixtures with organic solvents, and $CO_2$-organic solvent mixtures were used to determine the apparent pH of $CO_2$-based mobile phases.

## Acknowledgements

The authors gratefully acknowledge the financial support of the Project of the Czech Science Foundation (GAČR n. 25-15354S).

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# Development of Innovative Chiral Donor-Acceptor Stationary Phases for HPLC Enantioseparations

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## Introduction

In recent years, a wide range of chiral molecules has been investigated as potential chiral selectors (SOs) for enantiomeric separation by liquid chromatography. These chiral compounds exhibit substantial chemical and structural diversity, encompassing low-molecular-weight SOs, as well as macrocyclic compounds. Although a wide range of commercial chiral stationary phases (CSPs) is currently available, the development of novel and more versatile materials remains a major subject of research in the field of enantioselective separations. [1].

In this contribution, we report on the development of new donor–acceptor-type CSPs inspired by the structure of the commercially successful Whelk-O1 phase. Building on previous research, we introduce chiral stationary phases that incorporate the π-electron-rich tetrahydrophenanthrene (phenanthrone) unit and the π-electron-poor moiety, where we use the widely implemented 3,5-dinitrobenzoyl group, which exhibits superior efficiency in comparison to other electron-deficient aromatic units [2]. As in the original Whelk selector, the phenanthrone moiety is substituted with an alkyl chain that serves as a linker unit, covalently bonding the selector onto the silica carrier. The main structural modification lies in the variation of this anchoring moiety, potentially providing further interaction site for the analyte. Furthermore, we introduce an innovative method for anchroing the chiral SOs onto the modified silica. Additionally, by modifying the size of the silica particles and the length of the column hardware, we successfully developed highly functional chiral columns.

## Experimental

The prepared chiral sorbents were slurry-packed into stainless steel chromatographic columns of various dimensions. The resulting chiral columns were evaluated using HPLC system ECS05 (ECOM spol. s.r.o., Czech Republic) controlled by Clarity chromatographic workstation (DataApex, Czech Republic). The instrument was equipped with an integrated PC unit, a binary pump, degasser, photodiode array detector (PDA), solvent tray and an autosampler.

## Results

Systematic evaluation of the new CSPs employing HPLC demonstrated excellent chiral recognition across multiple classes of analytes. By adjusting the mobile phase and utilizing both enantiomers of the stationary phase, we were able to fine-tune retention factors, selectivity, and the elution order of analyte enantiomers. Among the most promising results, we developed the chiral separation of novel fentanyl analogs – compounds with potent pharmacological activity and increasing relevance in forensic toxicology. Further noteworthy outcomes include the successful separation of other important chiral analytes, including β-blockers, cannabinoids, and sulfoxides among others (**Figure 1**).

## Conclusion

We successfully synthesized several chiral stationary phases, which exhibited chiral separation of a diverse range of chiral analytes. Enantioseparation using these CSPs can be operated in various chromatographic modes and is comparable to that of commercial donor-acceptor type columns. Structural modifications of the current design aimed to increase the enantioseparation are subject of a future research.


## Acknowledgements

This work is supported by TACR, project no. TQ15000270.


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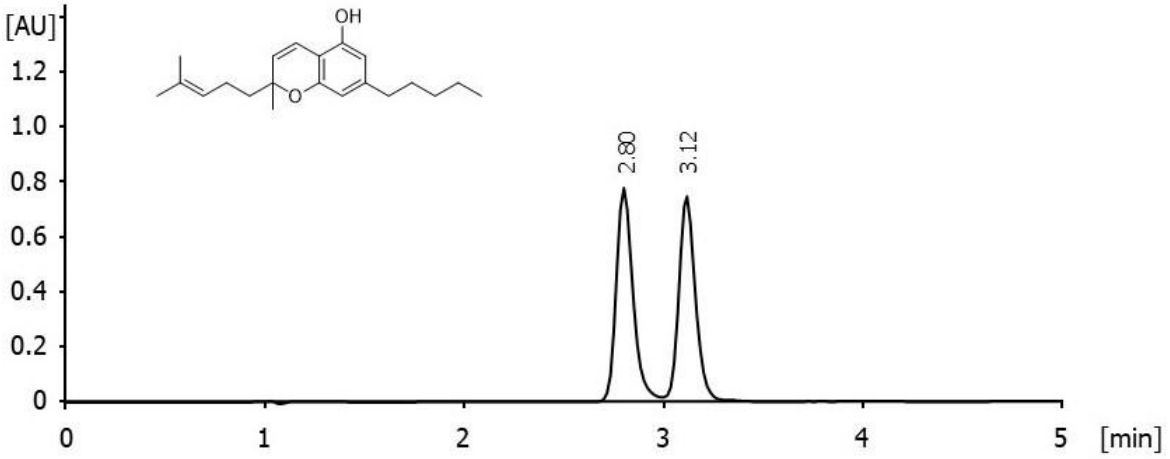
**Figure 1.** Chiral resolution of cannabichromene (CBC) on a prototype CSP.



# Preliminary Design of Enhanced NanoCEasy CE-MS Interface

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## Introduction

The nanoCEasy interface, introduced by Prof. Neusüß group (Aalen University, Germany), has proven to overcome the key limitations of existing capillary electrophoresis-mass spectrometry (CE-MS) coupling strategies: low sensitivity of conventional coaxial sheath-liquid interfaces caused by high dilution of the CE effluent; cumbersome manipulation and mechanical fragility of fitting-based nanoflow electrospray assemblies; complicated observation due to weak spray contrast in daylight; complicated adaptation of CE-MS interfaces to different MS instruments. The nanoCEasy interface counters these limitations through: a rapid fitting-free setup of capillaries, electrode, and glass emitter, all inserted into transparent 3D-printed housing enabling visual inspection and retaining nanoESI-level sensitivity; a two-capillary switching-valve functionality for flexible rinsing and matrix diversion; compatibility with high-electroosmotic flow separations; a mounting design compatible with MS instruments from multiple vendors (Bruker, Thermo, Agilent). The utility of the interface has been proven across a range of analytical applications: peptide mapping [1], monoclonal antibody charge-variant analysis [2], multi-platform metabolomics combined with CE-MS [3], and flow-rate characterization studies [4], confirming it as an easy-to-use, sensitive, and robust nanoflow CE-MS interface suitable for the most demanding analytical applications.

The present work reports further progress on nanoCEasy interface development, in particular, mechanical enhancements: incorporation of compact XYZ positioning stage, miniaturization of a motorized translation stage for both the separation and sheath-liquid capillaries, and an enclosure enabling the interface operation isolated from the ambient environment.

## Experimental

Design requirements were established through consultations with Prof. Neusüß's research group members, including demonstrations of the nanoCEasy interface under operating conditions, from which user demands, design constraints, and technical requirements were systematically collected. Preliminary sketches and the overall mechanical concept were developed in Trimble SketchUp 2019. Models of commercial off-the-shelf components were obtained from the respective manufacturers' online repositories and imported into the SketchUp environment for evaluation and further use. Production-ready designs, supplementary drawings, and manufacturing-ready files were subsequently finalized in SolidWorks 2025.

## Results

A mechanically enhanced nanoCEasy interface was developed as a direct engineering elaboration of the original design, preserving its analytical performance while substantially increasing engineering refinement. The enhanced design integrates: a manual XYZ tip-tilt micrometric stage for reproducible alignment of the emitter; a motorized axial translation stage for each capillary, enabling automated switching between separation and conditioning modes. Emitter replacement is further simplified by a dedicated tilting mechanism and an openable enclosure. Nanoelectrospray monitoring and capillary positioning are supported by a dual digital microscope observation setup employing epifocal illumination alongside an embedded laser diode beam, permitting precise visual alignment towards the MS inlet, real-time assessment of the emitter state, and increased spray contrast via laser-assisted illumination.

## Conclusion

The presented design, while retaining the original interface layout, introduces several targeted engineering improvements. The latter are expected to improve the repeatability of emitter alignment, optimize automated capillary switching during long measurement sessions, enhance visual observation of the nanoelectrospray plume, and enable operation in stable environmental conditions, collectively advancing the nanoCEasy interface towards fully automated, routine CE-MS analysis. Further work is required, including manufacturing of custom-designed components, device assembly, and experimental characterization.

## Acknowledgements

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# Capillary micellar electrokinetic chromatography – a powerful method for achiral and chiral separations of brefeldin A and its derivatives

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## Introduction

Brefeldin A (BFA), see Fig. 1, is a 13-carbon macrolide lactone antibiotic possessing antitumor, antiviral, and antifungal activities. It was first isolated (with the original name decumbin) from *Penicillium decumbens* by Singleton et al. [1]. Although BFA exhibits strong cytostatic activity accompanied by low toxicity, it could not be established as an anticancer agent because of its low bioavailability and slow pharmacokinetics. Therefore, it was necessary to prepare its derivatives [2] with cytostatic activity similar to that of BFA but with improved *in vivo* stability and solubility in aqueous systems. Six of such derivatives are shown in Fig.1. The aim of this work was to analyze and characterize them and to separate their diastereomeric forms.

## Experimental

All compounds were analyzed by capillary micellar electrokinetic chromatography (MEKC). in Beckman-Coulter MDQ analyzer equipped with diode array UV-absorption detector set at 200 nm, and bare fused silica capillary, ID/OD 50/375 μm, total/effective length 399/297 mm. The diastereomers were separated using various concentrations (10-50 mg/mL) of chiral selector, hydroxypropyl-β-cyclodextrin (HP-β-CD), in the background electrolyte (BGE) composed of 20 mM Tris, 5 mM $H_3PO_4$, 50 mM SDS, pH 8.1. For determination of electrophoretic mobilities and retention factors, methanol, and quinine were used as electroosmotic flow (EOF) and micellar phase markers, respectively.

## Theory

The purity of the *i*-th component of the sample, $P_{CA}(i)$, was quantified using relative corrected peak area of this component [3] defined by equation (1)

$$P_{CA}(i) = \frac{A_c(i)}{\sum A_c(i)}, \quad i = 1, \ldots, n \qquad (1)$$

where $A_c(i)$ is corrected (migration time normalized) area of the i-th peak and n is the number of sample components.

The effective electrophoretic mobility of the analyte A, $m_{A,eff}$, was calculated from MEKC experimental data using the equation (2):

$$m_{A,eff} = \frac{L_{tot}L_{det}}{U_{sep}}\left(\frac{1}{t_{mig,A}} - \frac{1}{t_{eof}}\right) \qquad (2)$$

where $t_{mig,A}$ and $t_{eof}$ are the migration times of the analyte and of EOF marker (DMSO), respectively, $L_{tot}$ and $L_{det}$ are the total and effective capillary lengths, respectively, and $U_{sep}$ is the applied separation voltage.

Retention factor *k* was calculated using equation (3):

$$k = \frac{t_{mig,A} - t_{eof}}{t_{eof}\left(1 - \frac{t_{mig,A}}{t_{mic}}\right)} \qquad (3)$$

where $t_{mig,A}$, $t_{eof}$, and $t_{mic}$ are the migration times of the analyte A, and the EOF and micellar phase markers, respectively.

## Results

The sample purity varied from 32.9 % to 92.3 %. All pairs of diastereomers were baseline separated with resolutions higher than 1.5, see e.g. Fig. 2. The effective electrophoretic mobilities varied in the range from -36.9 to -34.9 [$10^{-9}$ $m^2V^{-1}s^{-1}$] in BGE free of chiral selector and in the range from -18.9 to -5.2 [$10^{-9}$ $m^2V^{-1}s^{-1}$] in the BGE containing 60 mg/mL of HP-β-CD. Retention factors of the brefeldin A and its derivatives varied from 15 to 126.

## Conclusion

MEKC with SDS as micellar pseudostationary phase proved to be a suitable method for analyses of neutral compounds. Achiral and chiral separations of brefeldin A and its derivatives were achieved using different concentrations of HP-β-CD in BGE.

It was found that the migration order of diastereomers **3** and **4** is dependent on the concentration of HP-β-CD in BGE (see Fig. 3).


## Acknowledgements

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**Figure 1**. Chemical structures of BFA and its derivatives.

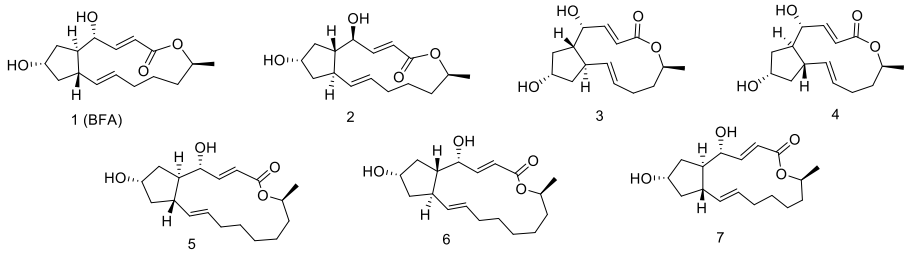



**Figure 2.** MEKC separation of the mixture of analytes **4, 1, 7,** and **6** at concentration 30 mg/mL of HP-β-CD in BGE

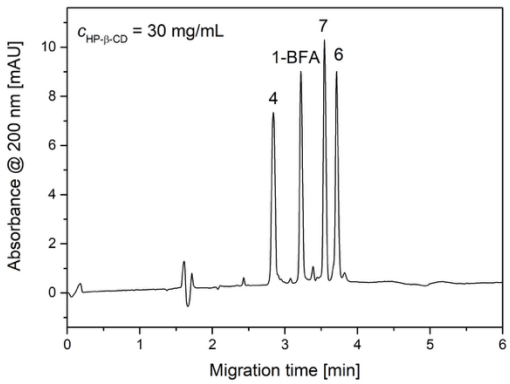
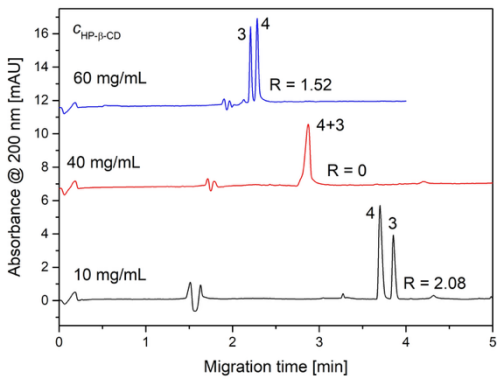



**Figure 3.** MEKC separation of diastereomers **3** and **4** at concentrations 10, 40 and 60 mg/mL of HP-β-CD in BGE; R – resolution.



# Applications of Magnetic Particles with Immobilized Albumin in Combination with CE for Plasma Protein-Drug Binding Studies

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## Introduction

Serum albumins play a key role in the transport and distribution of drugs in plasma; therefore, understanding the interactions between drugs and albumins is essential for evaluating pharmacokinetic and pharmacodynamic properties as an integral part of new drug development [1]. Magnetic particles have been previously used in protein-ligand studies, for example with immobilized enzymes [2].

## Experimental

Two different approaches based on magnetic microparticles with immobilized bovine serum albumin (MP-BSA) were tested to determine drug-albumin interactions, with an emphasis on low sample consumption and high throughput: a) affinity capillary electrochromatography (ACEC) on a fritless capillary MP-BSA column and b) drug extraction using MP-BSA in CE vials followed by magnetic separation and analysis of its free form by micellar electrokinetic chromatography (MEKC).

## Results

MP-BSA produced via glutaraldehyde coupling was selected for such studies using both above mentioned approaches due to its higher yield of immobilized BSA while maintaining drug-binding properties compared to the method using carbodiimide.

The MP-BSA loading into the capillary for ACEC column creation was optimized to achieve large capacity while maintaining column permeability. In addition, the binding capacity of the ACEC column was increased by using a longer bed of MP-BSA, which was held in the capillary by 25 pairs of permanent magnets in an attractive orientation within a 3D-printed holder placed in the capillary cassette. The resulting BSA containing and blank (without BSA) columns were stable under rinsing and analysis conditions. The columns were used to determine the equilibrium binding constant of the model drug – phenylbutazone using frontal electrokinetic injection. Differences of breakthrough times at different phenylbutazone concentrations allowed its calculation.

In the second approach, MP-BSA were incubated with different concentrations of warfarin in in glass inserts in CE vials. The extraction of MP-BSA together with bound drug fraction was after sufficient incubation achieved by insertion of small neodymium magnet inside the vial near the insert and the amount of bound drug was after magnetic separation determined indirectly by analysing its free form by means of MEKC. This setup allowed two-buffer systems application: physiological phosphate buffer for incubation and phosphate-borate, SDS containing buffer as background electrolyte. All of these steps were performed directly within the CE system. This combination offers advantages such as a rapid and simple separation of the protein-drug complex from the incubation mixture, relatively low reagent consumption, and speed of analysis with possible automatization.

## Conclusion

The proposed MP based approaches could serve in high-throughput assays as low sample consumption alternatives that can be used not only for the binding parameter determination but also to screen for promising protein ligands from different type of samples.

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# Microplastics without borders: A Cross-Regional Study of Microplastic Morphology and Composition

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## Introduction

Microplastic (MP) pollution has emerged as a pervasive global environmental threat, yet the characteristics and transport dynamics of these particles differ significantly across diverse hydrographical systems [1, 2]. While marine environments act as ultimate sinks for plastic debris, inland freshwater systems serve as critical conduits and secondary reservoirs [3]. This study provides a comparative characterization of microplastics in two contrasting geographical regions: the Canary Islands and the inland waters of Slovakia. The Canary Archipelago, located within the North Atlantic Subtropical Gyre, represents a marine hotspot where MP accumulation is driven by large-scale oceanic currents and localized seasonal tourism pressure. In contrast, Slovakian freshwater bodies—representative of Central European land-locked systems—are primarily influenced by riverine hydrodynamics and point-source anthropogenic inputs from urban and industrial activities. By employing harmonized analytical techniques to assess polymer composition, morphology, and size distribution, this research evaluates how distinct environmental stressors and transport mechanisms influence the MP profile. Understanding these regional variations is essential for developing targeted mitigation strategies and refining global plastic budget models.

## Experimental

Microplastic (1–5 mm) and mesoplastic (up to 10 mm) samples were systematically collected from eleven beaches across the eastern Canary Islands and Ruzin water reservoir areas. Laboratory processing involved manual debris removal and categorization by morphology and color. Chemical characterization was performed via Attenuated Total Reflectance Fourier Transform Infrared Spectrometry (ATR-FTIR). Spectra were recorded in the range of 4000–500 $cm^{-1}$, with a spectral resolution of 4 $cm^{-1}$, averaging 16 scans per measurement samples processed using Hummel Polymer Library.

## Results

The comparative analysis reveals distinct microplastic (MP) profiles and transport dynamics between the two study regions. In the Canary Islands, MP distribution is primarily governed by large-scale oceanographic drivers, specifically the Canary Current and North Atlantic trade winds. North and Northeast-facing beaches function as significant accumulation hotspots, with concentrations exceeding 500 items/$m^2$, characterized predominantly by white/transparent fragments (>50%) and weathered resin pellets. Polymer identification confirmed that polyethylene (PE) and polypropylene

(PP) constitute 91–98% of the total plastic mass, reflecting global production trends and the long-range transport of buoyant polyolefins.
In contrast, data from Slovakian inland waters indicate a high abundance of smaller debris, particularly in the 40–999 μm (509 items) and 1–1.9 mm (420 items) size classes. While PE (819 items) and PP (730 items) remain the primary contaminants, the Slovakian samples exhibit a significantly higher prevalence of polystyrene (251 items), largely in the form of T/W foam. This suggests that while Canarian pollution is shaped by oceanic "sinks" and seasonal tourism, Slovakian freshwater bodies are heavily influenced by localized riverine transport, urban runoff, and point-source industrial inputs.

**Conclusion**
To address the ecological risks of these particles acting as chemical vectors, future research will focus on **absorbed organic pollutant analysis**. Quantifying the presence of pharmaceuticals, pesticides, and industrial chemicals adsorbed onto different polymer types is essential for assessing the total toxicological burden and developing region-specific mitigation strategies for both marine and freshwater ecosystems.

**Acknowledgements**
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# Advanced Characterization of New Fossil Resins: From Screening to Molecular Fingerprinting

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## Introduction

Amber is a complex fossilized resin primarily composed of polymerized terpenoids [1, 2]. Its chemical composition reflects both its botanical origin and the environmental conditions during its long maturation [1, 3]. Standard macroscopic observations are often insufficient to distinguish between different geological types or to determine their precise origins [1, 4, 5]. Therefore, advanced analytical methods are essential for a reliable characterization. This study employs a multi-instrumental approach to investigate amber samples from six distinct regions: the Dominican Republic, the Baltic region, Myanmar (Burmite), Hungary (Ajkaite, Iharkút), and Romania (Telegdite). We focused on comparing their molecular structures and diagenetic maturity.

## Experimental

The analytical workflow was designed from non-destructive to destructive techniques. Initial screening was performed using infrared micro-spectroscopy (FTIR) on a Lumos II instrument (Bruker). We used an ATR ZnSe crystal and a TE-MCT detector to record spectra from 4000 to 670 $cm^{-1}$. Measurements were taken with 32 accumulations at a resolution of 4 $cm^{-1}$. Raman microspectroscopy was performed on a DXR2 system (Thermo Scientific). This system was equipped with a 785 nm laser operating at 15 mW power. The analysis utilized a 2-second exposure time and 60 sample exposures.

Finally, the molecular composition was analyzed via Py-GC/MS. The configuration included an Agilent 8890 GC coupled with a 5977B MSD and a Frontier Lab multi-shot pyrolyzer. Microsamples of approximately 0.1 mg were combined with 1 µL of HMDS for in situ silanization. Pyrolysis was conducted at 480 °C for 6 seconds. The GC oven program started at 40 °C for 5 min, ramped at 10 °C/min to 320 °C, and was held at 320 °C for 1 min. Identification of compounds relied on the NIST20 library and published results from previous amber studies.

## Results

The analytical results revealed distinct molecular "fingerprints" for each amber type. FTIR spectroscopy identified key differences in the carbonyl region. Baltic and Dominican ambers showed diagnostic shifts at 1700 $cm^{-1}$. In contrast, the Hungarian (Ajkaite, Iharkút) and Romanian (Telegdite) samples showed absorption near 1686 $cm^{-1}$. Moreover, the region of "Baltic shoulder" between 1160–1250 $cm^{-1}$ highlighted key differences among the samples. Raman data allowed us to track the maturation of the resins through the $I_{1645}/I_{1450}$ intensity ratio. The youngest samples from the Dominican Republic (15–20 Ma) showed the highest ratio (~0.80), indicating the lowest maturity, whereas Burmite (~100 Ma) exhibited advanced maturation with a significantly lower ratio (~0.40). The Hungarian Ajkaite and Iharkút resins occupied an intermediate position between 0.55 and 0.60.

Py-GC/MS provided conclusive evidence for resin provenance. Baltic amber was characterized by succinic anhydride and abietane derivatives typical of the *Pinaceae* family. These markers were completely absent in the Hungarian samples. Instead, the Ajkaite and Iharkút profiles were dominated by specific sesquiterpenoids, such as cedrol, widdrol, and calamenene. This distribution suggests a botanical affinity with the *Araucariaceae* or *Cupressaceae* families. Furthermore, Hungarian samples showed a high abundance of $C_2$-alkyl phenols and aromatic markers, such as phenanthrene and ionene. This profile reflects a lignitic depositional environment within the Ajka Coal Formation alongside advanced geological coalification.

**Conclusion**

The combination of micro-spectroscopic methods and Py-GC/MS is a highly effective tool for studying fossil resins. ATR-FTIR and Raman screening provide rapid, non-destructive insights into maturity and functional groups. However, Py-GC/MS remains essential for identifying specific geochemical and botanical markers. This integrated strategy was crucial for successfully characterizing Ajkaite and Iharkút. It clearly distinguished these variants from Baltic succinite and confirmed their *Araucariaceae* or *Cupressaceae* origin. Ultimately, this demonstrates the vital role of a multi-instrumental approach in archeometric and geological research.

**Acknowledgements**

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# Determination of the binding constants of complexes of cucurbit[7]uril with aromatic cationic ligands by pressure-assisted affinity capillary electrophoresis

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## Introduction

The cucurbit[*n*]urils (CB[*n*], *n* = 5–8, 10) are a family of cyclic host molecules comprised of *n* glycoluril units bridged by 2*n* methylene groups [1]. The CB[6], CB[7], and CB[8] are similar to α-, β-, and γ-cyclodextrins (CDs) but only rarely they have been applied as selectors in affinity capillary electrophoresis (ACE) [2, 3], mainly due to their limited solubility in water. The goal of this work was to study the strength of the CB[7] complexes with aromatic cationic ligands **1**–**5** (see Fig. 1A) [4] in mixed hydro-organic solvent (water/acetonitrile) by pressure-assisted ACE (PA-ACE).

## Experimental

PA-ACE analyses were carried out in CE 7100 analyzer (Agilent, Waldbronn, Germany) in hydroxypropyl cellulose coated fused silica capillary (id/od 50/375 μm, total/effective length 500/415 mm) using UV-vis absorption detector set at 200 and 260 nm. The background electrolyte (BGE) was composed of 9.5 mM NaOH, 6.25 mM $H_3PO_4$, pH 7.10, 25% v/v acetonitrile, and contained various concentration of CB[7] (0–1 mM). Separation voltage was 25 kV, electric current 8-9 μA, and temperature 25 °C. To speed up the measurements, external pressure of 20 mbar was applied at the capillary inlet end. The ligands at 0.1 mM concentration were introduced into the capillary hydrodynamically (10 mbar × 10 s, 3.4 nL).

## Results

The interaction between ligand L and receptor R with 1:1 stoichiometry results in formation of complex LR. The strength of this complex is characterized by the binding constant of the complex LR, $K_b$, defined by the equation (1):

$$K_b = \frac{c_{LR}}{c_L c_R} \tag{1}$$

where $c_L$, $c_R$, and $c_{LR}$ are the equilibrium concentrations of the ligand L, receptor R, and complex LR, respectively.

Dependence of the effective mobility of the ligand L, $m_{eff,L}$, on the concentration of the receptor R, $c_R$, in the BGE is described by equation (2):

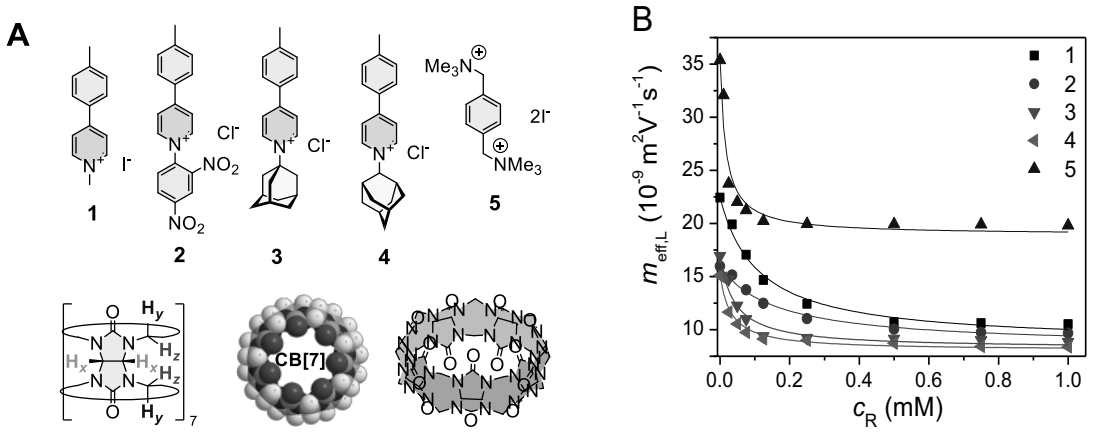
$$m_{eff,L} = \frac{m_L + m_{LR} K_b c_R}{1 + K_b c_R} \tag{2}$$

where $m_L$ is the mobility of ligand L in BGE free of the receptor R and $m_{LR}$, is the mobility of complex LR in the BGE containing receptor R. The effective mobility of ligand L, $m_{eff,L}$, is measured by PA-ACE in the BGE containing various concentrations of receptor R. The mobility of free ligand L, $m_L$, is determined by CZE of ligand L in BGE free of receptor R. The binding constant, $K_b$, and the mobility, $m_{LR}$, of the LR complex, are parameters of the equation (2). They are obtained by non-linear regression analysis of this dependence (see Fig. 1B) using equation (2) as the fitting function [5]. They are presented in Table 1.

**Table 1.** The binding constants, $K_b$, and the electrophoretic mobilities, $m_{LR}$, of the ligand-receptor (LR) complexes, and the electrophoretic mobilities, $m_L$, of the ligands in the BGE free of receptor R. $r^2$, coefficient of determination.

| Ligand | $K_b \pm$ SD (L mol$^{-1}$) | RSD (%) | $m_{LR} \pm$ SD ($10^{-9}$ m$^2$V$^{-1}$s$^{-1}$) | $m_L \pm$ SD ($10^{-9}$ m$^2$V$^{-1}$s$^{-1}$) | $r^2$ |
|---|---|---|---|---|---|
| **1** | $(9.10 \pm 1.19) \times 10^3$ | 13.0 | $8.69 \pm 0.49$ | $22.43 \pm 0.08$ | 0.98776 |
| **2** | $(6.14 \pm 0.93) \times 10^3$ | 15.1 | $8.36 \pm 0.35$ | $15.96 \pm 0.06$ | 0.98577 |
| **3** | $(2.39 \pm 0.51) \times 10^4$ | 21.5 | $8.23 \pm 0.41$ | $16.93 \pm 0.05$ | 0.95641 |
| **4** | $(3.13 \pm 0.74) \times 10^4$ | 23.6 | $8.05 \pm 0.35$ | $15.12 \pm 0.01$ | 0.94849 |
| **5** | $(1.13 \pm 0.07) \times 10^5$ | 0.1 | $19.56 \pm 0.09$ | $35.37 \pm 0.04$ | 0.99877 |

**Figure 1.** A) Structures of aromatic cationic ligands **1**–**5** and receptor CB[7]. B) Graphs of the nonlinear regression analysis of dependence of the effective mobilities, $m_{eff,L}$, of ligands L on the concentration, $c_R$, of the receptor R in the BGE.




## Conclusions

PA-ACE proved to be a suitable method for quantitative evaluation of the strength of noncovalent interactions of CB[7] receptor with aromatic cationic ligands. The binding constants of CB[7]-ligand complexes were rather high, in the order of $10^3$–$10^5$ L mol$^{-1}$.


## Acknowledgements

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# Investigation of acid-base properties of secondary amides and 2-(4-butoxyphenyl)acetic acid by capillary electrophoresis

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## Introduction

Amides are an important class of nitrogen containing compounds in organic chemistry and also potential precursors for the synthesis of numerous natural products, potent pharmaceuticals, and bioactive polymers. The aim of this work was to investigate acid-base and electromigration properties of secondary amides and 2-(4-butoxyphenyl)acetic acid (compounds **1**–**6** in Fig. 1) using capillary electrophoresis (CE). Their acidity constants, actual and limiting ionic mobilities, and hydrodynamic radii should be determined.

## Experimental

CE experiments were performed on the ProteomeLab PA 800 analyzer (Beckman Coulter). For analysis, internally uncoated fused silica capillaries with the total/effective length of 40.5/30.1 cm, and the ID/OD of 50/375 μm were utilized. For data acquisition and evaluation, ProteomeLab PA 800 software version Karat (Beckman Coulter), the Clarity station (DataApex), Origin Pro 9.1 (OriginLab Corp.) and freeware Anglerfish were used. The analytes were dissolved in methanol at concentrations 0.18-0.29 mg/200 μL and detected by UV at 200 nm. The electrophoretic mobilities were measured at constant temperature (25°C) and ionic strength (25 mM).

## Results

The acidity constants of compounds **3**–**6** were estimated to be <0 as they were neutral in the pH range of 0.95–9.25. At pH > 9.25 they started to decompose. For the determination of acidity constants of compounds **1** and **2**, their effective electrophoretic mobilities, $m_{\mathrm{eff}}$, were measured in aqueous background electrolytes (BGEs) in the pH ranges 1.47–6.75 and 7.24–11.23, respectively. The dependence of effective mobility, $m_{\mathrm{eff}}$, of univalent acid HA on pH is described by equation (1) [1]:

$$m_{eff} = \frac{m_{A^-}}{1+10^{pK_a^{mix}-pH}} \qquad (1)$$

where $m_{A^-}$ is the actual ionic mobility of anion $A^-$ and $pK_a^{mix}$ is the mixed acidity constant. The Eq. (1) was used as regression function to fit the pH dependence of the effective mobility gained by CE. The inflection point of the obtained sigmoidal-shaped curve corresponded to the $pK_a^{mix}$ value of the acid HA. Then, the thermodynamic acidity constant, $pK_a$, was calculated from the following relation:

$$pK_a = pK_a^{mix} - log\gamma_{A^-} \qquad (2)$$

CE data was used for the p$K_a$ determinations with the assistance of two programs, commercial program Origin and freeware AnglerFish [2] (see Table 1). The latter program enabled to determine also limiting ionic mobilities, $m_{A^-,lim}$, from which the hydrodynamic radius, $r_{A^-}$, of anion $A^-$ could

be calculated:

$$r_{A-} = \frac{q_{A^-}}{6\pi\eta m_{A^-,lim}} = \frac{z_{A^-}e}{6\pi\eta m_{A^-,lim}} \quad (3)$$

where $q_{A^-}$ is the effective charge and $z_{A^-}$ is the charge number of anion $A^-$, $e$ is the elementary charge, and $\eta$ is the viscosity of the BGE.

## Conclusion

CE proved to be a suitable method for the investigation of acid-base properties. The p$K_a$ values of compounds **1** and **2** determined from CE data using program Origin were 4.06 and 9.41, and with program AnglerFish 4.08 and 9.41, respectively. The p$K_a$ values of compounds **3**–**6** were estimated to be <0. The theoretically calculated p$K_a$ values of the amide NH group of compounds **5** and **6** were 20.6 and 11.0, respectively [3]. The hydrodynamic radii of compounds **1** and **2** were 0.39 and 0.38 nm, respectively.


## Acknowledgements

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**Figure 1.** Molecular structures of 2-(4-butoxyphenyl)acetic acid and secondary amides.

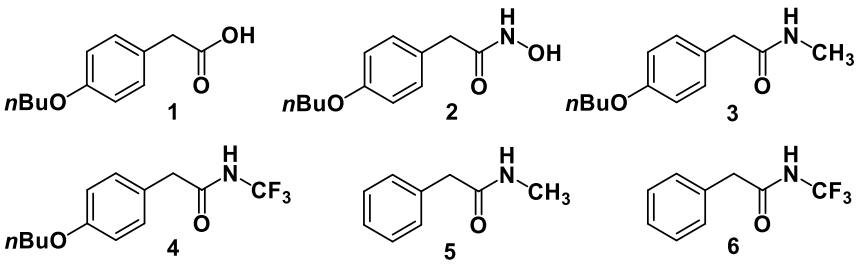


**Table 1.** Thermodynamic acidity constants of compounds **1** and **2**, and actual and limiting ionic mobilities and hydrodynamic radii of their anionic forms, and estimated $pK_a$ values* of compounds **3**–**6** (undissociated in pH range of 0.95–9.25).

| Comp. | p$K_a$ ± SD[a] | p$K_a$ ± SD[b] | $m_{A^-}$ ± SD[a] [$10^{-9}$ m$^2$ (Vs)$^{-1}$] | $m_{A^-,lim}$ ± SD[b] [$10^{-9}$ m$^2$ (Vs)$^{-1}$] | $r_{A^-}$ [nm] | Charge number |
|---|---|---|---|---|---|---|
| **1** | 4.06 ± 0.02 | 4.08 ± 0.01 | -20.2 ± 0.1 | -24.8 ± 0.1 | 0.39 | -1 |
| **2** | 9.41 ± 0.06 | 9.41 ± 0.09 | -20.4 ± 0.6 | -25.1 ± 0.9 | 0.38 | -1 |
| **3-6** | <0* | <0* | 0 | 0 | - | 0 |

[a]p$K_a$ determined by CE using program Origin. [b]p$K_a$ determined by CE using free software AnglerFish. [a] $m_{A^-}$, the actual ionic mobilities at ionic strength of 25 mM (determined by CE using program Origin). [b]$m_{A^-,lim}$, the limiting ionic mobilities at zero ionic strength (determined by CE using program AnglerFish).

# Alternative approach to using diazomethane for GC/MS: derivatization reaction in the gas phase

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## Introduction

Diazomethane is one of the most reactive methylating agents used both for organic synthesis and analytical derivatization. The standard procedure for $CH_2N_2$ derivatization consists of two steps: 1) preparation of $CH_2N_2$ solution, usually in diethyl ether, and 2) performing the reaction by mixing prepared $CH_2N_2$ solution with sample. [1,2] This protocol creates the need to prepare, store and manipulate with a solution of carcinogenic and explosive gas in highly volatile and flammable solvent. Also, the sample must be dry or diluted in suitable solvent (such as diethyl ether, toluene, dioxane, etc.) to prevent instant degradation of $CH_2N_2$, which limits the use of this method for aqueous samples. For these reasons, possibilities of utilizing gas phase reaction of analyte with $CH_2N_2$ for derivatization followed by GC/MS analysis were explored. This approach enables combining both steps into one while eliminating the use of diethyl ether and avoiding the risk of direct exposure to $CH_2N_2$. The aim of this communication is to introduce a novel method for generation of diazomethane and its application for derivatization of volatile compounds in the gas phase. The method is suitable for detection of acetic acid in aqueous samples.

## Experimental

For preliminary experiments 20 mg of N-nitroso-N-methylurea (NMU, diazomethane precursor) was weighted into the 40 ml headspace vial and closed with screw cap with septum. Diazomethane was released by careful injection of 1 ml of 1M NaOH through the septum. After 2 min equilibration 2 ml of the gas phase containing diazomethane was withdrawn with injection syringe and transferred to 40 ml headspace screw capped sample vial containing trace amount of acetic acid vapour in gas phase of zero air.

The apparatus needed for performing the gas phase reaction with aqueous sample was very simple and assembled from common laboratory equipment. First, 10 ml of aqueous sample was placed in the standard 40 ml headspace vial with magnetic stirring bar. Then a small glass container with 20 mg of NMU was secured above the water surface* inside the larger vial. The larger headspace vial was tightly closed with screw septum cap and then the water sample was acidified by addition of 0.5 ml of 1M $H_2SO_4$ through septum using injection syringe. The acidified sample was left stirring for at least 20 min to achieve equilibrium. Using gastight syringe, 2 ml of gas phase aliquot was analysed by GC/MS as blank sample.

To perform the derivatization reaction, diazomethane was generated *in situ* by decomposition of the precursor. The septum was pierced by injection syringe and 6M KOH was added to the small container drop by drop until all NMU dissolved. Without delay, 2 ml of gas phase aliquot was analysed by GC/MS for detection of methyl acetate.

* **Two setups were tested** for securing the small precursor container inside the larger vial. One option is using a glass insert placed in a holder that is hanged on the edge of the vial and fastened

with the screw cap. A second option is using a 2 ml crimp top glass vial propped up by a piece of plastic-covered metal wire twisted around the neck.

**Results**

For preliminary experiments in this study a small amount of the gas phase containing diazomethane was injected into the headspace vial with gas sample containing trace amount of acetic acid vapours. Preliminary experiments revealed that acetic acid reacts with diazomethane in the gas phase readily and that resulting methyl acetate can be detected even at concentrations that are far below the detection limit of acetic acid itself.

Simple and very efficient gas phase preparation of methyl ester and significantly better response of methyl acetate relative to free acetic acid provide a fundamental advantage in the determination of trace amounts of acetic acid in aqueous samples. For this purpose, sample of water was placed in 40 ml headspace vial equipped with glass container carrying diazomethane precursor. Sample was closed with septum screw cap, placed on magnetic stirrer and acidified with sulfuric acid. Septum was pierced with injection syringe and diazomethane was released by suitable amount of potassium hydroxide solution that was carefully added dropwise to the precursor container. Direct analysis of the headspace phase was performed immediately. Gas phase derivatization and GC/MS analysis enable detection of trace levels of acetic acid in aqueous samples in ppm level without any further sample pretreatment or extraction steps.

**Conclusion**

Gas phase derivatization, where analyte and derivatizing agent react in the headspace phase of an airtight vial, seems to be a promising approach to sample preparation for GC/MS analysis. Diazomethane is highly reactive and gaseous by nature, which makes it an ideal derivatization agent for this approach. Acetic acid is volatile and present in the gas phase in sufficient amount. The product of the derivatization, i.e. methyl acetate, provides better response during GC/MS analysis, thus derivatization by diazomethane leads to lower limit of detection.

**Acknowledgements**

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# Advanced Pollen Profiling by ASAP-MS Using Standard and Modified Capillary: Chemical Characterization of Intact and Modified Pollen

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## Introduction

Chemical characterization of pollen grains is challenging due to the chemically resistant nature of sporopollenin.[1] ASAP-MS allows the direct and rapid analysis of plant material without the need for solvent extraction, which is often associated with analyte loss and the formation of artifacts. Combined with a high-resolution analyser and the capability for tandem MS experiments, this technique enables detailed structural identification of the present metabolites.

The ASAP-MS technique has long been utilized in the analysis of synthetic polymers and is therefore potentially suitable for characterizing the biopolymers that constitute the pollen exine. This contribution explores the possibilities of metabolic profiling of intact pollen, as well as the characterization of the pollen exine after acid hydrolysis. Furthermore, it evaluates the use of ASAP-MS method as an alternative procedure for palynological expertise in recent and potentially also historical materials.

## Experimental

For ASAP-MS experiments, a modification of a previously published method[2,3] was utilized. This involves adapting of a commonly used glass capillary allowing the direct monitoring of targeted chemical modifications of the pollen and processes occurring during the mass spectrometry experiment. The methodology was optimized using commercial bee pollen and botanical *Corylus avellana* pollen samples. To study the sporopollenin skeleton, samples underwent pre-treatments including defatting and acid digestion (HCl, $H_3PO_4$). A Waters SELECT SERIES Cyclic IMS High-resolution mass spectrometer (Waters, Milford, USA), equipped with a cyclic ion mobility cell and an ASAP source operated in positive and negative ion mode, was used. The effect of vaporization temperature on the spectra appearance was studied in the range 200–600 °C. A corona current of 3 μA was applied.

## Results

Experiments with intact pollen confirm that ASAP-MS is a suitable technique for the analysis of low-to-medium polar low-molecular-weight metabolites present in the pollen intine. Through chemical hydrolysis of the pollen most active metabolites in the intine can be removed to reveal the structure of the exine. In the hydrolysed material, naringenin was detected and subsequently confirmed via tandem MS experiments and comparison with an authentic standard. This polyphenol and its glycosides (e.g., naringenin deoxyhexoside, Fig. 1) are present in all studied pollen materials with relatively high intensity and are released from the exine polymer structure under ASAP-MS conditions. Furthermore, structures containing coumaric and kojic acid were preliminarily identified, which are likely also constituents of the sporopollenin skeleton.

## Conclusion

A method has been developed for the direct analysis of intact pollen and pollen exine, serving as a supportive tool for the palynological expertise of recent pollens. Given the stability of the pollen exine, the method is potentially applicable to the study of historical pollen remains. Targeted experiments for profiling components of the pollen exine are ongoing, focusing on the analysis of minor signals in the spectra. Additionally, a method for studying metabolites ether-bound to sporopollenin is currently under development.


## Acknowledgements

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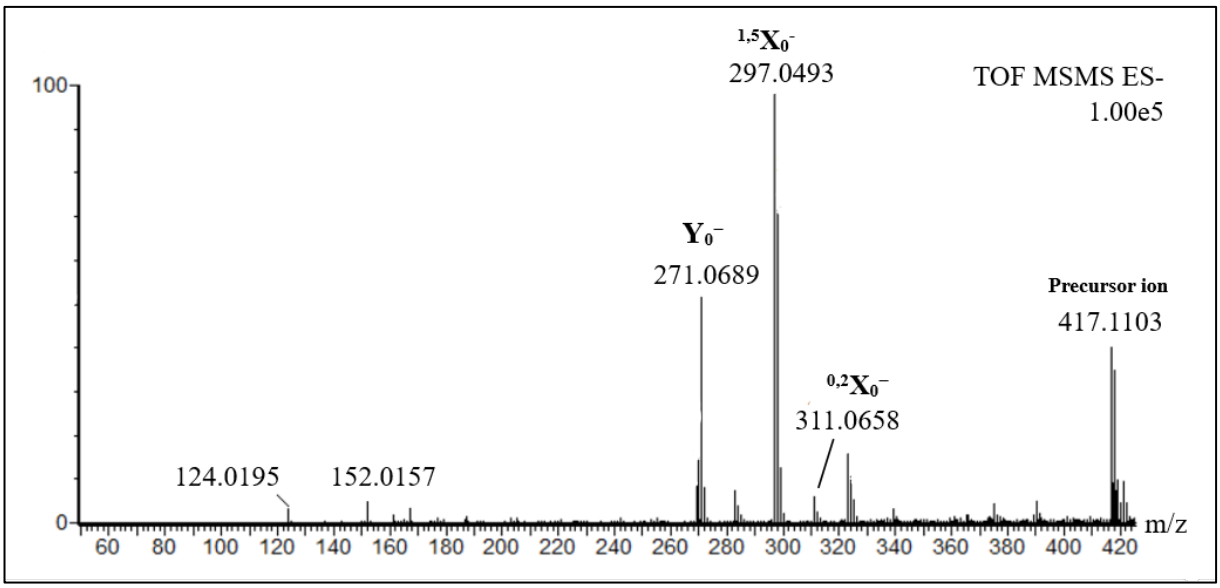
**Figure 1.** *High resolution MS/MS spectrum of a naringenin-deoxyhexoside released from the bee pollen exina*



# Towards the Structure of Melanin in Legume Seeds

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## Introduction

Plant melanins are structurally diversified polymeric pigments produced through the oxidative polymerization of phenolic precursors, including catechols, hydroxybenzoic acids, and hydroxycinnamic acid derivatives, catalyzed by polyphenol oxidase [1, 2]. These are found in various plant organs but the most often in seeds. It has been proposed that these pigments play protective, structural, and adaptive roles, enhancing plant survival under harsh conditions [3]. In legumes, dark pigmentation is prevalent in the seed coat, hilum, or both. This study reports the characterization of melanin isolated from the seed coats of black faba beans.

## Experimental

The mature faba bean (*Vicia faba*) black seed coats (20 g) were separated, milled and extracted in 0.5 M NaOH (300 mL) at 40°C for 36 h [4]. The filtrate was acidified to pH 2 with HCl, and the precipitate collected as crude melanin. Purification involved HCl hydrolysis [5] (6 M, 100°C, 2 h), organic solvent washing, alkaline redissolution, and reacidification. The purified melanin was characterized by attenuated total reflectance – Fourier transform infrared spectroscopy (iS50, Thermo Fisher Scientific) and mass spectrometry (Synapt G2-S, Waters) with laser desorption ionization (positive mode) and electrospray (negative mode) ionization sources.

For oxidative depolymerization [6], melanin (4 mg) was dissolved in 1 M NaOH (0.225 mL) and deionized water (2.442 mL), treated with 30% $H_2O_2$ (1.33 mL), and incubated at 50°C for 24 h, then neutralized with 1 M HCl. The product was purified with SPE (Strata S-DBL, Phenomenex) and analyzed with ESI-MS.

As an alternative decomposition method, butanolysis was employed by subjecting melanin (5.38 mg) to heating with n-butanol (10 mL) and concentrated $H_2SO_4$ (0.1 mL) at 100°C for 5 hours [7].

## Results

The ATR-FTIR spectrum revealed stretching vibrations of OH and $NH_2$ groups, C=O stretching or aromatic C=C stretching, N–H deformation and C–N stretching, C–O stretching of phenols, C=O stretching, and a methoxy group. These functional groups are indicative of melanin, in accordance with the previously reported studies dealing with different plant materials [4]. The ESI-MS spectra of the decomposed sample of melanin revealed the presence of fragments of the dihydroxyindole structure, namely pyrrole-2,3-dicarboxylic acid and pyrrole-2,3,5-tricarboxylic acid. The LDI-MS spectra of solid melanin exhibited phenolic compounds, including catechin, phenolic acid oligomers, L-3,4-dihydroxyphenylalanine (L-DOPA) dimers, and their combinations with L-DOPA. Further characterization of melanin and its degradation products is ongoing.

## Conclusion

ATR-FTIR, ESI-MS, and LDI-MS analyses provided complementary insights into the chemical structure of melanin isolated from faba bean seed coat. The identified functional groups, dihydroxyindole-derived degradation markers, and phenolic constituents collectively indicate that faba bean melanin is a heterogeneous polymer in which both nitrogen-containing and phenolic units are present, contributing to a better understanding of its chemical structure.


## Acknowledgements

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# Unravelling Complex Asian Lacquer Matrices using LC-MS/MS and Chemometrics

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## Introduction

Asian lacquers are traditional natural polymeric coatings derived from the sap of several tree species in the *Anacardiaceae* family, used for millennia in East and Southeast Asia for their exceptional durability and aesthetic appeal. These saps are complex water-in-oil emulsions primarily composed of substituted catechol derivatives (60–70%), water, polysaccharides, glycoproteins, and the enzyme laccase, which triggers an oxidative polymerization process to form a highly cross-linked, insoluble network. Three principal lacquer types are distinguished by their specific geobotanical origins and unique lipid biomarkers: urushiol (C15 side chains) from *Toxicodendron vernicifluum*, laccol (C17 side chains) from *Toxicodendron succedaneum*, and thitsiol (C17 and ω-phenylalkyl chains) from *Gluta usitata* [1]. While pyrolysis-gas chromatography/mass spectrometry (Py-GC/MS) is the established technique of choice for identifying the geobotanical origin of hardened lacquer films, infrared spectrometry (FTIR) serves as a common complementary tool for structural characterization, and atmospheric solids analysis probe mass spectrometry (ASAP-MS) has recently emerged as a promising method for their rapid differentiation [1,2], liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) has emerged as a powerful tool for profiling the soluble monomeric and oligomeric fractions [3]. LC-MS/MS offers superior separation and high selectivity, enabling the structural elucidation of specific catechol congeners based on their characteristic fragmentation pathways. This analytical approach is critical for determining vegetal provenance, distinguishing authentic oriental lacquers from imitations, and supporting the conservation of cultural heritage objects, effectively overcoming the challenges associated with the scarcity of pure commercial standards.

## Experimental

Urushiol, laccol and thitsiol lacquer samples were obtained from previously identified sources (two bowls and one art material). Chromatographic separation was performed on a Waters ACQUITY UPLC system using a Kinetex 2.6 µm C18 100A (100 × 2.1 mm) column (Phenomenex, Torrance, CA, USA) maintained at 30 °C. The mobile phase consisted of 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B). A linear gradient was executed at a flow rate of 0.200 mL/min as follows: initial conditions at 50% B; 0–10.0 min, linear ramp to 98% B; 10.0–22.5 min, isocratic hold at 98% B; 22.51–25.0 min, re-equilibration at 50% B. Mass spectrometry measurements were carried out on a SYNAPT G2-S Q-ToF mass spectrometer (Waters, Milford, MA, USA) equipped with an electrospray ionization (ESI) source operating in both positive and negative ion modes. The optimal source parameters were established as follows: capillary voltage of 2.7 kV for positive mode and 2.3 kV for negative mode, cone voltage 40 V, source temperature 120 °C, and desolvation temperature 450 °C with a desolvation gas flow of 800 L/hr. High-resolution MS spectra were recorded over an *m/z* range of 50–1200 with an instrument resolution of approximately 20,000 FWHM. For structural determination (LC-MS/MS), collision-induced dissociation (CID) was employed. Real-time mass correction was performed via a LockSpray interface using Leucine

Enkephalin as the reference compound (*m/z* 556.2771 in positive mode and *m/z* 554.2615 in negative mode), sampled at 10 s intervals. Raw mass spectrometry files were converted to the open .mzML format using MSConvert 3.0. Advanced data alignment, total ion chromatogram (TIC) normalization, and blank subtraction were subsequently performed using an in-house Python script to prepare the final feature matrix for Principal Component Analysis (PCA).

**Results**

The employment of untargeted LC-MS combined with Principal Component Analysis (PCA) provided exceptional chemometric discrimination of urushi, laccol, and thitsiol lacquers based on botanical origin. In positive mode (ESI+; 61.7% explained variance), clustering was driven by prominent markers (m/z 173.1348, 301.2202) and protonated $[M+H]^+$ urushiol congeners (e.g., m/z 315.20), whereas negative mode (ESI-; 44.9% variance) showed absolute separation along PC1 governed by deprotonated $[M-H]^-$ phenolic lipids, including urushi markers at m/z 315.19 (15:2) and 317.21 (15:1), and a laccol marker at m/z 345.28 (17:1). These findings were validated through targeted LC-MS/MS. In ESI+, alkyl catechols consistently yielded the universal dihydroxytropylium ion ($C_7H_7O_2^+$) at m/z 123.04. Thitsiol's unique phenylalkyl architecture (with parent ions like m/z 311.23 and 355.25) was verified by an intense tropylium fragment ($C_7H_7^+$) at m/z 91.06 and methylphenol signal (m/z 107.09). ESI- spectra were dominated by aliphatic chain elimination yielding stable benzofuran-phenate (m/z 135.05 and m/z 122.03), catechol core ions (m/z 108.02, 107.02), phenolate (m/z 93.03), and carbon monoxide-loss (m/z 79.06) fragment. These complementary findings demonstrate that both ionization modes provide robust, diagnostic chemical fingerprints essential for the unambiguous classification of historical lacquer materials.

**Conclusion**

LC-MS has emerged as a powerful technique for the sensitive characterization of the soluble fractions of Asian lacquers. LC-MS/MS offers significant advantages, including superior separation with high selectivity, allowing for the identification of specific molecular congeners (e.g., urushiols 15:3, 15:2, and 15:1) which serve as critical geobotanical indicators [4]. However, a major limitation of LC-MS is its unsuitability for the direct analysis of fully hardened, poorly soluble lacquer films, as the technique requires the analytes to be in solution. Furthermore, the high complexity of these natural mixtures and the presence of degradation products in historical samples can complicate data interpretation [5]. This study successfully utilized both positive and negative ionization modes, achieving excellent chemometric separation and unambiguous discrimination of the individual lacquer types.

**Acknowledgements**

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# Free Drug Concentrations in Plasma: Optimization of Ultrafiltration and Validation of an LC-MS Method for Clinical Purposes

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## Introduction

Determination of the free drug concentration in plasma has great importance in clinical pharmacology, as the unbound fraction provides more accurate information about the actual pharmacological effect (i.e. drug efficacy and safety). Principal importance of free (unbound) fraction is reported for drugs with high plasma protein binding and narrow therapeutic range, especially in vulnerable groups of patients (elderly, children, organ failure, hemodynamic instability). [1] The most useful fluid for estimating free drug concentrations appears to be plasma or serum, with subsequent treatment of the sample to separate free and bound drug by an appropriate technique. The two most widely used methods are equilibrium dialysis and ultrafiltration. Of these two, ultrafiltration has the greatest clinical utility because it is rapid and relatively simple.

Ultrafiltration belongs to conventional methods to disclose drug-protein binding characteristics. Unbound drug fraction is separated through the membrane with a specific molecular weight cut-off. The process is accelerated by centrifugation (approx. 15 min). However, this process could suffer from non-specific binding or leaky membranes.

This study aimed to optimize centrifugation parameters for plasma ultrafiltration, validate a robust LC-MS/MS method for quantifying the free fractions of the antiepileptic drugs lamotrigine (LTG) and valproate (VPA), and apply the optimized workflow to clinical samples from epilepsy patients.

## Experimental

Ultrafiltration optimization was performed with plasma saturated by drugs in study (an hour incubation at 37 °C). For functional test of centrifugal filters (MWL 30 000; Amicon Ultra, 0,5 ml, Millipore) three proteins of different relative molecular weight were centrifuged at preset conditions. First, it was human serum albumin (66,5 kDa), ovalbumin (45 kDa) and myoglobin (17,8 kDa) at a concentration of 1 mg/ml in normal saline. Filtrates and retentates were analysed via gel permeation chromatography (Superose™ 12, UV detection at 280 nm). Suitable centrifugation parameters were established by comparing experimental unbound fractions with literature data [2]. Plasma filtrates were prepared using a single-step protein precipitation/extraction with methanol:acetonitrile:water (70:20:10, v/v/v) containing isotopically labelled internal standards.

After centrifugation (14,000 rpm, 10 min, 4 °C), supernatants were analysed using a Luna Omega Polar C18 column on a UPLC system with MS/MS detection in ESI+ mode. Method validation was conducted in accordance with European Medicines Agency (EMA) guidelines. Clinical application

was verified using plasma samples from patients treated at the Brno Epilepsy Centre (St. Anne's University Hospital and Faculty of Medicine; Ethical Committee approvals No. 32/2022 and 03V/2023).

**Results**

Ultrafiltration has the advantage of simplicity and speed compared to other equilibrium-based methods. These characteristics are well appreciated when taking part in sample preparation workflow. Gel permeation chromatography confirmed excellent membrane integrity under the experimental conditions; human serum albumin and ovalbumin were completely retained in the retentate, while only myoglobin passed into the filtrate. The optimal centrifugation parameters were determined to be 1500 × g for 15 min at 37 °C, which minimized non-specific binding and prevented temperature-dependent shifts in protein-drug equilibrium.

The in-house LC-MS method validation successfully passed all EMA validation criteria. Linearity was confirmed over the clinical range (LTG: 0.2-25 µg/ml, $R^2$=0.9988 and VPA 0.2-50 µg/ml, $R^2$=0.9981), and both within-run and between-run accuracy and precision fell well within the acceptable ±15 % limits. Matrix effects were negligible, and the analytes demonstrated excellent stability.

In clinical application, the calculated free fractions about 45 % (n=7) for LTG and 12 % (n=4) for VPA responded well to literature data, confirming the accuracy of the pre-analytical ultrafiltration setup.

**Conclusion**

The optimized ultrafiltration protocol was successfully integrated into the pre-analytical sample preparation workflow, ensuring precise separation of the unbound drug fraction without compromising membrane integrity. Coupled with the validated LC-MS/MS method, this optimized methodology provides a robust, high-throughput platform for the accurate quantification of free LTG and VPA concentrations in clinical plasma samples. Consequently, the free VPA concentration data will be directly applied to the subsequent phases of the project focused on determination of the drug in different biological matrices.

**Acknowledgements**

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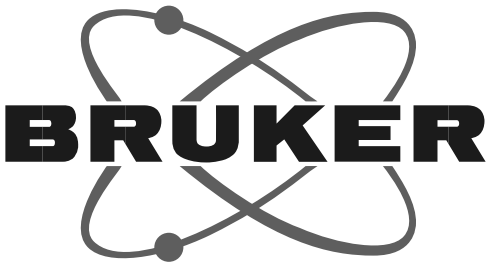


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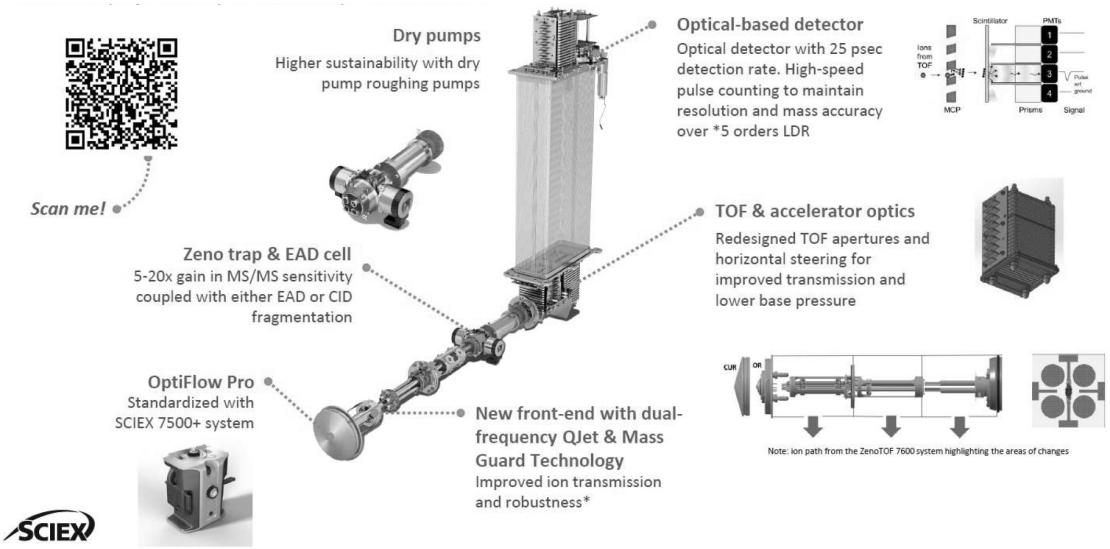


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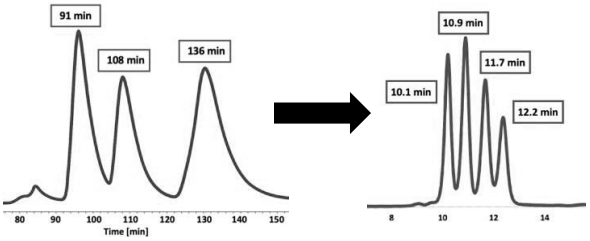


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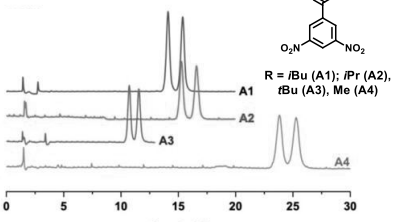





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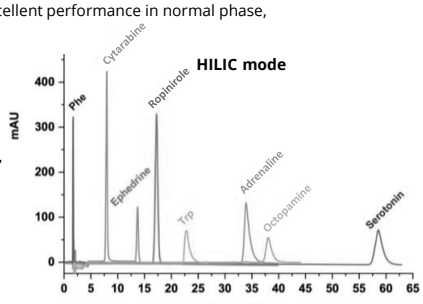


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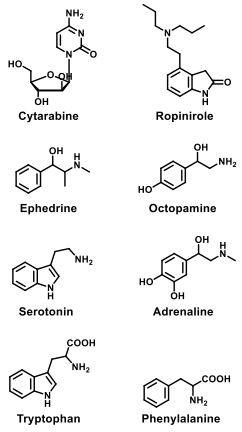
| Temp.: | 20 °C |

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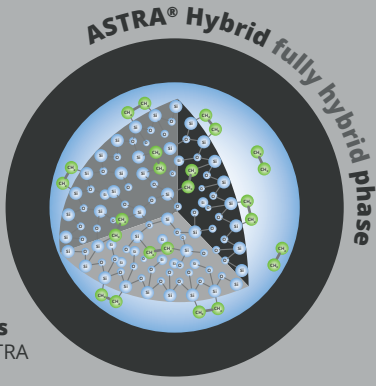


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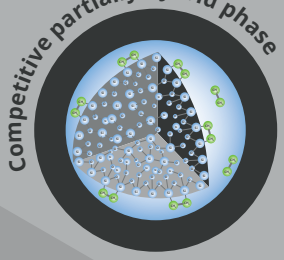





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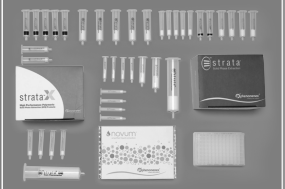

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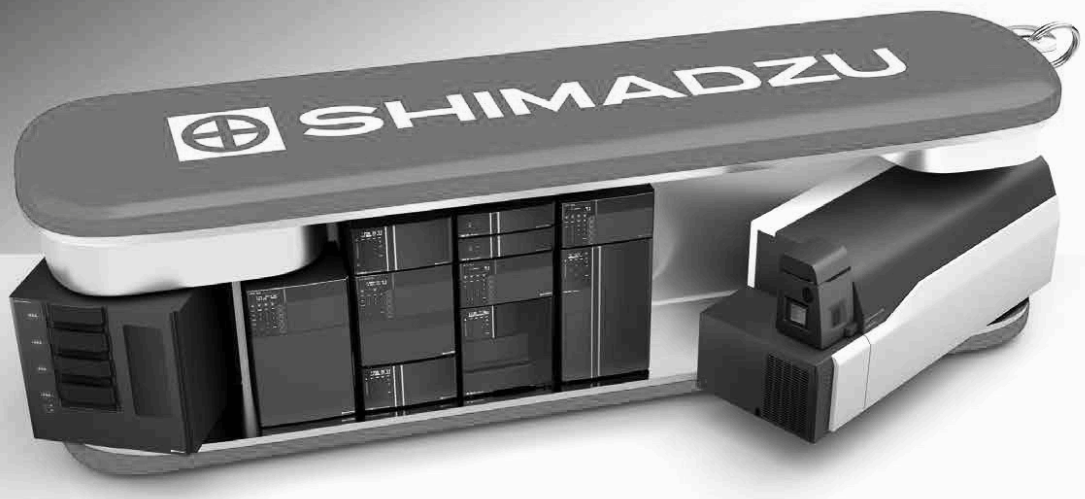


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Připojte se k TAPI! Jsme globální společnost se 13 závody a 5 vývojovými centry. Po celém světě pomáháme zachraňovat životy. V České republice máme dlouholetou tradici. V Opavě vyvíjíme, vyrábíme a prodáváme účinné farmaceutické látky a rostlinné extrakty. V TAPI Czech Industries pracuje více než 600 zaměstnanců, přes 70 z nich je na Výzkumu a vývoji.

Hledáme absolventy oboru chemie nebo přírodních věd. Uplatnění najdete v chemické výrobě, R&D, kvalitě, engineeringu a dalších odděleních. Nabízíme trainee programy, stáže, exkurze a příležitost účastnit se mezinárodních výzkumných projektů.

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zaměstnanců

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Waters™

LC | LC-MS | LC kolony | Automatizace

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- Nová generece Waters UHPLC (827 bar)
- Robustní a citlivý
- Jednoduchý převod metod
- Redukuje chybovost obsluhy až o 40%

## Charged Aerosol detektor (CAD)

- Optimalizováno pro Empower™ a MassLynx™
- Široký dynamický rozsah
- Univerzální detekce bez UV omezení
- Kompaktní a robustní design

## Xevo MRT

- délka doby letu 4m, vícenásobný odraz
- rozlišení 100 000 FWHM
- mass accuracy < 0.5 ppm
- rychlost skenování 50 Hz (MS) a 100 Hz (MS/MS)
- vysoká rychlost bez ztráty citlivosti a rozlišení
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- flexibilita použití (Unispray, DESI XS)
- vhodný pro target i untarget screening
- ideální pro metabolomiku a lipidomiku

## Xevo TQ Absolute XR

- vysoká citlivost ,široký dynamický rozsah a vysoká přesnost
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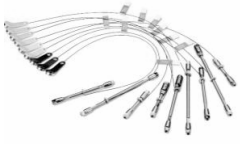
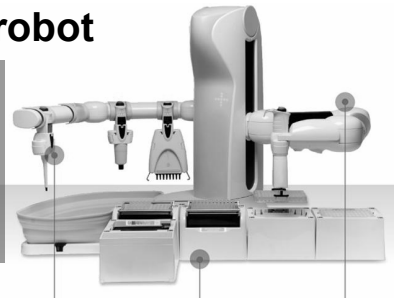
- umožňuje analyzovat až šestinásobně více vzorků mezi čištěními
- flexibilita použití (Unispray, DESI XS, APGC)
- software waters_connect
- plně automatické ladění

## LC kolony



- Osvědčené sorbenty pro RP i HILIC
- Malé molekuly i biomolekuly, MaxPeak HPS technologie
- Velikosti částic pro HPLC i UPLC
- Plně porézní i s pevným jádrem
- Preparativní kolony, SEC kolony

## Andrew+ robot



- Fleixibilní pipetovací robot
- Dynamický pipetovací rozsah 0,2ul a 10 ml
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