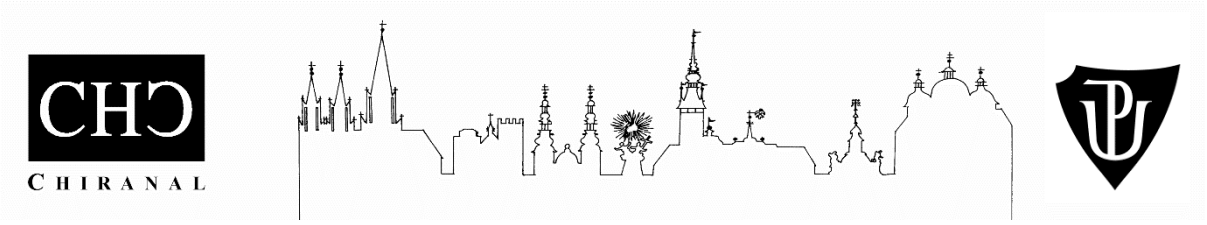
ADVANCES IN CHROMATOGRAPHY AND ELECTROPHORESIS & CHIRANAL 2024

BOOK OF ABSTRACTS AND PROGRAM



OLOMOUC, JUNE 17th–20th, 2024

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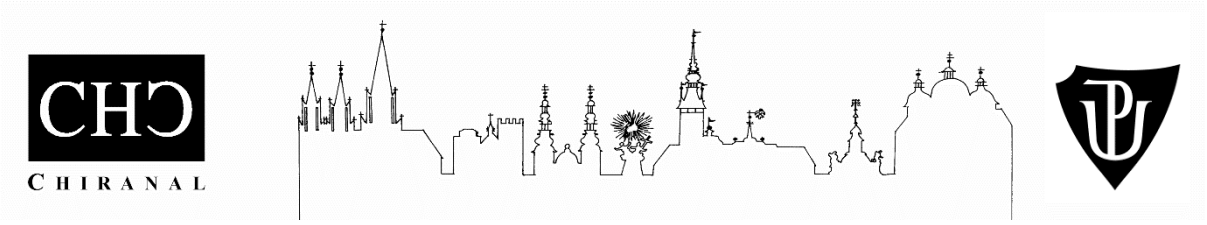

BOOK OF ABSTRACTS AND PROGRAM

organized by:

Department of Analytical Chemistry

Faculty of Science

Palacký University Olomouc

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PREFACE

Dear colleagues,

It is our honor to welcome you to traditional conference Advances in Chromatography and Electrophoresis & Chiranal. We are thus continuing the tradition of this conference series, which dates back to 1997. This conference has always aspired to serve as a platform for fostering connections and collaboration among experts from academia and industry who deal with separation science. In the spirit of "Chiranal", we have always tried to create a meeting that will bring generations together and provide space for young researchers and students to present their work at the same time. This year's symposium is proud to host over 140 registered participants.

In the separation sciences, we are witnessing great progress, whether in the field of stationary phases and selectors, miniaturization and microfluidics, or in detection methods. Application development in industrial analytical chemistry is also strong, covering cheap and robust solutions for routine monitoring as well as highly sophisticated analytical methods facilitating creation of new products. Separation methods together with modern data analysis are the driving force behind the development of all "omics". We are looking forward to learning about innovations in these areas, as well as other branches, from the lectures, posters and company communications presented at our symposium.

Allow us to say a few words about the program. Opening of the conference in Laudon Hall of the Fort Science will include plenary lecture of professor Sebastiaan Eeltink and ceremonial launch of the book of Lucie Nováková, Michal Douša, Petr Česla and Jiří Urban, Modern HPLC separations in theory and practice, which will certainly rank among the important textbooks of our field. On Tuesday we will move to the main venue – auditorium, foyer and seminary rooms of the main building of the Faculty of Science, Palacký University where the main scientific program will take place. The scientific program will be accompanied by a rich social one. A bowling tournament of conference participants in Bowland Bowling Center has become a lovely tradition. In the frame of cultural program, you can visit the Jesuit College and listen to a concert of vocal choir Muzikúra in Corpus Christi Chapel. We can also look forward to ceremonial consecration of the Eureka University Brewery and excursion of the conference participants to the brewery and to the Department of Analytical Chemistry FS UP.

Authors participating on the symposium are invited to submit their work presented at the conference to the special collection of papers of Journal of Separation Science. Further information can be found at conference web page https://chiranal2024.upol.cz/.

A big thank you goes to our sponsors and partners – without their support it would not have been possible to organize our symposium in the traditionally high quality. Our conference was supported by both major companies from the field of pharmaceutical and chemical production as well as manufacturers and suppliers of analytical instrumentation and laboratory material.

At this point, let us thank to the members of the organizing committee from the Department of Analytical Chemistry FS UP and to other colleagues who in any way contributed to the realization of the conference. Without their input, the meeting could hardly be organized in sufficient extent and quality.

We want to dedicate this year's conference to an excellent scientist and a great person, prof. Eva Smolková-Keulemansová, who recently left us forever.

Ladies and gentlemen, welcome in Olomouc.

Petr Bednář, Petra Krejčí, Lukáš Kučera and Ondra Kurka

on behalf of the organizing committee

Olomouc, June 17th, 2024

ADVANCES IN CHROMATOGRAPHY AND ELECTROPHORESIS & CHIRANAL 2024
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 17th, 2024)

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), presence 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*
Sebastiaan Eeltink – Toward unrivaled chromatographic resolving power: Design and development of comprehensive spatial three-dimensional liquid-phase separation technology

17:30–18:00 Ceremonial launch of the book of Lucie Nováková, Michal Douša, Petr Česla and Jiří Urban: Modern HPLC separations in theory and practice

18:00–?? Welcome party + banquet (Fort Science, Laudon Hall, ground floor)

Tuesday (June 18th, 2024)

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

09:00–11:20 *Lectures I (Faculty of Science, assembly hall, 2nd floor): Electromigration techniques, ion exchange chromatography; chairmen: Michael Lämmerhofer, Petr Bednář*

09:00–09:30 **Anne Varenne** – Electrokinetic methodologies for diagnostics development
09:30–10:00 **Marián Masár** – Advances in microchip electrophoresis for the analysis of environmental and biological samples
10:00–10:20 **Václav Kašička** – Determination of effective charge and ionic mobilities of highly sulfated cyclodextrins by capillary isotachophoresis and zone electrophoresis

10:20–10:40 **Katarína Maráková** – Capillary zone electrophoresis – mass spectrometry for intact protein analysis in biological fluids – Possibilities and limitations

10:40–11:00 **Petr Kubáň** – Microcontrollers and their application in constructing analytical instrumentation

11:00–11:20 **Jan Soukup (Metrohm)** – Adsorbable organic fluorine (AOF) – a sum parameter for non-targeted screening of per- and polyfluorinated alkyl substances (PFASs) in waters

11:20–11:40 Coffee break (Faculty of Science, foyer, 2nd floor)

11:40–13:40 *Lectures II (Faculty of Science, assembly hall, 2nd floor): Approaches to method development in chromatography and mass spectrometry, applications of capillary chromatography; chairmen: Anne Varenne, Petr Barták*

11:40–12:10 **Davy Guillarme** – How to successfully analyse oligonucleotides with liquid chromatography?

12:10–12:40 **Zeineb Aturki** – Recent applications of nano-liquid chromatography: Analysis of molecules affecting human health

12:40–13:00 **Jiří Urban** – Personal insights on the design of experiments

13:00–13:20 **Egidijus Machtejevas (Merck)** – How to make chromatography more sustainable

13:20–13:40 **Aiko Barsch (Bruker Daltonics)** – Automated workflow for derivatized analyte analysis in LC-TIMS-MS/MS 4D-metabolomics data: Incorporating in-silico derivatization, CCS prediction, and in-silico fragmentation

13:40–14:40 Lunch (university cafeteria, Šmeralova 12)

14:40–16:00 ***Poster session I (even poster numbers)*** and coffee break (Faculty of Science, foyer, seminary rooms, 2nd floor)

16:00–18:00 *Lectures III (Faculty of Science, assembly hall, 2nd floor): Current trends in chromatography and mass spectrometry, chairmen: Václav Kašička, Ondřej Kurka*

16:00–16:30 **Michael Lämmerhofer** – Isomer separations in metabolomics and lipidomics

16:30–17:00 **Michal Holčapek** – Novel approaches in the analysis of lipids, glycosphingolipids, and metabolites

17:00–17:20 **Barbora Papoušková** – Unveiling the power of deep UV LAESI/LAESCI dual ambient ionization: Towards comprehensive molecular MSI

17:20–17:40 **Luka Milivojević (Pragolab / Thermo Fisher Scientific)** – Advanced small molecule mass spectrometry

17:40–18:00 **Alex Muck (Waters)** – Employing ballistic gradients, vacuum jacketed columns and prototype benchtop multi reflecting time-of-flight (MRT) to increase lipidomic analytical throughput whilst maintaining highly confident identifications

18:00–19:00 Dinner (university cafeteria, Šmeralova 12)

19:15–21:00 Ceremonial consecration of the Eureka University Brewery, excursion of the conference participants to the brewery and to the Department of Analytical Chemistry

Wednesday (June 19th, 2024)

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

09:00–11:20 *Lectures IV (Faculty of Science, assembly hall, 2nd floor): Advances in analysis of bioactive molecules, heritage science; chairmen: Sebastiaan Eeltink, Lukáš Kučera*

09:00–09:30 **Radim Kučera** – Advances in chiral separation of carboranes – new phenyl ring isosteres for drug development
09:30–10:00 **Petra Štěrbová-Kovaříková** – Analysis of selected drugs in whole blood collected with a volumetric absorptive microsampling device
10:00–10:20 **David Friedecký** – Translation of omics into clinics
10:20–10:40 **Paola Lucero-Gomez** – Py/GC-MS characterization of oil paintings to correlate museum environment and materials condition over time for SMARTMUS-e project
10:40–11:00 **Ondřej Novák (on behalf of Altium)** – Recent chromatographic advances in plant hormone profiling methods
11:00–11:20 **Luděk Vlk (Chromservis)** – New era in amino acid analysis

11:20–13:00 ***Poster session II (odd poster numbers)*** and coffee break (Faculty of Science, foyer, seminary rooms, 2nd floor)

13:00–14:00 Lunch (university cafeteria, Šmeralova 12)

15:00–17:00 ***Social/sport events:***
- Bowling at Bowland Olomouc
- A guided tour of Jesuit College, followed by an interactive concert of vocal choir Muzikúra in Corpus Christi Chapel

18:00–?? Social dinner (Faculty of Science, foyer, 6th floor)

Thursday (June 20th, 2024)

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

09:00–11:00 *Lectures V (Faculty of Science, assembly hall, 2nd floor): Matrix effects, chiral separation, advances in stationary phases, supercritical fluid chromatography; chairmen: Zeineb Aturki, Petr Fryčák*

09:00–09:30 **Lucie Nováková** –Matrix effects in LC-MS: Importance of correct calculation approach

09:30–10:00 **Michal Kohout** – New trends in ion exchange-type chiral stationary phases and their chiral recognition mechanisms

10:00–10:20 **Ghaid W. A. Abualzulof** – Multiple heart-cutting achiral-chiral LC-LC analysis of branched-chain amino acids in food supplements

10:20–10:40 **Ivan Petřík** – Rapid profiling of bioactive compounds using supercritical fluid chromatography coupled with tandem mass spectrometry

10:40–11:00 **Jan Vlasák (Phenomenex)** – Symbiosis of core-shell and fully porous particles: Implementing both for better HPLC and UHPLC results

11:00–11:20 Coffee break (Faculty of Science, foyer, 2nd floor)

11:20–13:30 *Lectures VI (Faculty of Science, assembly hall, 2nd floor): Selectivity in chromatography and mass spectrometry and its optimization, analysis of natural products; chairmen: Lucie Nováková, Tomáš Pluháček*

11:20–11:50 **Kevin Schug** – Targeted and untargeted analysis of psilocybin mushrooms using LC-MS

11:50–12:10 **Deirdre Cabooter** – On the potential of two-dimensional liquid chromatography for environmental analysis

12:10–12:30 **Volodymyr Pauk** – Leveraging ion mobility spectrometry-mass spectrometry data using machine learning reveals provenance of indigo dyes

12:30–12:50 **Petr Česla** – Numerical optimization of gradient separations in RP and HILIC using non-linear retention models

12:50–13:10 **Rutuja Hiraji Patil** – Do we need separations in clinical diagnostics?

13:10–13:30 **Mariateresa Maldini (SCIEX)** – Metabolomics analysis of *Moringa oleifera* leaves: Correlation between in vitro effect on C2C12 myotubes cell line and geographical origin

13:30–14:10 Closing of conference and farewell drink (Faculty of Science, foyer, 2nd floor)

14:10–15:10 Lunch (university cafeteria, Šmeralova 12)

LIST OF POSTERS

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

1. Isolation of lectin from *Musa acuminata* by affinity chromatography as potential Therapy against Biofilm Forming Pathogens
   Summra Ahmed, Muneera Naz Baloch, Syed Faraz Moin, Hina Musa

2. Spark-generated nickel oxide nanoparticle modification of carbon fiber microelectrodes for enhanced detection of piperazine antihistamine drugs in HPLC
   Zeynab Belbasi, David Jirovský, Jan Hrbáč

3. Comparison of extraction efficiency of quercetin complex from onion peel using NADES and organic solvents
   Karolína Benešová, Lea Lojková, Jhonny E. Alba-Mejía, Helena Pluháčková, Radim Cerkal

4. Using mass spectrometry for study of salicylic acid metabolism in plants
   Beata Budíková, Jitka Široká, Asta Žukauskaitė, Ondřej Novák

5. CZE-MS/MS method development for simultaneous quantification of eight β-Lactam antibiotics and two β-Lactamase inhibitors in plasma samples
   Ivana Čižmárová, Peter Mikuš, Juraj Piešťanský

6. Development and validation of microchip isotachophoresis method for the analysis of cardiovascular drugs
   Marta Ďuriš, Jasna Hradski, Roman Szucs, Marián Masár

7. RP-HPLC separation of amino sugars in blood serum samples
   Róbert Góra, Renáta Górová

8. Optimized HPLC-guided separation and purification of 1,3,5-triazine derivatives containing amino acids with non-polar side chain as potential bioactive molecules
   Mária Bodnár Mikulová, Michal Hanko, Peter Mikuš

9. Middle-up quantification of a monoclonal antibody in pharmaceutical matrix by capillary electrophoresis-mass spectrometry
   Jana Havlíková, Katarína Maráková, Peter Mikuš

10. Methods for GC-MS analysis of pyrolytic compounds in different matrixes
    Danylo Holub, Barbora Přibylová, Petr Barták

11. Chiral separations of new potential pharmacophores: *nido*-$[7,8\text{-}C_2B_9H_{12}]^-$ and $[Co(C_2B_9H_{11})_2]^-$ derivatives
    Ondřej Horáček, Lucie Nováková, Bohumír Grüner, Radim Kučera

12. An insight into deschloroketamine metabolism – identification of minor hydroxy metabolites in human blood serum and urine
    Andrea Horniaková, Juraj Piešťanský, Vítězslav Maier

13. Separation and determination of carboxylic acids in complex samples by microchip electrophoresis coupled with ion mobility spectrometry
    Jasna Hradski, Marta Ďuriš, Roman Szucs, Marián Masár

14. Detailed LC-MS/MS analysis of sugars and their alditols for diagnosis of inherited metabolic disorders
Eliška Ivanovová, Eva Hlídková, Vojtěch Bekárek, Hana Janečková, David Friedecký

15. The use of carbon-based columns in the analysis of structurally similar herbicides
Michal Kašpar, Petr Česla

16. Chiral separation of ketamine and its metabolites from rat plasma samples
Renáta Konášová, Dušan Koval, Petr Tůma, Václav Kašička

17. Development of the metod for therapeutic drug monitoring of selected benzodiazepines by liquid chromatography
Kateřina Krátká, Pavel Šištík

18. *In vitro* pea seed feeding and analysis of labelled metabolites in monolignol pathway
Petra Krejčí, Jana Balarynová, Barbora Klčová, Petr Smýkal, Petr Bednář

19. Optimization of saliva sampling methods for the analysis of bile acids by HPLC-MS
Petr Kubáň, Věra Dosedělová, Markéta Laštovičková, Štefan Konečný, Jiří Dolina

20. Ion-exchange chromatography of anions in environmental analysis
Lukáš Zima, Kristýna Machálková, Vojtěch Vrána, Lýdia Stoyalová, Marta Farková, Přemysl Lubal

21. Enantioselective separation of piperazine derivatives via capillary electrophoresis with sulfated β-cyclodextrin in methanolic background electrolytes
Navid Niaei, Jan Petr

22. Desorption electrospray ionization coupled to cyclic ion mobility in analysis of new psychoactive substances
Marianna Nytka, Jiahao Wan, František Tureček, Karel Lemr

23. Off-line microelution SPE as a sample pretreatment step in quantitation of intact proteins in biological fluids using CZE-MS
Martina Opetová, Radovan Tomašovský, Peter Mikuš, Katarína Maráková

24. Quantification study of selected polyphenols during pea seed maturation
Aneta Pátková, Petra Krejčí, Jana Balarynová, Barbora Klčová, Petr Smýkal, Petr Bednář

25. Cardiovascular event risk assessment using the CERT score
Barbora Piskláková, Aleš Kvasnička, Jakub Rozhon, Miloš Táborský, David Friedecký

26. Verification of the microfluidic device design for single cell detection and isolation using FEM and SPICE analysis
Oleksandr Prystopiuk, Petr Fryčák, Kateryna Trach, Petr Bednář

27. GC/MS analysis of binders in a single microsample from František Emler painting
Barbora Přibylová, Matěj Buřinský, Danylo Holub, Petr Barták

28. Bridging microsampling with microextraction for doxorubicin and doxorubicinol determination
Adam Reguli, Hana Bavlovič Piskáčková, Olga Lenčová, Petra Kollárová-Brázdová, Martin Štěrba, Petra Štěrbová-Kovaříková

29. Separation of selected catecholamines and determination of binding constants of their complexes with HS-β-CD by capillary electrophoresis
Petra Sázelová, Jiří Jiráček, Václav Kašička

30. Application of the multi-component integrated calibration method to study the origin and concentration of polycyclic aromatic hydrocarbons in the Katowice (Poland)
Paweł Świt, Joanna Orzeł, Sławomir Maślanka

31. Analyses and physico-chemical characterization of peptides and lipopeptides regulating food intake by capillary electrophoresis and isotachophoresis
Veronika Šolínová, Adéla Křížková, Lenka Maletínská, Václav Kašička

32. Quantitative analysis of two therapeutic peptides using multiple sample injection in a hydrodynamically closed system capillary electrophoresis
Ondrej Štefánik, Peter Mikuš, Juraj Piešťanský

33. Separation of cyclic diadenosine diphosphorothioate and the diastereomers of its difluorinated derivative and estimation of binding constants of their complexes with 2-hydroxypropyl-β-cyclodextrin by affinity capillary electrophoresis
Sille Štěpánová, Petra Břehová, Václav Kašička

34. Development of simple and rapid CE-UV method for analysis of salivary lysozyme
Radovan Tomašovský, Peter Mikuš, Katarína Maráková

35. Characterization of retention with ternary mobile phases in HILIC chromatography
Sára Mrvková, Barbora Tošovská, Petr Česla

36. Effect of degradation processes on polyphenol content in wine
Jonáš Trach, Petra Krejčí, Petr Bednář

37. Using microchip electrophoresis for the determination of melamine in infant formula
Peter Troška, Lucia Šottníková, Marián Masár

38. Unlocking seed coat composition with Raman microscopy: Complementary tool to mass spectroscopy
Kateryna Trach, Notburga Gierlinger, Petr Smýkal, Petr Bednář

39. Electromembrane extraction of methadone on-line coupled with capillary electrophoresis by flow-gating interface
Petr Tůma, František Opekar

40. Targeted, suspect and non-targeted liquid chromatography-high resolution mass spectrometry for identification of degradation products of selected pollutants in water samples
Lucia Hojová, Martin Vrška, Marian Marton, Tomáš Mackuľak, Roman Grabic, Marian Vojs, Andrea Vojs Staňová

41. Characterization of Asian lacquers by atmospheric solids analysis probe high resolution tandem mass spectrometry coupled with cyclic ion mobility separation (ASAP-cIMS-HRTMS)
Vojtěch Zemek, Radek Ryšánek, Petra Krejčí, Lukáš Kučera, Jana Nádvorníková, Adéla Tůmová, Helena Heroldová, Adriana Stříbrná, Petr Bednář

42. Wagon or no wagon: The new insight in Early Iron Age burial custom
Katja Hagemann, Doris Mischka, Bernd Mühldorfer, Štěpán Dostál, Zbyněk Žingor, Lukáš Kučera

LECTURES

Toward unrivaled chromatographic resolving power: Design and development of comprehensive spatial three-dimensional liquid-phase separation technology

Sebastiaan Eeltink

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Spatial comprehensive three-dimensional chromatography (3D-LC) entails a novel chromatographic concept that promises unprecedented resolving power in terms of peak capacity and sample throughput. Spatial 3D-LC separations will be performed by making analytes migrate to different positions in a three-dimensional body. The maximum peak capacity is the product of the three individual peak capacities. Due to parallel analysis in the second and third dimensions the analysis time is greatly reduced, overcoming the fundamental limitation of coupled-column multi-dimensional approaches, in which sampled fractions are analyzed sequentially. This makes the technology ideally suitable for high-throughput screening of a multitude of samples, and consequently highly relevant for biomarker-validation studies and to monitor therapeutic regimens.

This contribution focuses on the design aspects of the microchip for spatial 3D-LC and the selection of orthogonal separation modes to enable the analysis of intact proteins. The design considerations for the flow distributor and channel layout are discussed, along with various approaches to confine the flow during the subsequent development stages. Additionally, the integration of stationary phases into the microchip is addressed, and interfacing to mass-spectrometry detection is discussed. According to Pareto-optimality, the integration of isoelectric focusing, size-exclusion chromatography, and reversed-phase chromatography in a spatial 3D-LC approach is predicted to achieve an exceptional peak capacity of over 30,000 within a 1-hour analysis, setting a new benchmark in chromatographic performance. During the presentation I will show different spatial 3D-LC chip prototypes and will discuss how feasible it is to establish such technology.

Electrokinetic methodologies for diagnostics development

Anne Varenne

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The design and development of diagnostics for biomedical applications, such as the vast domain of proteins (proteomics, protein-corona, etc) and nano-probes, implies various expertises among which analytical sciences. Electrokinetic methods represent an interesting tool for this purpose, however some challenges still remain to reach the expected performances. We will present here some of our strategies and developments for this objective:

- the design and electrokinetic characterization of nano-objects, nano-platforms and selective agents in terms of (colloidal) stability, biocompatibility, biodistribution [1,2]
- the quantitative electrokinetic characterization of non-covalent interactions in solution (with targets or plasma proteins) [3]
- the development of miniaturized electrokinetic tools integrating various analytical steps or multi-functional nano-probes [4]

These strategies will be highlighted with different targets for different applications going from interactomics, proteomics to analytical diagnostics and imaging.

References

[1] L. Trapiella-Alfonso, G. Ramirez-Garcia, F. d'Orlyé, A. Varenne, Trends Anal. Chem. 84 (2016) 121.
[2] F. D'Orlyé, L. Trapiella-Alfonso, C. Lescot, M. Pinvidic, B.T. Doan, A. Varenne, *Molecules* 26 (2021) 458.
[3] G. Ramirez Garcia, F. D'Orlyé, C. Richard, N. Mignet, A. Varenne, Analyst 146 (2021) 5245.
[4] L. Trapiella Alfonso, T. Pons, N. Lequeux, L. Leleu, J. Grimaldi, M. Tasso, E. Oujagir, J. Seguin, F. D'Orlyé, C. Girard, B.T. Doan**,** A. Varenne, ACS Appl. Mater. Interfaces 10 (2018) 17107.

Advances in microchip electrophoresis for the analysis of environmental and biological samples

Marián Masár, Jasna Hradski, Peter Troška, Roman Szucs, Marta Ďuriš

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Introduction

Considerable progress in life sciences and environmental protection creates growing demand for modern analytical systems. Aligned with the principles of green analytical chemistry, such as reagent, waste and energy reduction, there is a growing interest in miniaturized separation techniques for their potential to meet most of these objectives. Microchip electrophoresis (MCE) has significant benefits in terms of high-speed, high separation efficiency, high-throughput, reduced consumption of solvents and production of waste, easy automation, and low running costs [1]. The aim of this contribution is to present some advances of the use of the MCE for the analysis of complex environmental and biological samples.

Experimental

MCE separations were carried out on an inhouse constructed equipment with two main operational units: electrolyte and electronic unit. Electrolyte unit consists of peristaltic micropumps and membrane driving electrodes. Electronic unit delivers stabilized driving current to the electrodes and controls the peristaltic micropumps. A poly(methyl methacrylate) microchip used in this work consisted of two separation channels equipped with two conductivity detectors. Miniaturized surface enhanced Raman spectrometer and spectrometer working at visible wavelength (490 nm) were implemented directly on the microchip; ion mobility spectrometer was coupled online using interface based on thermal spray.

Results

Theoretical and instrumental aspects of MCE as well as its integration with various detection techniques are presented. Practical examples of the separation and the determination of the analytes present in complex samples of biological, pharmaceutical and environmental origin are demonstrated. Microchips equipped with coupled channels facilitate combination of different electrophoretic separation techniques. For example, preconcentration and sample pretreatment potential of isotachophoresis (ITP) combined with zone electrophoresis (ZE) is very effective tool for trace analysis [1]. In this instance, different separation mechanisms can be implemented in each channel, while independent monitoring of separation is achieved using conductivity detectors [2]. In addition to the integrated conductivity detection, other detection techniques such as UV-Vis detection, surface-enhanced Raman spectroscopy (SERS) or ion mobility spectrometry (IMS) can be combined with the MCE. In this context, the benefits of the use of electrophoretic microchips with coupled channels and different miniaturized detectors for determining trace analytes in complex ionogenic samples are shown. For example, (1) the ZE-ZE method for the separation and determination of metabolic organic acids in the cerebrospinal fluid, biomarkers of some neurological diseases in urine [2]; (2) the ITP-ZE method, preceded with microsolid phase extraction for the determination of traces of nitrite and

nitrate in cerebrospinal fluid for the purpose of indication of various neurological diseases [3]; (3) the ZE method, performed on the microchip with a photometric detector operating at 490 nm, for determination of carminic acid, natural red food dye, present in various food and pharmaceutical samples [4]; (4) the ITP-SERS method, capable of detecting traces of synthetic dyes in various pharmaceuticals [5]; and (5) the ZE-IMS and ITP-IMS methods for the determination of carboxylic acids in various food, pharmaceutical and biological samples [6,7] were developed. The microanalytical methods delivered good repeatability, accuracy and low limits of detection for biologically and environmentally important compounds.

Conclusion

MCE performed on the microchip with coupled channels is a multifunctional analytical tool, which facilitates online integration of sample pretreatment with two-dimensional separation and utilization of various detection techniques for the analyses of complex biological and environmental samples.

Acknowledgements

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Determination of effective charge and ionic mobilities of highly sulfated cyclodextrins by capillary isotachophoresis and zone electrophoresis

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Introduction

Sulfated cyclodextrins (SCDs) belong to the most popular chiral selectors in capillary electrophoresis [1, 2]. SCDs are available as mixtures of randomly highly sulfated species (HS-CDs) or as well defined single isomer compounds (SI-CDs). For the randomly HS-CDs, only an average degree of substitution (DS) is estimated but the isomeric heterogeneity and the charge distribution are not known. In the SI-CDs, the sulfatation positions and the DS are well specified. However, due to close vicinity of the sulfate groups in both types of SCDs, counterion condensation can occur in their molecules and their actual effective charge can be lower than number of sulfate groups. For that reason, the aim of this work was to estimate the degree of charge reduction in both types of SCDs using the procedure based on separation of SCDs and reference compounds by capillary isotachophoresis (CITP) and zone electrophoresis (CZE).

Experimental

CITP and CZE experiments were performed in Agilent CE7100 analyzer (Agilent Technologies, Waldbronn, Germany) equipped with a UV-Vis diode array detector (DAD) and contactless conductivity detector (C4D). The separations were run in fused silica capillaries with outer polyimide coating (Polymicro Technologies, Phoenix, AZ, USA, 50/375 μm id/od, 424 mm total length, 279 mm. effective length 1 (to C4D) and 339 mm effective length 2 (to DAD). The inner capillary wall was permanently coated with hydroxypropyl cellulose (HPC). HS-CDs were obtained from Sigma Aldrich and SI-CDs were prepared as described in [3].

Theory

The effective charge number of the *i*-th charged species, $z_{\mathrm{eff,i}}$, was calculated using Eq. (1) derived earlier [4-6]:

$$z_{\mathrm{eff,i}} = z_{\mathrm{ref}} \frac{\Delta t_{\mathrm{i}} c_{\mathrm{ref}} V_{\mathrm{ref}}}{\Delta t_{\mathrm{ref}} c_{\mathrm{i}} V_{\mathrm{i}}} \frac{\mu_{\mathrm{i,z}} \left(\mu_{\mathrm{ref,z}} + \mu_{\mathrm{c,z}} \right)}{\mu_{\mathrm{ref,z}} \left(\mu_{\mathrm{i,z}} + \mu_{\mathrm{c,z}} \right)} \tag{1}$$

where subscripts *i*, *ref*, and *c* refer to the species of interest, reference compound, and counter ion in the ITP electrolyte system, respectively; *z* stands for the charge number, Δt is the zone length in time units, *c* is the molar concentration, *V* is the injected sample volume, and μ is the actual ionic mobility, i.e. mobility of ion at actual ionic strength and temperature.

Results

The effective charges of three SI-α-, β-, and γ-CDs and three randomly sulfated HS-α-, β-, and γ-CDs were determined from their ITP zones lengths, ionic mobilities, and molar concentrations, and from the same parameters of the reference compounds, formic acid and dichloroacetic acid, respectively, using the Eq. (1). The ITP zone lengths of both types of SCDs and reference compounds were obtained from their CITP analyses in anionic mode using the

leading electrolyte (LE) composed of 10 mM HCl, 20 mM histidine, pH 6.2, and the terminating electrolyte (TE) containing 20 mM sodium glutamate, pH 7.0. The records of representative CITP separations of SI-α-CD and formic acid and of HS-α-CD and dichloroacetic acid are shown in Fig. 1A and Fig. 1B, respectively. The actual ionic mobilities of the SI-CDs and HS-CDs and singly charged reference compounds were determined by their CZE separations in the BGE of the same composition as the LE. The effective charge numbers of the SI-CDs were in a good agreement with theoretical values (numbers of sulfate groups in single isomer CDs). On the other hand, the effective charges of randomly HS-CDs were significantly (26.4-31.9%) reduced as compared to the theoretical values (average numbers of sulfate groups (DS) in randomly HS-CDs). The actual ionic mobilities of the SI-CDs achieved values (35.5–37.5) × 10^{-9} $m^2V^{-1}s^{-1}$ and in accordance with the theory, due to their smaller charge numbers, they were significantly lower than the actual ionic mobilities of the more multiply charged randomly HS-CDs that covered a narrow range of (43.5–44.1) × 10^{-9} $m^2V^{-1}s^{-1}$. They were in a good agreement with the ratio of their effective charges and relative molecular masses.

Conclusion

CITP and CZE methods proved to be powerful tools for determination of the effective charge and the ionic mobilities of single isomer and randomly highly sulfated CDs. No counterion condensation was observed at single isomer CDs but relevant reduction (26.4-31.9 %) of charge was found at randomly highly sulfated CDs.

Acknowledgement

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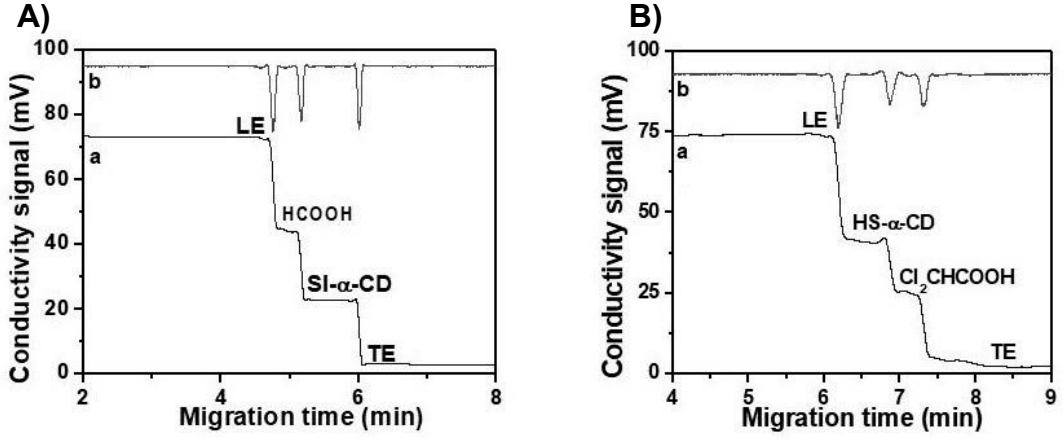
Figure 1. CITP separation of A) single isomer α-cyclodextrin (SI-α-CD) and formic acid, and B) randomly highly sulfated α-cyclodextrin (HS-α-CD) and dichloroacetic acid. Trace a), signal of conductivity detector; trace b), the first derivative of the conductivity signal. LE, leading electrolyte: 10 mM HCl, 20 mM histidine, pH 6.2; TE, terminating electrolyte: 20 mM sodium glutamate, pH 7.0. For other conditions, see the section Experimental.



Capillary Zone Electrophoresis – Mass Spectrometry for Intact Protein Analysis in Biological Fluids - Possibilities and Limitations

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Introduction

Most of the quantitative protein assays based on mass spectrometry (MS) use the bottom-up methodologies, where proteins are subjected to proteolytic cleavage prior to instrumental analysis. Direct quantitation of proteins in their intact form from complex biological matrices by MS is still a very challenging task. Currently, liquid chromatography (LC) in a combination with MS detection is the most frequently employed separation method in protein analysis. However, the high-performance capillary electrophoresis (CE) has emerged as complementary technique to LC. CE methods provide highly efficient separations, require low sample amounts and volumes per single analysis and they are more environment friendly than LC methods [1,2]. However, there are still several challenges connected with an application of CE methods for intact protein analysis: protein adsorption on the inner wall of the fused-silica capillaries and the lower concentration sensitivity due to the low injected sample volume. This can be a serious drawback if the analyte is present in complex biological matrices and at very low concentration level.

Experimental

Capillary electrophoresis Agilent 7100 CE System coupled to triple quadrupole mass spectrometer Agilent 6410 Series using a sheath-liquid electrospray ionization interface (ESI) was applied for our analyses. The analyses were performed in uncoated bare fused silica capillary with 50 μm ID and 85 cm in total length. The separation voltage was set at 20 kV. The optimum background electrolyte was 500 mmol/L formic acid with an addition of 5% (v/v) acetonitrile. The sheath-liquid was composed of 50/50 (v/v) MeOH/water with an addition of 0.1% formic acid and delivered at a flow rate 8 μL/min. The pressure of the nebulizing gas (nitrogen) was kept on 10 psi. Drying gas was delivered at the flow rate 10 L/min and its temperature was 300°C. The voltage on the ESI tip was set to 4500 V.

Results

19- to 127-fold increase in signal intensity was achieved by employing transient isotachophoresis (tITP) as an in-capillary preconcentration method. tITP was performed using 200 mM ammonium formate (pH 4.0) added to the sample matrix as leading electrolyte and optimized BGE as terminating electrolyte. Sample was introduced to the capillary using hydrodynamic injection for 250 s.

Off-line μSPE with various eluate treatment was evaluated to ensure the compatibility of the sample pretreatment method with the selected in-capillary preconcentration, CZE separation,

and MS detection. The Oasis Hydrophilic Lipophilic Balance® (HLB) µElution 96-well sample plate were used for µSPE extraction [3]. Achieved extraction recoveries of spiked proteins were in the range of 76–100 % for urine, 12–54 % for serum, 21–106 % for plasma, and 25–98 % for saliva when the eluate was evaporated and reconstituted into the solution of the leading electrolyte to achieve the tITP process [4].

Conclusion

The optimized method demonstrates good linearity, accuracy, and precision, suggesting their suitability for proteomic studies across various biological samples. Applications of these methods could include therapeutic drug monitoring and biomarker research. The use of CE-MS creates a pathway for more efficient and environmentally conscious analytical process, thereby advancing the field of proteomics.

Acknowledgements

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Microcontrollers and their application in constructing analytical instrumentation

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Introduction

Microcontrollers are small, yet powerful autonomous electronic platforms that find wide use in many do it yourself projects. However they can also find application as cheap, open source, analytical instruments. They have been for example used in construction of pumps, electrochemical sensors, spectrophotometers and various other instruments. We have been using Arduino microcontrollers as parts of our instrumentation, specifically the instrumentation that can be used in separation techniques, such as capillary electrophoresis (CE) or liquid chromatography. In this lecture, we shall outline some basic principles on using microcontrollers in constructing and controlling in-house built analytical instruments and show examples developed in our laboratory: an open source data acquisition unit, a temperature controlled sampler for collection of exhaled breath condensate (EBC) and a CE autosampler.

Experimental

Arduino microcontrollers (Arduino Nano, Uno and Mega) were purchased from the e-shop on the internet. All electronic components used for the construction of the instruments were purchased from the local electronic store. The chassis of the EBC sampler and the CE autosampler were printed using a Prusa MK3 printer. A homemade CE system with either contactless conductivity or laser induced fluorescence was used in the presented work. All Arduino codes were written in the Arduino IDE. All source codes, schemes, list of electronic parts and 3D models are available in the published articles [1-3].

Results

In the first part of this lecture, we will shortly discuss how Arduino microcontrollers work, their basic programing structures and eventually discuss the three devices developed in our laboratory. First, several simple data acquisition (DAQ) devices based on Arduino Nano microcontroller and various analog-to-digital converter modules with resolution in the range from 16 to 24 bit will be shown. We demonstrate that for the CE system with contactless conductivity detection, the best results were obtained with the developed 24-bit DAQ device, the performance of which was comparable to a commercial, high-end 24-bit DAQ device (ORCA 2800). Second, an Arduino Uno controlled EBC sampler will be presented. This sampler uses Peltier elements to cool the collection tubes and the feedback control from a temperatures sensor to regulate the collection temperature within 1 degree Celsius. We shall show the use of this device for collection of large volumes of exhaled breath condensate and its

analysis. Third, an autosampler for a modular CE system will be presented. The entire system is driven by an Arduino Mega microcontroller, which manages hydrodynamic sample injection, capillary flushing and external control of the high voltage power supply and data acquisition. The instrument manual control and programming is enabled through a keypad and an LCD display. The functionality of the autosampler was tested on analysis of anions. Performance of the autosampler was comparable to commercial instrument (Agilent 7100 CE) and with lower errors if compared to the same in-house-built CE system but with manual sample injection.

Conclusion

The open source paradigm is gaining significance in analytical chemistry as it allows the scientists to share ideas on constructing cheap analytical instrumentation. In this lecture we have presented three examples of devices that were developed in our laboratory and that fall within the open-source instrument category, i.e. we have published all necessary information for their easy construction in the respective articles [1-3]. We hope that our efforts will contribute to the wide availability of cheap and simple analytical instrumentation and that many similar articles and ideas will follow.

Acknowledgements

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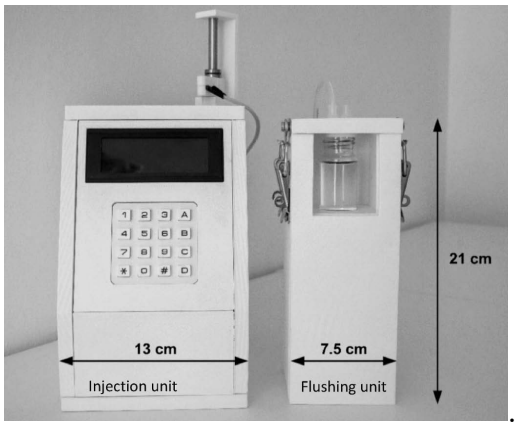
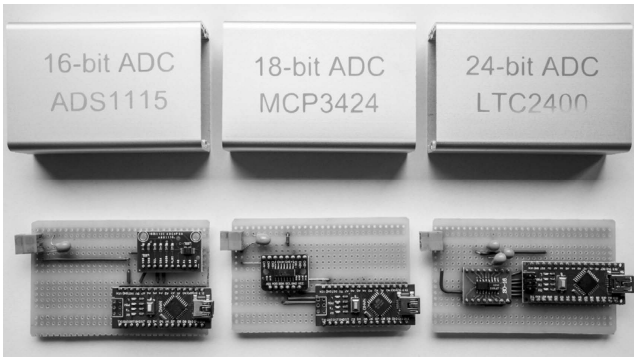
Figure 1. A 3D-printed CE autosampler (left), various data acquisition devices (right).





Adsorbable organic fluorine (AOF) – a sum parameter for non-targeted screening of per- and polyfluorinated alkyl substances (PFASs) in waters

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Introduction

The prevalence of per- and polyfluorinated alkyl substances (PFASs) and other perfluorinated compounds (PFCs) that persist and accumulate in the environment (as well as in our own bodies) is becoming an increasing international concern [1]. PFASs are a class of nearly 10,000 different compounds more commonly known as «forever chemicals» due to their stability [2]. They are a challenge to monitor individually and quantify in low concentrations. Expensive analytical instrumentation and experience is required to determine a small selection of individual PFASs, and such analyses can be time-consuming and difficult to validate.

Experimental

The adsorbable organically bound halides were analyzed by preconcentrating the analytes on activated carbon and analyzing this activated carbon by means of combustion IC. The adsorption on the activated carbon was performed automatically using the APUsim from Analytik Jena. 100.5 mL of sample (100 mL sample + 0.5 mL nitrate stock solution/nitric acid nitrate stock solution) were passed through 2 activated carbon columns arranged in series at a flow rate of 3 mL/min. The columns were then washed with 25 mL of nitrate wash solution (AOF) at a flow rate of 3 mL/min.

For analysis, the activated carbon and the wool were pushed out of the column into a quartz boat. The two columns were analyzed individually. No preconcentration column and inline matrix will be used because of the use of ultrapure water as absorber solution and to minimize blanks coming from the system.

Results

All samples were analyzed in replicates (n=4). All waters contained trace concentrations of AOF ranging from an average of 6.52 µg/L to 9.70 µg/L, with lower concentrations found in surface water compared to wastewater (Table 1). Although concentrations of AOF are generally low and sample preparation can be complex, the automation of sample processing and the analysis guarantees excellent repeatability. For the replicates, RSDs of 3.6–5.3% were achieved (n=4). For routine analysis, the method blank was determined to be 1.1 µg/L for AOF (based on ultrapure water and including all sample preparation and combustion steps).

Conclusion

The AOF sum parameter is more comprehensive to estimate the overall impact of organofluorine substances in water samples. It is a good indicator to initiate detailed targeted analyses if values are high. This can be especially helpful to determine whether water treatment processes have been effective enough in municipal water facilities and wastewater treatment plants to sufficiently remove harmful fluorinated substances before they are released to the environment or to the general water supply.

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Table 1. Results of the AOF analyses for surface water and wastewater samples. The table shows AOF results for the four measured replicates of each sample, the average and standard deviation (SD), and the relative standard deviation (RSD).

sample	AOF^1 [µg/L]	AOF^2 [µg/L]	AOF^3 [µg/L]	AOF^4 [µg/L]	Avg. ± SD [µg/L]	RSD [%]
Surface water	6.26	6.27	6.79	6.77	6.52 ± 0.30	4.6
Wastewater 1	10.23	10.03	9.31	9.21	9.70 ± 0.51	5.3
Wastewater 2	7.36	6.99	7.61	7.21	7.29±0.26	3.6

How to successfully analyse oligonucleotides with liquid chromatography?

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The emergence of new DNA or RNA-based therapies is opening up new perspectives for the treatment of genetic diseases. Among them, therapeutic oligonucleotides are enjoying growing success due to their high specificity for their target and their improved pharmacokinetic properties. Thus, although considered by regulatory agencies as small molecules, they also share characteristics with therapeutic proteins. Oligonucleotides are therefore a new class of pharmaceutical compounds requiring specific considerations.

In order to ensure the safety and efficacy of these new therapeutic molecules, their characterisation is essential and requires adapted and robust analytical methods. Reverse phase liquid chromatography with added ion pairing agents (IP-RPLC) is the reference method for the analysis of oligonucleotides, and HILIC is gaining in popularity.

In the present work, various strategies will be exposed to improve sensitivity, throughput, and selectivity when analyzing therapeutic oligonucleotides in IP-RPLC and HILIC modes. The goal of this presentation will be to highlight i) the importance of bioinert columns to limit adsorption of oligonucleotides, ii) the interest to use alternative column chemistries to improve selectivity, iii) the possibility to work with ultra-short columns of only a few mm to achieve high throughput separations, and iv) the use of pressure as an additional parameter to tune selectivity.

Recent applications of nano-liquid chromatography: Analysis of molecules affecting human health

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The global acceptance of sustainability led to the development of emerging areas at that time, such as "Green Chemistry". In the field of analytical chemistry, the main efforts have been focused on the reduction or elimination of hazardous reagents, the decrease of waste, the development of automatized and miniaturized techniques that diminish the costs, analysis times and reagents consumption [1].
In this regard, nano-liquid chromatography (nano-LC) shows many advantages as a separation technique. Analytes separation is carried out into capillary columns (internal diameter 100 μm) with flow rates in the range of a few hundred nanoliters per min, resulting in high separation efficiency, good resolution, and enhanced sensitivity. The miniaturized dimensions of the chromatographic system allow the reduction of solvent consumption, stationary phase and small sample volume required, which reduce analytical costs compared to HPLC. In addition, the reduced flow rate provides a good compatibility with mass spectrometer [2].
For these purposes, nano-LC becomes a very promising tool in a wide research area (e.g. omics, food, environmental, biomedical).
In this presentation, two different miniaturized analytical methods, concerning the determination of contaminants and biomolecules in different matrices are reported.

A dispersive liquid-liquid microextraction combined with nano-liquid chromatography was optimized for the analysis of seven neonicotinoids in honey matrices and biological samples.
Neonicotinoids are a relatively novel class of insecticides widely used in the global market owing to their broad-spectrum efficacy against insects, systemic action, and relatively long residual activity. Despite the low toxicity, their widespread use has resulted in their transfer and accumulation in the environment and food chain, with increasing negative effects on health [3].
The analytical method was developed for the simultaneous determination of seven neonicotinoids (dinotefuran, nitenpyram, thiamethoxam, imidacloprid, clothianidin, acetamiprid, and thiacloprid) investigating the chromatographic and extraction conditions. Two different ultrasound assisted dispersive liquid-liquid microextraction (UA-DLLME) procedures were optimized for the neonicotinoids in honey samples and urine samples, respectively. The parameters affecting DLLME (pH and ionic strength of the aqueous phase, type, and volume of the extractant and dispersive solvent, vortex, and centrifugation time) were studied for each matrix. The reverse phase nano-LC-UV method was optimized by using a X-BridgeTM C18 column allowing the complete separation of the selected neonicotinoids within 25 min under isocratic elution mode. The methods were validated in terms of precision, sensitivity, linearity, and recovery. The combination of a large injection volume with the DLLME procedure resulted in a good method sensitivity.

In this study, the profiling polyphenol composition in plant extracts by means of nano-liquid chromatography is reported.

Plant extracts have always been the primary source of medicine and food for humans in the world. They are abundant sources of key secondary metabolites including polyphenols, alkaloids, terpenes, vitamins, possessing biological activity. For those purposes, plant extracts because of the wide variety of bioactive components, have been known for various health-promoting benefits like antioxidants, anti-inflammatory, anticancer, hypoglycemic, antiobesity [4].

A nano-LC-UV method was developed for the polyphenolic profile of selected plant extracts analyzing simultaneously ten phenolic acids and six flavonoids. The experiments were carried out with a capillary column with an internal diameter of 100 μm, packed with a reverse stationary C18 and a mobile phase composed by a mixture of water/acetonitrile (ACN) and formic acid (0.1 % v/v) with a step gradient elution.

For the extraction of polyphenols from dried plants, two approaches were tested: solvent extraction supported by ultrasound bath and dynamic maceration. Different compositions of extraction solvent were also tested. The extraction procedure was also investigated optimizing the extraction time, the volume and nature of solvent.

This presentation shows that the optimized miniaturized analytical methods (separation and extraction procedure) are promising approaches, rapid, cost-effective, eco-friendly and can be complementary to the conventional analytical tools.

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Personal insights on the design of experiments

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The results of each analytical method depend on the setup of the experimental conditions and their influence on the registered signal. One of the most basic approaches to finding the optimal conditions is incremental optimization, where the operator varies the settings of these conditions and monitors their effect on the response. This approach is relatively simple and requires only knowledge of the physicochemical laws of the method. On the other hand, in the case of stepwise optimization, the mutual effects of experimental factors may not be revealed, and – although simple – this approach may be more time-consuming and, as a result, more expensive.

Experimental designs are a suitable alternative. In these cases, the levels of experimental conditions (e.g., pH range, analysis temperature used, reaction mixture composition) are first defined, which must be based on knowledge of the method and physicochemical constraints. This is followed by a description of the experimental space given by the selected experimental design. After the proposed experimental conditions have been measured, a mathematical model is created by regression analysis to predict the outcome of those experimental conditions that were not part of the original plan. The advantage is also the easy visualization of the effect of the conditions on the response. This procedure can be repeated for more narrowly selected experimental conditions if necessary.

The lecture will describe and demonstrate the optimization of experimental conditions using an experimental design approach. A utilization of experimental design in the preparation of polymer-based monoliths will be described, focusing on optimizing porous structure, tailored surface modification, and development of enzymatic reactor. Optimization of sensitivity in LC-MS analysis will also be mentioned.

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How to make chromatography more sustainable

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Liquid chromatography is a widely utilized analytical technique across various industries including pharmaceuticals, biotechnology, food and beverage, and environmental monitoring. The most prevalent form is reversed-phase chromatography, constituting over 75% of applications. However, traditional methods are not considered environmentally friendly, largely relying on acetonitrile and large column dimensions (250x4.6mm). To mitigate these environmental impacts without compromising performance, several approaches can be employed. Firstly, adopting eco-friendly solvents like water and ethanol, which are non-toxic, biodegradable, and have minimal environmental footprints, can significantly reduce environmental impact. Water, especially in reversed-phase chromatography, stands out as a readily available and cost-effective green solvent. Ethanol, derived from renewable sources, further exemplifies this sustainability trend.
Additionally, method optimization plays a crucial role in reducing solvent consumption and waste generation. This includes adjusting column length, injection volume, and gradient conditions. Embracing greener equipment, designed for energy efficiency and solvent recycling, can further enhance sustainability efforts. Waste generated during chromatographic processes can also be recycled or reused, contributing to overall environmental preservation.

Presentation will delve into various strategies researchers can employ to minimize their environmental footprint. This includes utilizing smaller ID columns and higher efficiency shorter columns while maintaining accuracy, as well as transitioning to non-toxic mobile phases like bioethanol. It's essential to note that while green solvents offer promising alternatives, their suitability depends on specific method requirements. We will discuss these options comprehensively, providing multiple examples to illustrate their efficacy and impact on chromatography.

Automated workflow for derivatized analyte analysis in LC-TIMS-MS/MS 4D-metabolomics data: Incorporating *in-silico* derivatization, CCS prediction, and *in-silico* fragmentation

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Analyzing hydrophilic metabolites in the TCA cycle poses challenges due to low LC retention and sensitivity. Chemical derivatization enhances LC retention and detection sensitivity but complicates data interpretation. Our automated workflow combines in-silico derivatization, CCS prediction, and in-silico fragmentation for confident analysis of derivatized metabolites. Using 3-nitrophenylhydrazine (3-NPH) as an exemplar reagent, we annotated 9 TCA cycle metabolites in SRM 1950. Additionally, we will demonstrate the workflow's utility in assessing changes in plasma samples from individuals with inborn errors of metabolism, including methylmalonic aciduria. Confidence in annotation was bolstered by closely matching CCS values (average deviation of 0.36% between two instrument platforms used).

In brief, our automated approach streamlines the interpretation of derivatization data for both targeted and non-targeted metabolomics using LC-TIMS-MS.

Isomer separations in metabolomics and lipidomics

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Introduction

Lipidomics and metabolomics are analytical approaches widely adopted in biomedical research with the aim to obtain mechanistic insights in biochemical processes, to find diagnostic and prognostic biomarkers, and generate new hypothesis about biological processes or validate concepts from other experimentation. Although common untargeted methodologies allow simultaneous analysis of hundreds of metabolites, they often fall short in full coverage of pathways in which isomeric species play a major role, e.g. the phosphoinositide network, glycolysis and pentosephosphate pathways. This presentation will focus on challenging lipid and metabolite classes in targeted and untargeted lipidomics and metabolomics, respectively, for which biological interpretations rely on a separation of their structural isomers.

Results

In the first part, the focus will be on the phosphoinositide pathway. Phosphoinositides are regulating key metabolic processes including signal transduction via phospholipase C and phospshoinositide-3-kinase pathways. Arising from dynamic phosphorylation/dephosphorylation on the inositol ring of phosphatidylinositols (PI), phosphoinositides (PIPx) comprise seven classes according to phosphate position and number. Combined with various fatty acid side chains high structural complexity arises. For a comprehensive understanding of the phosphoinositide signaling, analytical coverage of the entire metabolic network is highly desirable including besides above lipid classes also the corresponding hydrophilic inositolphosphate metabolites, such as the second messenger inositol triphosphate (I(1,4,5)P3). However, the analysis of the entire PIPx network remains extremely challenging due to their low abundance, high negative charge density, presence of regioisomers, and extremely wide polarity range of the metabolites involved in the network, including PI and diacylglycerols (DAG) (lipophilic), PIPx (amphiphilic), and inositol phosphates (IPx) (hydrophilic). Here, we describe an integrated workflow for these metabolites using a single sample aliquot.

In the second part, the challenges of an integrated workflow covering the entire glycolysis and pentosephosphate pathways with their numerous phosphorylated carbohydrate structural isomers will be discussed. In spite of new HILIC columns with deactivated hardware surface, HILIC separations of the entire sugar phosphate isomer sets remained troublesome. Published works typically focus on selected metabolites. Moreover, HILIC separations were reported to be poorly repeatable with significant shifts in retention time. In this presentation, an integrated workflow with HILIC separation will be presented which overcomes these problems. All metabolites of the glycolysis and pentose phosphate pathway are covered with good peak shapes and isomer selectivity and a simple solution to the reported repeatability problem of HILIC will be presented.

Novel approaches in the analysis of lipids, glycosphingolipids and metabolites

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The field of lipidomic, glycolipidomic, and metabolomic analysis is developing quite rapidly, owing to the increased interest of the scientific community and the implementation of new analytical technologies, which allow the development of new methods providing higher sensitivity, isomeric resolution, and reliable quantitation. There are two major strategies in the area of mass spectrometry coupling with separation techniques in liquid or supercritical states, which are referred to as lipid class and lipid species separation approaches [1]. The lipid class separation techniques are primarily based on interactions between the polar head group of lipid molecules (e.g., phospholipids or sphingolipids) with the polar functionality embedded in the stationary phase (e.g., hydroxyl group in case of silica gel). The effects of the length of fatty acyl chains and the number of double bonds are minor, which results in the separation of lipid classes according to the type of polar head group, whereas the minor separation within the lipid class could be observed as well. The typical separation modes are hydrophilic interaction chromatography (HILIC) or HILIC-like mode in ultrahigh-performance supercritical fluid chromatography (UHPSFC). The strategy is ideal for quantitation because the exogenous internal standard coelutes with lipid species from the same class, resulting in identical matrix and suppression effects and, hence, robust and high-throughput quantitation [2]. However, some isomers may not be separated, and the structural information is limited to the level of lipid species. Examples of the lipid class quantitation approach are the use of UHPSFC/MS in the screening of pancreatic cancer [3] or HILIC/MS methods used for detailed characterization of complex glycosphingolipids [4] including the isomeric separation (glucosyl vs. galactosyl). The high level of automation can be achieved due to semiautomated data processing using LipidQuant 2.1 software [5]. The second strategy is the lipid species separation, which is based on the use of reversed-phase UHPLC/MS, which offers higher separation selectivity resulting in the detection of more than 500 lipid species at the fatty acyl level [6], but the method has lower throughput and requires more manual supervision during the data processing. Some ionic metabolites and lipids, for example, nucleotides containing one to three phosphate groups, cause serious problems due to the peak tailing caused by the interaction of the ionic group with metal ions on the column surface. Our new method based on the bioinert system and bioinert chromatographic column, significantly improves the peak shapes and allows the analysis of more than 130 metabolites, e.g., proteinogenic amino acids, including leucine/isoleucine/norleucine isomers, carnitines, nucleotides, and nucleosides. Glycosphingolipids (GSL) are another group of complex molecules containing a hydrophobic ceramide part and a hydrophilic (oligo)saccharide part, which could be neutral, typically with 1-4 hexose units or anionic, e.g., gangliosides or sulfatides. We use HILIC/MS either for the analysis of intact GSL molecules or also for enzymatically hydrolyzed glycan parts. The last approach deals with the development of new comprehensive online RP-

UHPLC×UHPSFC/MS/MS method and the application to the characterization of a wide range of lipids in human plasma samples. The reversed-phase UHPLC with C18 column (150 x 0.5 mm, 1.9 μm) applied in the first dimension (1D) allows the separation of lipids with low flow rate of mobile phase (8 μL/min). UHPSFC in the second dimension (2D) is used due to the high speed of the analysis (35 s, 10 x 2.1 mm, 1.7 μm column). Continuous 4D lipidomic analysis represents a novel strategy for the comprehensive analysis of more than 300 lipid species from 16 lipid subclasses. This is the first connection of UHPLC and UHPSFC in this configuration for lipidomic analysis, using very short columns in 2D (10 mm), short sampling time (0.55 min), and the gradient elution in both dimensions. The gas-phase separation by ion mobility provides an additional separation dimension in addition to chromatography and tandem mass spectrometry (MS/MS). Examples of ion mobility separation by TIMS-Ultra ion mobility from Bruker Daltonics will be presented.

Acknowledgments
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Unveiling the power of deep UV LAESI/LAESCI dual ambient ionization: Towards comprehensive molecular MSI

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Introduction

Ambient molecular mass spectrometry techniques have revolutionized our ability to analyze complex biological surfaces under native conditions. The laser ablation electrospray ionization (LAESI) has emerged as a promising technique due to its capability to directly sample surfaces with minimal preparation [1]. However, traditional LAESI instruments often suffer from limitations such as poor spatial resolution, limited analyte coverage, or inefficiency in desorption due to the sample composition [2, 3]. Here, we introduce a new deep UV LAESI/LAESCI dual ambient ionization source (Fig. 1) with "in-house" adjustable interface, enabling simultaneous acquisition of complementary LAESI/LAESCI mass spectra.

Experimental

All LAESI/LAESCI-MS and LAESI/LAESCI-MSI experiments utilized the Analyte G2 laser ablation unit (Photon Machines, USA) equipped with a 193 nm excimer laser with a variable laser spot size (3 – 160 μm), sampling frequency (up to 100 Hz), and fluence (0.6 – 12 J/cm^2). The device was connected to a Synapt G2-S hybrid Q-TOF mass spectrometer (Waters Corporation, UK) via a custom interface. Spot analysis conditions included optimization variable parameters such as repetition rate, spot size, scan speed, ESI/ESCI parameters. For imaging experiments, parameters were adjusted for various lateral resolutions and the data were processed using Ilaps software [4] for laser ablation data reduction and imaging.

Results

The newly developed deep UV LAESI/LAESCI dual ion source was tested for its ability to efficiently ionize analytes of different molecular masses and polarities, with ablated material transported from the remote device to the dual probe for ionization. Representatives of PAHs, waxes, lipids, purine derivatives, designer drugs and others were selected to demonstrate the detection potential of LAESI and LAESCI ionization for different types of analytes. For example, LAESCI exhibited 2- and 7-times higher sensitivity to squalene and PEG 600 in positive mode, while LAESI showed two times higher sensitivity to selected designer drugs, mephedrone or cathinone. The technique also enabled molecular mass spectrometry imaging with variable laser spot size, enabling comprehensive analysis of complex samples such as latent fingerprints. For instance, the visualization of another designer drug, naphyrone, on latent fingerprints demonstrated its forensic potential by aiding in personal identification through reliable papillary line patterns reconstruction. The achievable limits of detection were as low as 0.5 amol per pixel. LAESI/LAESCI-MSI methods have proven to be effective in detecting characteristic features even in overlapping fingerprints, enabling the linking of molecular features with biometric information for forensic analysis and personal identification.

Conclusion

We present a novel deep UV LAESI/LAESCI dual ambient ionization source, applicable for comprehensive molecular mass spectrometry in both spot analysis and mass spectrometry imaging. This developed source combines the notable advantages of LAESI, such as minimal sample preparation, versatility, rapid analysis and minimal sample consumption with wide analyte coverage and improved spatial resolution, offering a potentially highly valuable tool for diverse applications in biomedicine, environmental analysis, and materials science. By addressing critical challenges in ambient ionization, this work aims to contribute to advancing molecular analysis techniques and interdisciplinary research worldwide.

Acknowledgements

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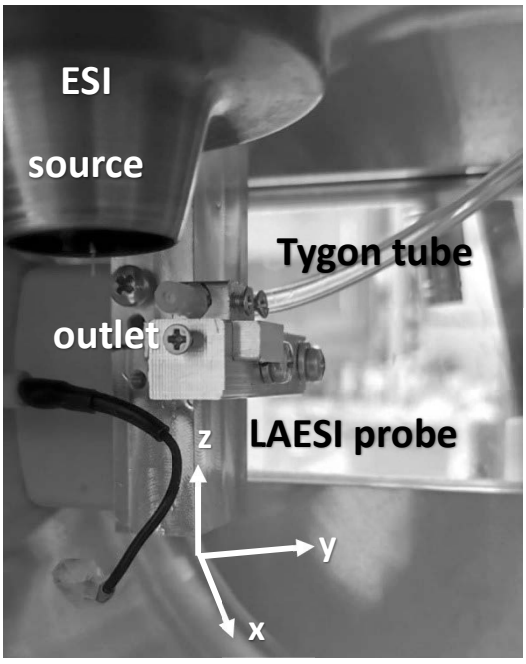
Figure 1. Deep UV LAESI/LAESCI dual ambient ionization source interface



Advanced small molecule mass spectrometry

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Soon after discovery, first commercialization and later hyphenation with different chromatographical separation techniques, mass spectrometry showed potential for expansion of analytical workflows towards larger analyte panels, more complex matrixes, and unknown compounds identification. In the world of small molecules, mass spectrometers hyphenated with gas chromatographs (GC-MS) were proven early on to be useful for compound identification by the means of comparing acquired mass spectra with spectral libraries. This is possible mainly due to inert carriers used in gas chromatography, stable conditions in the ion source and standardization of ionization energy. Resulting GC-MS spectra are repeatable between different instruments and methods and have relatively low number of interferences. Mass spectrometers hyphenated with liquid chromatographs (LC-MS) suffer much more in that respect since variability of mobile phases is much bigger, there are number of impurities introduced already with the mobile phases and source conditions can also vary a lot. Routine unknown compound identification with LC-MS was therefore always challenging.

Innovations in the field of mass spectrometry, like new analyzers and optimized ion optics enabled mass spectrometers to achieve higher spectral resolution, higher scan speed, better mass accuracy over longer time periods and broader dynamic range. At the same time higher processing power of affordable computers and cloud-based data storage opened the door for development of high-performance data processing software packages and large on-line spectral tree libraries. With that, high quality data from mass spectrometers can be effectively used for routine one run qualitative/quantitative workflows, fast large analyte panel analysis, unknown screening and confident identification, differential analysis and more.

Employing ballistic gradients, vacuum jacketed columns and prototype benchtop multi reflecting time-of-flight (MRT) to increase lipidomic analytical throughput whilst maintaining highly confident identifications

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Introduction

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

Experimental

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

Results

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

samples could be separated based on abundance. Statistical analysis comprising of multi-variate statistics, resulted in similar profiles being generated via unsupervised principal component analysis (PCA), regardless of the chromatographic methodology employed. This ultimately resulted in the most statistically significant features between the patient groups being identified for 1mm and VJC-based data. Finally, methods were transferred to a 1mm vacuum jacketed column and when a 10-minute gradient was employed, peak capacity was shown to have increased when compared to conventional column formats, which facilitated a larger number of identified features.

Conclusion

The high scan speed afforded by the prototype benchtop multi reflecting time-of-flight (MRT) instrument allowed adequate profiling of sub-1 second peaks observed during VJC separations without compromising on mass accuracy at the ppb level and resolution. Application of VJC using a reduced gradient time compared favourably to conventional columns and separation time, allowing for a 30% increase in throughput whilst maintaining equivalent chromatographic performance.

Advances in chiral separation of carboranes – new phenyl ring isosteres for drug development

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Introduction

Boron cluster compounds form a unique class of highly stable abiotic molecules. Substituting BH^- units with CH^- groups forms icosahedral carboranes, which enhance hydrophobicity and stability compared to typical enzymatic systems. Both endohedral and exohedral modifications can yield asymmetric molecules. These structures exhibit chirality, deriving either from the overall asymmetry of the structure or from axial or helical chirality, which occurs when the molecule's rotation is limited or obstructed, as illustrated in Fig. 1. In medicinal chemistry, these clusters are explored as unconventional bioisosteres for phenyl rings. Regrettably, the chirality of these boron clusters had been largely ignored over the last six decades.

Results

Our team has recently developed quick and effective techniques to confirm chirality and assess the optical purity of these molecules. We focused on exploring the primary causes behind the failure of chiral separations of particularly anionic carboranes using HPLC. Cyclodextrin-based stationary phases were demonstrated to successfully separate enantiomers of anionic carboranes. Additionally, the enantioseparation of 7,8-dicarba-nido-undecaborate(1-) ions was accomplished for the first time via HPLC. Our findings also indicate that polysaccharide-based chiral selectors can be employed in RPLC settings for the chiral separation of both anions and zwitterions. Recently, SFC has proven to be effective for quick chiral baseline separations, completed in under 10 minutes, which significantly outperforms HPLC in terms of the volume of separated chiral carboranes, resolution, and analysis time.

Conclusion

Our research provides new and valuable insights into the strategy for developing chiral methods for anionic and zwitterionic carboranes, with applications in drug development. Furthermore, our findings can be applied broadly in many fields where the chirality of these fascinating molecules might be relevant.

Acknowledgements

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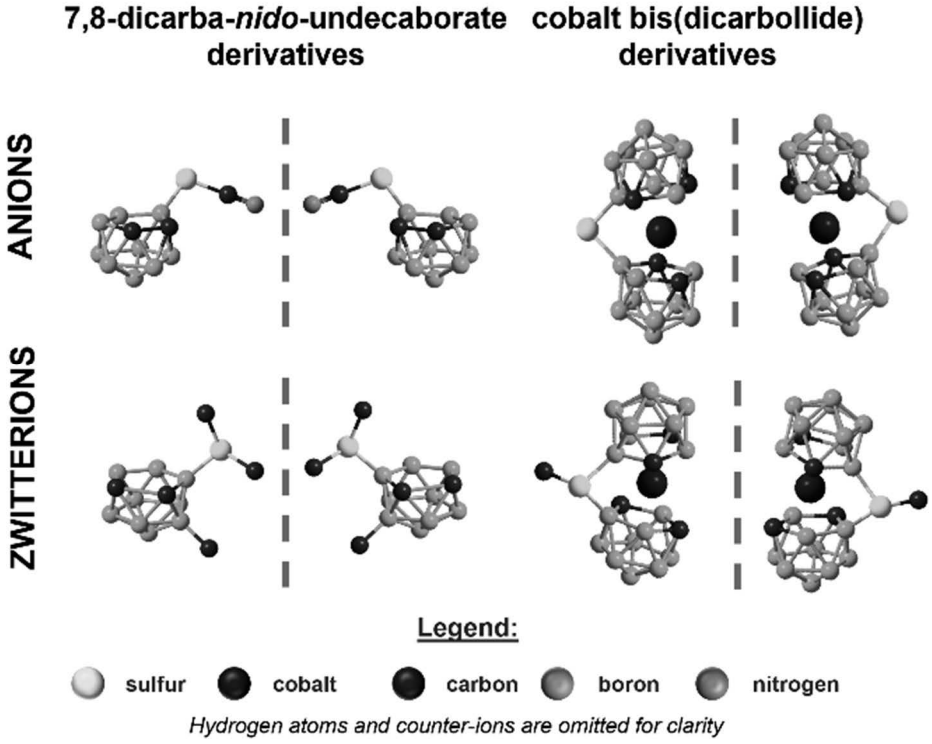
Figure 1. Chirality of studied carboranes [5].



Analysis of selected drugs in whole blood collected with a volumetric absorptive microsampling device

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Introduction

Preclinical research is an important part of novel drug development. However, ethical concerns surrounding the use of animals in research have led to the "3R" recommendation, which emphasizes replacement, reduction, and refinement [1]. Microsampling is a technique that aligns with the 3R recommendation by reducing the number of animals required for a study. This is achieved by repetitive collection of small blood samples from a single animal, which is particularly beneficial for rodents with low blood volumes. Microsampling also offers the advantage of a less invasive sample collection. Dried blood spot (DBS) is a commonly used microsampling technique but has the disadvantage of hematocrit (HCT)-dependent results [2]. Volumetric absorptive microsampling (VAMS) is a newer technique that significantly reduces the impact of HCT on the assay and requires even smaller sample volumes than DBS. VAMS devices consist of plastic sticks with tips made from a unique hydrophilic polymer, allowing for the collection of 10, 20, or 30 µl of blood. Analytes are commonly extracted from VAMS tips by direct desorption into an appropriate solvent facilitated by ultrasonication, agitation, or vortexing [2]. However, microextraction approaches have a high potential for the isolation of drugs from VAMS tips, offering an ethical and environmentally friendly sample cleanup solution.

This study focusses on developing different sample extraction procedures for isolating of selected drugs from blood microsamples collected with VAMS devices in preclinical studies with nude mice. The first group of analytes comprises doxorubicin (DOX), a clinically used anticancer drug and its metabolites (DOXol), which are commonly used in preclinical studies. The second group is cardioprotective agents, including dexrazoxane (DEX) and its more potent novel analogue ICRF-193, which are designed to prevent anthracycline-induced cardiotoxicity [3].

Experimental

The volumentric absorptive microsampling device (10 µl, Mitra®, Neoteryx) was used for all experiments. Electromembrane microextraction (EME) in a 96-well plate format was developed to extract DOX and DOXol from blood loaded on VAMS tips. The recovery, repeatability, matrix effect and environmental impact of EME were compared to those obtained from a direct extraction procedure. Dexrazoxane and ICRF-193 were isolated from blood on VAMS tips using ultrasonic-assisted desorption. All samples were analyzed using UHPLC-MS/MS (Agilent 1290 Infinity LC with Triple Quad LC/MS 6400). The assay used either isotopically

labelled internal standards (DOX and DOXol) or structurally closed analogues (DEX and ICRF-193). The methods were validated within the relevant concentration ranges and utilized for analysis of blood samples taken from preclinical pharmacokinetic studies in nude mice.

Results

Various supported liquid membranes were tested for EME of DOX and DOXol from blood absorbed on VAMS tips. Among the different membrane options, a mixture of 1-ethyl-2-nitrobenzene and 1-undecanol in a 1:1(v/v) ratio was found to be optimal for achieving higher recovery, lower matrix effects and acceptable reproducibility. The donor solution was formic acid, and acetic acid was used as the acceptor phase. The method was validated in a concentration range of 3.5 to 8600 nM for both DOX and DOXol in blood. After systematic optimization, a mixture of acetonitrile and formic acid was selected as the desorption solvent for DEX and ICRF-193. The method was validated for concentration ranges of 0.1-400 μM and 0.01-20 μM, respectively. In addition to common validation parameters, the possible effects of distinct HCT, the presence of anticoagulation agents in calibration samples, and the formation of analyte-iron complexes to assay results were tested. All were found to have only a minor impact on the assay of both groups of analytes. Concentration time profiles of all analytes in blood were determined in nude mice, and at the end of the experiment, the analyte concentrations were also assayed in plasma to estimate the blood/plasma ratio.

Conclusion

This study investigates various procedures for extracting analytes from blood microsamples loaded on VAMS tips. The results show that EME is a promising platform for a single-step, environmentally friendly treatment of VAMS microsamples containing anthracyclines. Additionally, an ultrasonic-assisted desorption procedure was developed for isolating cardioprotective agents from the same material. The application of these novel extraction approaches to the analysis of real biological samples from preclinical experiments has proven the reliability of the developed procedures. Further utilization of these procedures in preclinical studies provides benefits such as ethical treatment of animals, improves data quality by allowing a complete sampling profile from a single animal, and linking toxic effects to drug exposure in an individual animal.

Acknowledgements

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Translation of omics into clinics

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Introduction

The integration of omics technologies into clinical practice is pivotal for advancing personalized medicine. This presentation explores the translation of metabolomics and lipidomics into clinical settings, highlighting their application in diagnosing and managing complex diseases, including inborn errors of metabolism (IEM). Metabolomics and lipidomics provide comprehensive snapshots of metabolic and lipid alterations, respectively, which are crucial for understanding disease mechanisms at a molecular level.

Experimental

Our research successfully utilizes these technologies to identify biomarkers for early detection, prognosis, and therapeutic monitoring in metabolic disorders, cardiovascular, cancer, and particularly IEM. By profiling specific metabolites and lipids, we have been able to link subtle changes in the biochemical landscape of patients to specific disease states, thus enabling earlier and more precise interventions. This is especially crucial in IEM, where early detection and accurate diagnosis can significantly alter patient outcomes and treatment approaches.

Results

This presentation will discuss our latest findings in the clinical application of metabolomics and lipidomics, including case studies where these methods have directly influenced patient care. We will also address the technological advancements that have facilitated these applications, such as high-throughput analytical techniques and robust data analysis frameworks. Furthermore, challenges such as the integration of complex omics data into clinical workflows and the establishment of standardized protocols for clinical adoption will be examined.

Acknowledgements

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Figure 1. Visualization focusing on the artistic depiction of metabolomics and lipidomics concepts *(generated by ChatGPT)*.

Py-GC/MS characterization of oil paintings to correlate museum environment and materials condition over time for SMARTMUS-e project

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This paper lays its ground on an earlier three-year research project where material's preservation of art collections was correlated to the indoor environment of two Venetian museums (Gallerie dell'Accademia and Scuola Grande di San Rocco).
In SMARTMUS-e project the impact on materials conservation of 5 museums or historical sites in northern, southern and middle Europe (Viking Ship and Historical Museum of Oslo, Český Krumlov Castle in Czech Republic and Gallerie dell'Accademia in Venice) will be characterized to compare the impact of these very different environments in artwork conservation.
In order to do so mock-ups of oil painting on canvas and panel painting were executed using oil painting formulated in our laboratory with no modern additives (Fig. 1), in order to attain a similarity to the formulation of oil paintings used during XV to XVI centuries in Europe. Only one modern oil painting was applied on the mockups, as a way to confront the behavior of these two different formulations.
In Chiranal conference we will present the preliminary analytical characterization of this mock-ups using Py-GC/MS before actual ageing in these different environments takes place. These results will help us to distinguish potential changes due to the interaction between oil and pigments and those due to the materials and environmental conditions.

Figure 1. Mock-up of oil paintings on canvas with two different grounds: on the left ground formulated with calcium carbonate and animal glue, on the right ground formulated with gypsum and animal glue.

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Recent chromatographic advances in plant hormone profiling methods

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Introduction

Phytohormones play crucial roles in influencing various physiological processes. Whilst metabolism provides the building blocks for plant growth and development, phytohormonal groups are essential for controlling the rate of growth of individual plant parts and for integrating the activities of those parts. High-resolution measurements of phytohormones are therefore necessary for physiological studies of their mode of action.

Experimental

Plant tissue (approx. 10 mg FW or 1 mg of DW) samples were extracted with a mixture of stable isotope-labelled internal standards and various cold extraction solvents such as (i) 50 mM phosphate buffer (pH 7.0) with 0.1% sodium diethyldithiocarbamate [1], (ii) 1 M formic acid in 10% aqueous methanol [2], (iii) 10% ACN with 0.5% formic acid (v/v) [3] or a modified Bieleski solution consisting of 75% methanol, 20% water and 5% formic acid (v/v) [4]. Moreover, root tips or cell suspension cultures were used for analysis of individual cell types [4,5]. Fluorescently activated cells and/or organelles were then analysed and sorted using a BD FACS Aria III flow cytometer equipped with four lasers (BD Biosciences, USA). The extracts from minute amounts of plant material or sorted samples were immediately purified using solid-phase extraction (SPE) protocols based on mixed-mode cartridges (MCX; Waters, USA) or microSPE sorbents (SDB-XC/C18; Empore, USA). Phytohormone profiling was performed using (i) a 1290 Infinity LC system and a 6490 Triple Quadrupole LC/MS, or (ii) an 1260 Infinity II LC/SFC hybrid system coupled with an Agilent 6495B Triple Quadrupole, both equipped with Jet Stream and Dual Ion Funnel systems (Agilent Technologies, USA). MS conditions were optimized for each analyte and quantification was obtained by selected reaction monitoring (SRM) of precursors and the appropriate product ions.

Results

New analytical tools provide a comprehensive insight into plant hormone regulatory networks, such as the detailed distribution of plant hormones in specific tissues, cells and organelles. We have developed several fast-chromatographic separations and highly sensitive tandem mass spectrometry (LC-MS/MS) methods for simultaneous profiling of phytohormone metabolites [1–3]. Moreover, we recently presented a breakthrough analytical technique in the field of

phytohormone analysis based on supercritical fluid chromatography (SFC) coupled with MS/MS [4]. We also focused on efficient cell and organelle isolation, combining different approaches such as density gradient ultracentrifugation or fluorescence-assisted cell/organelle sorting (FACS/FAOS) with a simple one-step purification protocol based on in-tip micro-solid phase extraction and MS-based quantification [5,6]. Our preliminary data point out to the fact that phytohormone profiles in the plant cell are quite complex and include not only the expected active molecules but also other key representatives that covers phytohormone biosynthesis, conjugation and degradation.

Conclusion

By employing these novel methods, we are able to gain a much better understanding of how genetic and experimental manipulations affect plant hormone levels, which will foster a more complete understanding of how these hormones act.

Acknowledgements

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New era in amino acid analysis

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Introduction

Amino acids (AAs) play a significant role in clinical studies, mainly in metabolic disorders monitoring and as biomarkers for differential diagnostics. L-amino acids play an important role in the physiology of all living organisms. Their chiral counterparts, the D-amino acids, are increasingly recognized as essential molecules in many biological systems. There are many analytical techniques for AAs, where chromatography methods (GC-MS, LC-MS, HPLC) are the most common ones [1]. The new MetAmino® kit offers novel derivatization in both, GC-MS and LS-MS together with micro-solid-phase-extraction (MSPE) and liquid-liquid-micro-extraction (LLME). The sample preparation of chiral AAs has been extended with amidation of the arising low-polar derivatives, an evaporation step, re-dissolution, and final GC-MS analysis. The developed method was used for various biological matrixes, e.g. human biofluids, biologically active peptides containing chiral proline constituents, and collagen [2].

Experimental

25 µL of a biological sample with added internal standard is derivatized using 25 µL of reducing agent, 25 µL of basic medium, 50 µL of reagent solution and 50 µL of catalytic medium, following with LLME step. The final derivatives were converted to methylamides using a 50 µL amidation solution. The mixture was kept at 40°C for 15 min., evaporated under a gentle stream of nitrogen and finally, the residue was dissolved in 15 µL of chloroform before GC-MS analysis.

Results

The developed method was examined in the chiral GC-MS analysis of real samples. The derivatives obtained from the first step of derivatization were mostly applied to the quantitative AAs analysis; the combined derivatization and amidation method was tested for the determination of the secondary AAs enantiomeric purity and recalculation of a quantitative level for each corresponding enantiomer. D- and L-proline, D- and L-pipecolic acid were detected in pooled urine samples, whereas in pooled serum samples D- and L- proline and exclusively L-pipecolic acid were found. The quantification of total proline and pipecolic acid was performed in urine and serum samples (Tab. 1). Suitability of the method was also demonstrated for the determination of secondary amino acids in peptides after their hydrolysis. Furthermore, D- and L-forms of proline and trans-4-hydroxy-L-proline were found in collagen from human placenta.

Conclusion

A GC-MS method for the enantiomeric resolution of eight biologically important secondary amino acids (azetidine-2-carboxylic acid, proline, pipecolic acid, nipecotic acid, *cis*/*trans*-3-hydroxyproline, *cis*/*trans*-4-hydroxyproline) and for the chiral separation of *cis*/*trans*-5-hydroxy-L-pipecolic acid on Chirasil-Val was developed and validated. The method enables profiling of secondary AAs in biological samples after derivatization and LLME, where the absolute concentrations can be measured. Chiral separation was achieved after two-step derivatization. Enantiomeric ratios down to 0.1 % can be measured. Presented derivatization method proceeds rapidly under relatively mild conditions, is cost-effective and compatible with the aqueous sample environment.

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Table 1. Total content of secondary amino acids in human serum and urine (n=5)

Analyte – secondary acid	**Human serum [µmolL^{-1}]**	**Human urine [µmolL^{-1}]**
Pro	195.7 ± 8.6	28.6 ± 0.9
D-Pro	0.20 ± 0.01	0.51 ± 0.02
L-Pro	195.5 ± 8.6	28.1 ± 0.9
Pip	5.23 ±0.30	3.34 ± 0.10
D-Pip	NF	2.76 ± 0.08
L-Pip	5.23 ± 0.30	0.58 ± 0.02

NF = not found.

Matrix effects in LC-MS: Importance of correct calculation approach

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Introduction

Matrix effects represent a significant challenge in current liquid chromatography-mass spectrometry (LC-MS) analyses of complex matrices. They arise from sample components other than the analytes of interest and impact the ionization process in mass spectrometry. Their origin is from co-eluting compounds of endogenous or exogenous nature introduced from the analyzed sample itself or during analytical procedure. Matrix effects manifest as signal enhancement or suppression and as a consequence, method accuracy, precision, and sensitivity are negatively affected.

Matrix effects can be mitigated through various strategies such as optimizing sample preparation techniques, chromatographic separation, using different ionization techniques, and incorporating isotopically labeled internal standards to correct for signal suppression or enhancement. Determining matrix effects can be accomplished through various methods including (i) post-column infusion which is only qualitative approach, (ii) post-extraction addition as described by Matuszewski, or (iii) by comparing calibration curve slopes. The latter two are both quantitative approaches. Determination of matrix effects is an inherent part of method development and validation. However, the selection of correct procedure remains in question.

Results

In our study, we compared two commonly used matrix effects evaluation approaches, i.e., post-extraction addition and a calibration curve slope comparison approach using a set of analytes with different physicochemical properties analyzed in electrospray positive and negative modes. The results for the latter were substantially affected by the selected calibration model. Therefore, detailed examination of the effect of calibration model including linear model, logarithmic transformation, and models using weighing, on results has been carefully examined.

When compared the two approaches for matrix effects evaluation, no agreement has been achieved. Indeed, the calibration curve slope comparison strategy has demonstrated underestimated results corresponding to the results of the post-extraction addition approach obtained for higher analyte concentrations. Therefore, a new equation to tackle also the translational matrix effects has been suggested.

Conclusion

The calibration curve slope comparison strategy should not be used for matrix effects evaluation unless appropriate correction including translational matrix effect is involved in the calculation model.

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New trends in ion exchange-type chiral stationary phases and their chiral recognition mechanisms

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Introduction

Ion exchange-type chiral stationary phases represent a mature technology that is utilized in chiral resolution of enantiomeric mixtures of polar compounds.[1] Commercially available *Cinchona* alkaloids-based chiral weak ion exchangers are typically used for chiral resolution of organic acids, while zwitterion ion exchangers are efficient in resolution of acids, bases, and zwitterions. The latter possess in their structure a cation exchange unit, which alone can serve as a cornerstone of chiral strong cation exchangers facilitating chiral separation of various basic racemic mixtures. Although chiral strong cation exchangers (cSCX) are efficient CSPs, their structural variations have not been thoroughly studied thus far.

It has been assumed that the mechanism of chiral recognition of basic compounds by cSCX is based predominantly on π-π-interactions, hydrogen bonding and steric interactions.[2] Recently, we have shown that the π-π-interactions are not strictly necessary for effective chiral recognition by a chiral cation exchanger.[3] This finding prompted us to study the structural variations of these chiral stationary phases further.

Experimental

The new chiral stationary phases were packed into stainless-steel analytical columns (150 × 4.6 mm i.d.) in Chromservis (Prague, Czech Republic) using an isopropanol slurry and methanol as a packing solvent at constant pressure of 600 bar. The column screening experiments were performed on an HPLC system ECS05 (ECOM spol. s r.o., Prague, Czech Republic) equipped with a solvent tray, binary pump, column oven, diode array detector, an autosampler and integrated PC. The chromatographic instrument was controlled, and data acquired using Clarity Chromatography Software (DataApex, Prague, Czech Republic). The flow rate was set at 0.75 ml min^{-1}. The detection wavelength was 254 nm and the column temperature was set at 20 °C. Analytes were dissolved in methanol at the concentration of 1–2 $mg \cdot ml^{-1}$. Conformers of selectors in water were generated in CREST and optimized on B3LYP/6-311++g(d,p) level in Gaussian G09.

Results

We have utilized a previously described (and patented) aromatic cSCX (CSP 1) and synthesized its novel non-substituted homologue (CSP 2). In the next step, we replaced the central benzene ring by the non-aromatic (saturated) 1,4-disubstituted cyclohexane ring and synthesized two chiral selectors differing in the relative configuration (*cis*-/*trans*-) on the central cyclohexane ring (CSP 3 and CSP 4). The structure of another CSP was modified via inserting a glycine fragment in the molecular structure thereby placing a mobile fragment between the aromatic

ring and the chiral unit (CSP 5). The effect of structural variations of selectors (Figure 1) on their chiral recognition properties will be discussed on the basis of chromatographic results supported with molecular modeling.

Conclusion

Hereby, we studied the effect of structural variations on the chiral recognition power of chiral strong cation exchangers. Based on chromatographic results and molecular modeling, we propose the possible reason for unexpectedly high chiral recognition power of CSP 3. We also document very good chiral recognition properties of CSP 5, which could be based on double hydrogen bonding and higher conformational flexibility of the selector.

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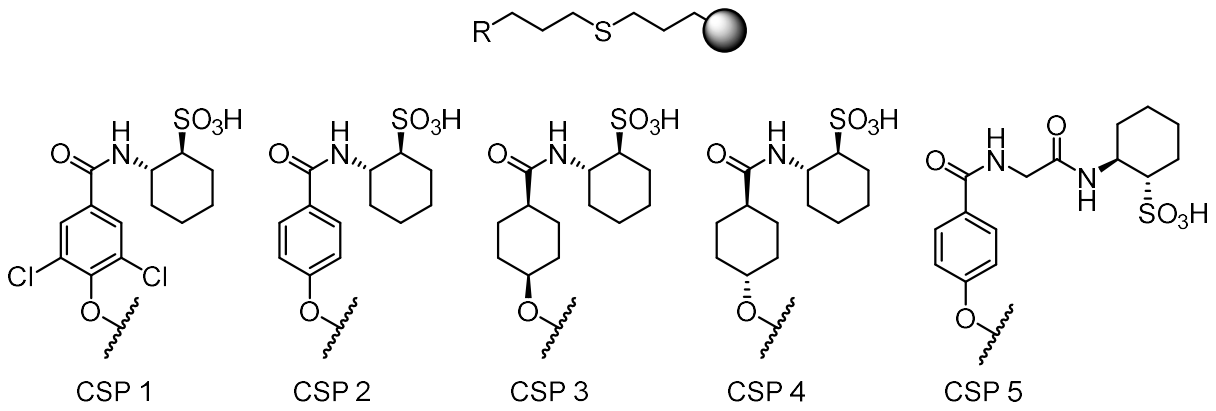
Figure 1. Chiral strong cation exchangers discussed in this contribution.



Multiple heart-cutting achiral-chiral LC-LC analysis of branched-chain amino acids in food supplements

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Introduction

The global market for dietary supplements has experienced rapid growth in recent years, particularly in the realm of proteins and amino acids (AAs). However, this market is increasingly susceptible to risks due to the prevailing lack of commitment to implement effective legislation [1,2]. To uphold consumer confidence in the quality of commercial products, there is a pressing need for new validated analytical methods that can identify and quantify bioactive substances in these products. Within the category of AA-based dietary supplements, the market for branched-chain amino acids (BCAAs), namely isoleucine (Ile), leucine (Leu), and valine (Val), is witnessing substantial growth in various regions worldwide [3], attributed to their health-promoting attributes [4,5].

Given the aforementioned factors, accurate analysis of BCAAs present in food supplements assumes paramount importance, both qualitatively and quantitatively, alongside the control of their stereochemistry. Notably, all commercially available food supplements claim to exclusively contain L-BCAAs, implicitly negating the presence of D-forms. However, it is now understood that D-BCAAs exert distinct physiological effects in humans compared to L-BCAAs [6]. Consequently, there arises a necessity to ascertain the enantiomeric composition of individual BCAAs within a food supplement, as their stereochemistry not only influences nutritional properties but also yields varying "pharmaco-toxicological" profiles [7,8].

With this objective, an easily deployable and cost-effective two-dimensional multi-liquid chromatography (2D mLC-LC) method has been devised for accurate achiral-chiral analysis of BCAAs as dansyl (Dns) derivatives in dietary supplements, particularly those formulated as tablets, shedding also light on enantiorecognition mechanisms.

Experimental

A Waters Xbridge RP C18 column (150 × 4.6 mm, 5 μm I.D.; 130 A pore size; from Waters Corporation) turned out to be the optimal choice for the HPLC-UV study. UV detection wavelength was set at 254 nm, whereas the flow rate was set at 1.0 mL/min. Injected samples were solubilized in a water/ACN 50/50 (v/v) solution to obtain a 0.021 mg/mL concentration. The injection volume was 20 μL for all the analyses. Mobile phase was composed of aqueous 40 mM NH_4OAc (A), and ACN (B). The following gradient program was optimized: 0–40 min 70% (v/v) A, 40–65 min 90% (v/v) A, 65–90 min 100% (v/v) A for column equilibration.

The enantioselective HPLC analysis (for both the in- and off-line second dimensions) was performed using a Chiralpak QD-AX columns (150 × 4.0 mm, 5 μm I.D.; 120 A pore size; from Chiral Technologies Europe). The identified best eluent system with this chiral stationary phase

(CSP) was water/ACN, 30/70 (v/v) with 50 mM NH_4OAc, pH 5.5. Analyses were performed at a 1.0 mL/min eluent flow rate. For the in-line 2D LC–LC analysis, a two-position six port switching valve assembled in our laboratory with a conventional Rheodyne injector including a 100 μL stainless steel loop was used. For the molecular modelling study, the simulations for each Dns-AA with the selected CSP were performed and investigated through the Desmond Molecular Dynamics System (version 7.3, Schrodinger, LLC, 2023) present in the Schrodinger Suite 2023-1 [9].

Results

In the first dimension, an achiral C18 column was used under gradient conditions with buffered aqueous solution and acetonitrile. The analytes were preliminarily derivatized with Dns-Cl applying a simple reaction protocol. At the end of the optimization study, a gradient program providing an elution order Dns-Val < Dns-Ile < Dns-Leu, with a full resolution between adjacent peaks (7.25 and 1.50 respectively for the pairs Val/Ile and the Ile/Leu) in less than 55 min was identified. This is a reasonable run time for analysis with conventional HPLC systems, considering the well-recognized difficulty to fully discriminate isomeric Ile and Leu.

A "research" validation study was performed revealing high accuracy (Recovery%) and precision (RSD%) of the achiral method using two external set solutions: respectively in the range 93.7 – 104.1%, and 0.4 – 3.2%. With the aim of developing a method capable of revealing the incidental presence of D-BCAAs in the tablet formulations, a chiral chromatography method relying upon the use of a quinidine-based Chiralpak® QD-AX column was optimized. A water/acetonitrile 30/70 (v/v) with 50 mM ammonium acetate (apparent pH of 5.5) eluent allowed getting the three enantiomers' pairs fully resolved: R_S equal to 4.3 for Dns-Val and Dns-Ile, and 1.7 for Dns-Leu. At this point of the study the two systems (that is, the achiral and the chiral one) were connected in series through a two position six-port switching valve, and an analysis of a real commercial tablet was performed. Application of the multiple heart-cutting achiral-chiral LC-LC (mLC-LC) method confirmed that the content of Val, Ile and Leu in the tablets was compliant with that labeled by the producer. Only L-enantiomers were indeed found in the food supplement, as confirmed by LC-MS/MS analysis.

Finally, to deeper understand the key driving forces involved in the enantiorecognition mechanism of DNS-AAs with the QD-based chiral selector, molecular dynamics simulations were performed. As a result, a key role of the conformational energies of the selector and selectands in ruling the enantiorecognition mechanism turned out.

Conclusion

In this study, an easy-to-set-up 2D mLC-LC method has been developed for the accurate achiral–chiral analysis of BCAAs in dietary supplements formulated as tablets, relying upon the use of two conventional HPLC-UV systems connected through a simple six-port switching-valve. The achiral column for the first dimension was a common C18 phase, whereas a QD-based CSP was used under RP conditions to realize the enantioselective analysis in the second dimension. The analytes were preliminarily derivatized with Dns-Cl applying a simple reaction protocol. Furthermore, it has been demonstrated that the developed chiral method can be easily transferred to a UHPLC–HRMS apparatus after only marginal modifications of the elution conditions. A deeper insight into the enantiorecognition mechanism through the application of a molecular dynamic simulation protocol has been simulated.

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Rapid profiling of bioactive compounds using supercritical fluid chromatography coupled with tandem mass spectrometry

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Introduction

Determination of plant hormones is still a very challenging analytical discipline, mainly due to their low concentration in complex plant matrices. Therefore, involvement of very sensitive high-throughput techniques is required. Cytokinins (CKs) are semi-polar basic plant hormones regulating plant growth and development. Modern methods for CK determination are currently based on ultra-high performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS), which enables the separation of CK isomeric forms occurring endogenously in plants [1]. Here, ultra-high performance supercritical fluid chromatography coupled with tandem mass spectrometry (UHPSFC-MS/MS) was used for the simultaneous determination of 37 CK metabolites [2].

Experimental

The work was performed using Agilent 1260 Infinity II LC/SFC hybrid system coupled with Agilent 6495B Triple Quadrupole equipped with electrospray ionization (ESI). The chromatographic conditions were tested on three different columns with various retention mechanisms. Hybrid silica modified with 2-picolylamine was selected as the stationary phase. Several parameters such as column temperature, back pressure regulation, mobile phase composition and make-up solvent were investigated to achieve efficient separation of CK isomers and reasonable sensitivity.

Results

Compared to UHPLC-MS/MS, a 9-min chromatographic analysis using a mobile phase of supercritical CO_2 and 5 mM ammonia in methanol represents a three-fold acceleration of total run time. The quantification limit of UHPSFC-MS/MS method was in the range of 0.03–0.19 fmol per injection and the method validation showed high accuracy and precision (below 15 % for most analytes). The method was finally applied to the complex plant matrix of the model plant Arabidopsis thaliana and the obtained profiles of CK metabolites were compared with the results from the conventional UHPLC-MS/MS method.

Conclusion

We have developed a new rapid method for the determination of endogenous CKs in plant tissues based on rapid and highly sensitive UHPSFC-MS/MS. The work brings new knowledge about SFC chromatography of semi-polar basic compounds on different types of columns in

combination with various composition of the mobile phase, column temperature, back pressure effect, injection parameters and the composition of make-up solvent. The developed method was validated with reasonable accuracy and precision. Compared to the well-established UHPLC-MS/MS method for CK profiling, the UHPSFC-MS/MS method proposed in this study offers a 7-min separation while maintaining sufficient resolution of all isomers of endogenous CK metabolites. Our data also showed that chromatographic parameters, such as peak symmetry and FWHM, are satisfactory for a rapid screening of CK metabolites using UHFSC as an analytical tool. Surprisingly, we detected a lower matrix effect for naturally occurring isoprenoid CKs in Arabidopsis samples in the novel UHPSFC mode rather than the conventional UHPLC method. Our findings open the opportunity to use the UHPSFC-MS/MS instrumentation for fast target plant hormonomics including other (not only non-polar) families of plant hormone [3].

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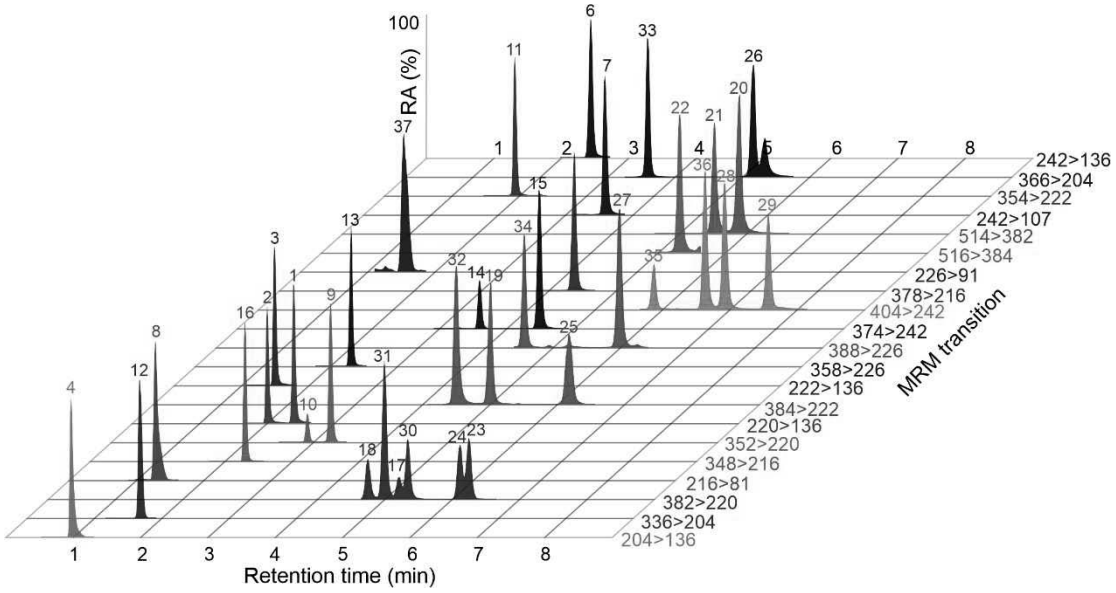
Figure 1. Separation of cytokinin standards by UHPSFC-MS/MS method using a Torus 2-PIC (3.0 × 100 mm, 1.7 µm) column. Multi-MRM chromatograms of 22 isoprenoid and 15 aromatic cytokinins including bases, ribosides, and N-/O-glucosides containing 0.1 pmol of each metabolite per injection. Relative abundance (RA%) is function of retention time (min) and MRM transition.



Symbiosis of core-shell and fully porous particles: Implementing both for better HPLC and UHPLC results

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Introduction

The presentation describes the basic characteristics of fully porous particles and particles with a solid core, the so-called "core shell". Both segments are represented and demonstrated by the Phenomenex brand, and both can give extra benefits, but you might not see full potential. The goal is to provide the audience with comprehensive information about both types of particles with respect to instrumentation and the nature of the substances tested.

Experimental

Experimental data for maximizing separation efficiency, finding the ideal length of analysis, retention and sample loading with respect to resolution and asymmetry factor for polar acids, polar bases. How to improve loading for basic substances and how to work with orthogonal selectivity.

Results

General chromatographic performance

Criteria/Need	Ranking	Caveat/Exceptions/Recommendation
Need maximum efficiency (Rs, H)	Kinetex > Omega	Benefits of core-shell diminish with particle size decrease
Fastest Analysis Time	Kinetex > Omega	
Need more sample loading	Kinetex < Omega	Usually not a factor with neutrals or acids
Need more retention (C18)	Kinetex < Omega	Can be offset with stationary phase selection
Injecting strong solvent	Kinetex < Omega	

System consideration

Criteria/Need	Ranking	Caveat/Exceptions/Recommendation
Standard HPLC-UV	Kinetex >> Omega	Benefits of core-shell diminish with high dwell volume
UHPLC/UPLC®-UV	Kinetex <= Omega	Core-shell will be faster usually
LC/MS	Kinetex = Omega	Very case-specific

Method development

Criteria/Need	Ranking	Caveat/Exceptions/Recommendation
Polar bases	Kinetex = Omega	Kinetex Biphenyl or Kinetex/Omega Polar C18
Polar acids	Kinetex < Omega	Omega PS C18 (Polar C18 phases are alternatives)
Best peak shape/loading for Bases	Kinetex < Omega	Omega PS C18 (Kinetex EVO C18 is alternative)
Stability at high pH	Kinetex > Omega	Kinetex EVO C18
"Orthogonal Selectivity" to C18	Kinetex > Omega	Kinetex Biphenyl & F5

Conclusion

Kinetex brand in general offers higher efficiency and shorter RT. For HPLC it is almost always beneficial to see more than double higher efficiency. Going to the UHPLC – retention, peak of asymmetry and loading capacity must be taken into consideration more because efficiency boost is not so significant. That is why Luna Omega brand usually dominates.

Targeted and untargeted analysis of psilocybin mushrooms using LC-MS

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Abstract

Psilocybin mushrooms have drawn recent attention due to their potential for the treatment of major depressive disorders and anxiety. A desire exists to continue the investigation of psilocybin treatments for mental health problems. Prior clinical studies have relied exclusively on the use of synthetic psilocybin. In order to provide a reliable dose of psilocybin and psilocin from a natural product "magic" mushrooms, reliable methodologies are needed to understand their composition. A liquid chromatography – triple quadrupole – mass spectrometry method was developed to study and establish the potency of different psilocybin mushrooms [1]. Mushrooms were milled using a Fritsch Pulverisette 11 blade mill; 50 mg of mushroom was extracted twice using 5 mL of methanol acidified with 0.5% acetic acid. A Shimadzu LCMS-8040 was used in reversed phase mode (5 cm Supelco Biphenyl phase). Confirmed also with interlaboratory testing at MilliporeSigma, a series of different psilocybin mushroom strains were established to contain between 0.9 – 1.2% of psilocybin and psilocin by weight. To further investigate the compositional differences among psilocybin mushrooms (and compared to regular mushrooms), an untargeted analysis was also performed using liquid chromatography – quadrupole – time-of-flight – mass spectrometry (Shimadzu LCMS-9030). Psilocybin mushrooms could be well differentiated from regular mushrooms based on the reversed phase analysis performed. Some chemical compound classes could be identified that clearly differentiate the different mushroom types. However, additional work is ongoing to elucidate the presence of other tryptamine alkaloids of interest, especially their potentially different levels of abundance in different psilocybin mushroom strains.

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On the potential of two-dimensional liquid chromatography for environmental analysis

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The presence of contaminants of emerging concern (CECs), such as pharmaceuticals, hormones, personal care products, pesticides, … in the environment is becoming increasingly problematic. These CECs are widespread in environmental waters due to their frequent use and discharge, but also because current wastewater treatment plants (WWTPs) are inadequately equipped to degrade them. This results in environmental concentrations of these CECs that can reach up to tens and even hundreds of µg/L. Because CECs can negatively impact aquatic organisms, but also have an effect on human health, their occurrence in the environment needs to be carefully monitored. Since environmental samples are typically complex, consisting of a variety of compounds with diverse physicochemical properties at trace levels, highly sensitive, high-resolution techniques are required for their analysis. Two-dimensional liquid chromatography (2D-LC) in combination with (high-resolution) mass spectrometry (MS) is increasingly used for the analysis of environmental samples. This presentation aims to give an overview of different 2D-LC-MS approaches that have been recently developed in our research group for the analysis of CECs in the environment. Difficulties and possible solutions inherent to the 2D-LC analysis of these samples will be discussed.

Leveraging ion mobility spectrometry-mass spectrometry data using machine learning reveals provenance of indigo dyes

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Introduction

Indigo, the renowned blue dye and pigment, was historically produced on an industrial scale from several plants grown in various parts of the world: *Isatis tinctoria* (woad) in Europe, *Indigofera tinctoria* (true indigo) in the Indian region, *Indigofera suffruticosa* (anil) in South and Central America and *Polygonum tinctorium* (Japanese indigo) in East Asia. Ongoing geographical explorations, discovery of new land and sea routes driven by economical motifs as well as technical progress affected the manufacture and availability of these pigments to dyers and artists. Tracing the biological source of a colorant remains a major focus because it could locate the origin of pigments and dyes used in clothes and artworks, help in assessing their age, or contribute to tracing of important trade routes.

The main color component of indigo, indigotin, is formed during the fermentation and oxidation of plant matter. The latter was mainly used in the dyeing industry during the vat process, i.e. the reduction of indigotin in an alkaline environment to water-soluble leucoindigotin, and the reverse oxidation to indigotin. The finely dispersed indigo collected from the dyeing vat was subsequently purified (grinding with chalk, boiled with vinegar, etc.) and used for artistic purposes. Both organic and inorganic components of raw indigo have been substantially modified or even destroyed during processing and inevitable degradation.

We have developed the first systematic untargeted workflow for the differentiation of indigoid dyes/pigments from different biological sources [1]. In the current contribution we have identified the most important markers of indigo origin and confirmed their identities by means of ion mobility spectrometry coupled to mass spectrometry.

Experimental

The analytical method ensures the collection of signals of the greatest possible number of potential markers present in the pigments. It is based on flow injection analysis of DMSO extracts and high-resolution mass spectrometry coupled with ion mobility spectrometry (Synapt G2-S, Waters). Data processing is followed by the selection of variables, training of the supervised classification models and their validation according to our original multi-method screening scheme. Combination of eight variable selection methods (information gain, information gain ratio, Gini decrease, ANOVA, Chi squared, ReliefF, fast correlation-based filter and significance analysis of microarrays) and five machine learning classification algorithms (k-nearest neighbors, support vector machines, random forest, neural network, and naïve Bayes) were evaluated using double (nested) cross-validation.

The implementation of the developed scheme was carried out in the Origin Data Mining

program and partially in the MetaboAnalyst web platform, which do not require knowledge of programming or advanced statistical procedures. In the analysis of art objects, it is not possible to use historical samples to create classification models due to the rarity of samples, the limited quantity of an individual sample and the absence of reliable information about its exact composition (type of binder or pigment). Therefore, the initial modeling was done with reference materials (synthetic indigo and pure pigments from various biological sources/manufacturers) and model samples (egg tempera and oil painting).

Results

The instrumental limit of detection was around 10 ng/mL of synthetic indigotin (50 pg injection). The reliable identification of pigment origin was successful for a 10 μg sample of oil paint with woad content of 50% or a 1 mg sample of egg tempera wall painting with woad content of 1% (woad contains the least indigotin). The best performance was achieved by the random forest algorithm across all variable selection methods (average classification accuracy 94%). The final classification model built on the 46 selected most stable variables was subjected to a permutation test (n = 10000) and had a p value $\leq 10^{-4}$, confirming that the performance of this model is not by chance.

Identification of the most important markers was carried out based on their fragmentation spectra and preliminary classes of substances were proposed using MSFinder. An indole alkaloid and an O-methylated tetrahydroxyflavonoid were suggested for true indigo and woad pigments, respectively. While the identity of the former marker was unambiguously confirmed as 2,2'-biindolyl by UPLC analysis of standards, the true nature of the latter could not be revealed due to poor separation of positional isomers, e.g. diosmetin (luteolin-4′-methyl ether) and chrysoeriol (luteolin-3′-methyl ether). Thanks to the added dimension of ion mobility spectrometry, identity of the true indigo marker was confirmed as chrysoeriol.

The presence of indigotin was confirmed in historical samples of a Slovak textile blueprint and an oil painting sample (both early 20th century), which were classified as originating from the indigo plant and anil, respectively. A textile sample of trousers from the 18th century contained indigo carmine (identified by an existing method and confirmed by standard analysis), which thus belongs to the oldest samples dyed with sulfonated indigo (known as Saxon blue). Other historical samples for which alternative methods demonstrated the presence of copper pigments or Prussian blue did not contain indigotine and were classified as “blank”. Therefore, it is very important that possible interferences from other pigments are not confused with signals from minor indigo components and thus do not cause false positives.

Conclusion

In the future, the presented procedure could be implemented for the systematic screening and classification of a wider range of pigments and dyes, required by analytical and forensic chemists as well as restorers, archaeologists or art historians.

Acknowledgements

The authors gratefully acknowledge the financial support by the project Arteca [ERDF/ESF CZ.02.1.01/0.0/0.0/17_048/0007378] from Ministry of Education, Youth and Sports of the Czech Republic. The authors are thankful to Magdalena Marjaková, Eliška Randůšková (Sklenářová), Lenka Zamrazilová and Kateřina Cichrová for the provided historical samples.

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Numerical optimization of gradient separations in RP and HILIC using non-linear retention models

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Gradient elution in liquid chromatography is a highly effective tool for increasing peak capacity, improving analyte resolution and enhancing quantitative characteristics of developed methods. To optimize the gradient profile, window diagram approach can be used, based on retention modeling under isocratic conditions. For compounds which exhibit non-linear retention behavior in dependency on mobile phase composition – typically convex dependencies of logarithms of retention factors on volume fraction of organic solvent in mobile phase are observed – the retention models cannot be simply integrated for calculation of gradient retention data. In this case, either empirical model suitable for explicit integration must be applied [1], or numerical integration must be used for calculation of gradient retention data [2]. In our previous work [2–4], we have shown that for both reversed phase separations [4] and hydrophilic interaction liquid chromatography [2,3], the non-linear isocratic retention behavior can be solved in gradient optimization using window diagram approach. In current work, both approaches are compared and extended for the optimization of ternary mobile phases containing various combinations of acetonitrile and methanol in HILIC mobile phases for fine tuning of separation selectivity in uni- and two-dimensional separation methods.

Acknowledgement
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Do we need separations in clinical diagnostics?

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Introduction

The separation of substances in time, space, and over time represents a critical aspect of all clinical diagnostic portfolios. In clinical chemistry, analytical chemists monitor characteristic disease features or patients' status biomarkers in chemically complex environments, often represented by bodily fluids and tissues. The limited dynamic range of cheaper diagnostic routines may present a challenge. Consequently, separation sciences must be implemented to meet the requested sensitivity and specificity of the clinical diagnostic approaches. If a diagnostic procedure is developed using costly instrumentation, then technology transfer is necessary to provide a high throughput, automated, and cost-effective commercialized procedure.

In response to the COVID-19 pandemic we developed "Infection Metallomics" portfolio used in the diagnostics of lung infections [1]. Our original aim was clinical, focused on noninvasive diagnostics of bacterial, mycobacterial, and fungal superinfections in the lungs, to which SARS-Cov-2 paved the way. The portfolio combines enrichment, separation, and electrospray ionization (ESI)/matrix-assisted laser desorption and ionization (MALDI)/inductively-coupled plasma (ICP) ionization techniques with diverse mass analyzers, supported by microbiology-driven CycloBranch software [2]. Later, functional studies in biofilms, structural biology, and microbial physiology applications emerged in the one-health concept. We have also expanded our diagnostic portfolio to include infections of the urogenital tract and central nervous system. Microbes secrete metal-containing secondary metabolites, called metallophores, which are microbial virulence factors synthesized during pathogen proliferation in high molecular copy numbers and excellent indicators of pathogen viability [3]. Their favourable molecular structures allow them to easily pass through barriers between tissue and blood and be eliminated by renal excretion. They are also ideal subjects for elemental and molecular mass spectrometry (MS) due to their metal-characteristic isotopic patterns [4].

MALDI Biotyper-type laboratory represents the current standard for routine characterization of microbes. Transfer from liquid chromatography (LC)/MS-based infection metallomics to MALDI is needed. This presentation will demonstrate the current status of our group's MALDI Biotyping of metallophores.

Experimental

We compared the efficiencies of two ionization techniques for four siderophores using MS on the same instrument: (MALDI) and (ESI). The siderophores were specific for fungal pathogens, *Aspergillus fumigatus* (triacetylfusarinine C), *Rhizopus microsporus* (rhizoferrin),

Scedosporium apiospermum, and *Lomentospora prolificans* (coprogen variants). The detection limits were assayed in urine samples.

Results

In the analysis of triacetylfusarinine C in urine using MALDI, we achieved the limits of detection (LOD) of 9.5 ng/mL and of quantitation (LOQ) of 28.9 ng/mL. In contrast, the combined use of LC and ESI provided a LOD threshold of 0.6 ng/mL [4] which enabled us to successfully map the time course of biomarker secretion in human aspergillosis. For coprogens, MALDI-MS provided a sensitivity (7 ng/mL) that was similarly poorer by one order of magnitude than that obtained by LC-ESI-MS (0.9 ng/mL). Whereas MALDI sensitivity was sufficiently high (LOD 5 μg/mL) to generate MS imaging (MSI) data of rhizoferrin and host neutrophil peptides in a rat model, for the analysis of human urine, LC-ESI was necessary so with the LOD and LOQ for ESI rhizoferrin 18 and 55 ng/mL, respectively. In the MSI experiment, rhizoferrin deprotonated molecule m/z 435.1258 was visualized with ± 3.3 ppm accuracy. Rat neutrophil peptide-3 protonated molecules m/z 3266.498 capsulated the infection (± 35.4 ppm). In summary, ESI in the positive ion mode, when combined with LC separations, yields a better detection limit in the order of one magnitude for triacetylfusarinine C and coprogen ferriforms compared to MALDI (without separation). The excellent correlation coefficient between MALDI and ESI indicates that MALDI represents a quantitative tool. However, the detection of rhizoferrin in MALDI still represents a challenge (5000 ng/mL).

Conclusion

The answer to the presentation title would be “Yes, but...” since MS with separations is critical for method development. The term “but” reflects the fact that MALDI-Time of Flight technology remains a common MS standard in smaller hospitals. For the final MALDI examination (without separation), the biomarkers can be enriched from human samples. Looking ahead, advancements in siderophore enrichment procedures hold promise for facilitating routine MALDI biotyping of siderophores in humans.

Acknowledgements

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Metabolomics analysis of *Moringa oleifera* leaves: Correlation between in vitro effect on C2C12 myotubes cell line and geographical origin

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Introduction

Moringa oleifera Lam. is an important, multipurpose plant, widely used traditionally for its nutritional and medicinal properties due to the presence of active biomolecules in its parts. In particular, the leaf extract is used for its ability to in regulate metabolism and its antioxidant activity.

It has been reported that *Moringa oleifera* leaf extract (MOLE) has beneficial properties that may mitigate pathological conditions including diabetes. MOLE treatment has been shown to increase oxidative energy metabolism and possibly favors mitochondrial biogenesis through the SIRT1/PPARα-pathway [1].

This work aims to test the antioxidant capacity of *Moringa oleifera* leaves from different geographical areas on C2C12 myotubes and correlate the phenotypic activity to the metabolomics profile.

Experimental

One gram of Moringa oleifera leaf powder (PureBodhi Nutraceuticals Ltd, UK) was sonicated (Vibra-Cell CV 18 SONICS VX 11, Sonics & Materials, CT, USA) in 10 mL of methanol 100% twice for 10 minutes at + 4°C. The extract was then centrifuged (2000 × g for 10 minutes at +4 °C), collected, and stored at –20 °C (stock solution).

Qualitative profiling of MOLE extracts was obtained by ultra-high performance liquid chromatography-quadrupole time-of-flight mass spectrometry ZenoTOF 7600 (AB SCIEX GmbH, Landwehrstraße 54, Darmstadt, Germany). Data obtained were processed using SCIEXOS Software 3.3 (AB SCIEXGmbH, Landwehrstraße 54, Darmstadt, Germany), and the SCIEX Natural Products 2.1 Library (AB SCIEX GmbH, Landwehrstraße 54, Darmstadt, Germany) was used for searching database compound spectra.

Results

An untargeted metabolomics analysis of MOLE samples from each different geographical area has been performed to identify primary and secondary metabolites that may be responsible for the biological activity. Accurate mass spectrometers, such as time-of-flight (TOF) instruments, are often used, given their capability for thorough identification and confident characterization using MS/MS data. Here, a sensitive quantitative and qualitative method has been optimized and developed by using a ZenoTOF 7600 system.

The generation of highly sensitive MS/MS spectra together with the high-quality fragmentation spectra enables confident identification of low abundant compounds using MS/MS library

matching. Their presence and/or variation resulted in finding marker compounds of the geographical origin and/or biological activity.

Conclusion

The results obtained in this work demonstrate that different MO specimens, despite being cultivated in the same environmental conditions, provide extracts with different qualitative characteristics, but with similar biological effects. This is of particular importance given that the qualitative differences depend entirely on the genetic difference between individuals from different geographical locations and not on the quality of cultivation. This therefore makes the use of extracts of this plant for nutritional purposes even more valid.

The zenoTOF MOLE Qualitative Profiling LC-MSMS used in this research is a much better performing method in the detection of metabolites in leaf extracts and allows for a much more accurate analysis of the bioactive molecules present. The metabolomics analysis highlighted the presence of glucosinolates/isothiocyanates as well as flavonoids, polyphenols, and phenolic acids in all samples but in different percentage.

Samples from Fiji and Reunion as the commercial UK were found rich in flavonoids and polyphenols, while samples from Colombia, Mexico, India and Kenia showed a much higher glucosinolate component content.

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POSTERS

Isolation of Lectin from *Musa acuminata* by Affinity Chromatography as Potential Therapy against Biofilm Forming Pathogens

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Introduction

Lectins, the omnipresent carbohydrate-binding proteins, can potentially function as an alternative therapy against biofilm-forming pathogens [1]. Antibiotic-resistant microorganisms, currently called "superbugs", pose a significant public health threat. Furthermore, the resistance is related to biofilm formation as it is one factor that decreases the efficacy of antimicrobial chemicals. Consequently, progress in treating such harmful microorganisms is critical [2]. The interaction of plant lectins and probiotics (beneficial groups of microorganisms) with the extracellular matrix of resistant pathogens might lead to novel treatment options. Hence, extraction and isolation of proteins from the banana pulp were performed to check the antibiofilm activity. The cell-free extract from isolated probiotics was also assessed, and the synergistic impact of both was monitored against the methicillin-resistant *Staphylococcus aureus* (MRSA) [3].

Experimental

The acetic acid extraction of banana proteins was performed, followed by affinity chromatography with Sephadex G-75. The molecular weight of the proteins was evaluated by sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE, 15%), and the antibiofilm activity was performed by crystal violet staining *in vitro* assay. Moreover, the DNA was extracted from an isolated probiotic strain using a GJC ® DNA purification kit. The PCR (ThermoFischer Scientific) amplification was performed using universal primers (Macrogen), and the amplified products were separated by electrophoresis in 2% agarose gel. The cell-free supernatant (CFS) was extracted from the isolated probiotic strains, and the antibiofilm activity against MRSA was performed using 96 well microtiter plates. The synergistic effect of banana proteins and CFS obtained from probiotics were also tested for the antibiofilm potential by crystal violet staining *in vitro* assay.

Results

The banana lectin (BanLec) was successfully isolated and appeared as a 14.5 KDa band after SDS-PAGE (15%), while multiple bands of unbound protein fractions were also observed. After

chromatography, the unbound protein fractions (Fig. 1, peak 1) showed inhibition of planktonic cells and the biofilm against MRSA (inhibition 84.3%), but BanLec exhibited no significant outcome. The isolated lactic acid bacteria were mainly from the Enterococcus genus. The CFS of *Enterococcus faecium* (LCM002), *E. lactis* (LCM003) and *E. durans* (LCM004 and LCM005) displayed antagonistic effects against MRSA (Biofilm inhibition 100%). The synergistic effect of CFS from *E. lactis* (LCM003) and unbound proteins showed inhibition of biofilm and pathogenic growth (biofilm inhibition 60–70%).

Conclusion

This study demonstrates the application of plant proteins and probiotics against bacterial biofilm formation. The synergistic effect of plant proteins and probiotics exerts considerable potential for tackling *Staphylococcus aureus* resistance.

Acknowledgement

The dean of the Faculty of Science, and the Department of Microbiology, University of Karachi, Pakistan, Dr. Zafar Husnain Zaidi National Center for Proteomics, University of Karachi, Pakistan, and Rutuja H. Patil (Institute of Microbiology, Prague, Czechia).

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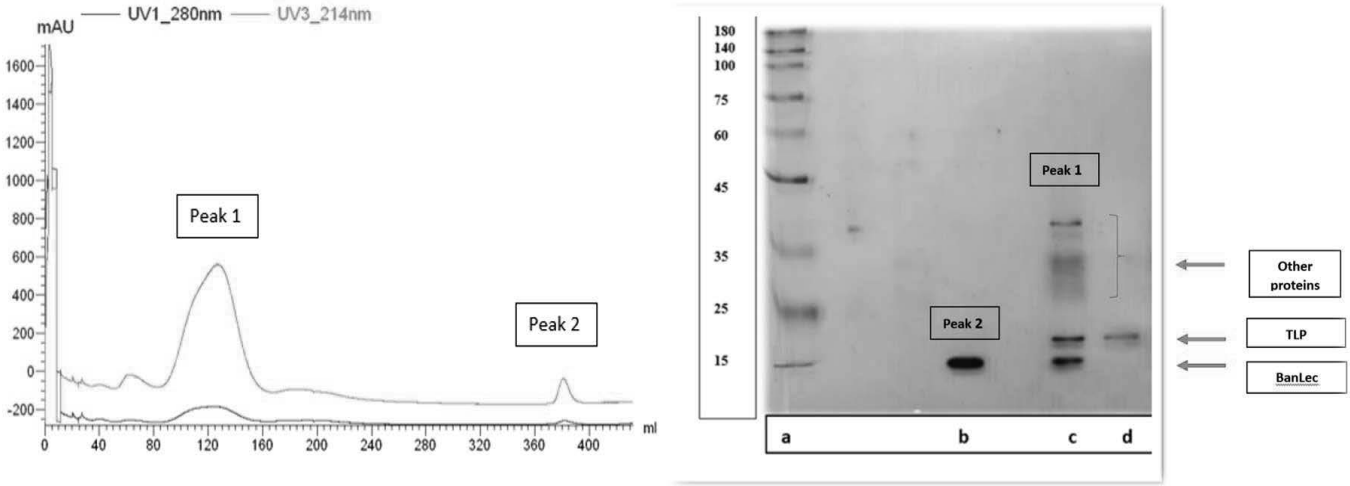
Figure 1. Chromatogram of banana proteins with Sephadex G-75 and gel electrophoresis illustrating banan pulp proteins (Lectins, Thaumatin like protein and other proteins).



Spark-generated nickel oxide nanoparticle modification of carbon fiber microelectrodes for enhanced detection of piperazine antihistamine drugs in HPLC

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Introduction

The class of H1-antihistamine piperazine derivatives, including cyclizine, chlorcyclizine, cetirizine, flunarizine, meclizine, and buclizine, are known for their efficacy in managing various conditions such as allergic rhinitis, motion sickness, nausea, and vertigo[1]. However, their usage can lead to side effects ranging from sedation to cardiac complications, highlighting the importance of accurate determination in biological samples [2–4]. High-performance liquid chromatography (HPLC) coupled with electrochemical detection offers a promising avenue for this purpose, providing enhanced sensitivity and selectivity compared to conventional methods. While various modified electrodes have been explored for the electrochemical determination of individual drugs like cetirizine, research on the electrochemistry of other piperazine antihistamines remains limited. In this study, we propose a novel approach utilizing a carbon fiber microelectrode modified with spark-generated nickel oxide nanoparticles for sensitive and selective detection of these drugs in blood samples. This method offers simplicity, sensitivity, and cost-effectiveness, representing a significant advancement in the field of pharmaceutical analysis.

Experimental

Antihistamine standards, including buclizine dihydrochloride (BCZ), meclizine dihydrochloride (MCZ), cetirizine dihydrochloride (CTZ), flunarizine dihydrochloride (FLZ), cyclizine hydrochloride (CZ), and chlorocyclizine (CCZ), were obtained from Sigma. Additionally, diclofenac sodium salt (DF) was used as an internal standard (IS). Carbon fiber microelectrodes (CFMEs) were fabricated from individual carbon fibers. Spark discharge was employed for electrode modification, conducted between the CFME and nickel wire electrode. The morphology of carbon fibers was examined using scanning electron microscopy (SEM), and energy-dispersive X-ray analysis (EDX) was used to determine the elemental composition. Electrochemical measurements, including cyclic voltammetry and amperometry, were conducted using a three-electrode system in a single-compartment cell. A leak-free Ag/AgCl electrode served as a reference electrode, and platinum wire acted as an auxiliary electrode. The HPLC system comprised an ESA isocratic pump, a Rheodyne manual injector with a 20 µL loop, and a Coulochem III potentiostat coupled to a self-designed amperometric flow cell equipped with a CFME. HPLC separations were performed using a reversed-phase ARION-CN 3 µm column, with a mobile phase consisting of 50 mM NaH_2PO_4 (pH 3)/MeOH (45/55, v/v). For sample preparation, plasma samples spiked with analytes and IS were subjected to protein removal by mixing with acetonitrile, followed by vortexing, sonication, and centrifugation. The supernatant was then diluted with phosphate buffer and injected into the

HPLC system.

Results

The activation of carbon fiber microelectrodes (CFMEs) involves removing any thin polymer coating and introducing oxygen-containing moieties to ensure defined and reproducible electrochemical behavior. In this study, CFMEs were sparked with carbon and various metals to assess their responses to the electrooxidation of cetirizine, a representative model compound for the studied antihistamine drugs. Among them, Ni-sparked CFMEs exhibited the most promising sensing properties and were further investigated. SEM images revealed presence of nickel oxide nanoparticles (NPs) attached to the carbon fiber surface, confirmed by EDX. The modification procedure, involving spark discharge between the CFME and a source electrode, was simple, efficient, and waste-free.

Cyclic voltammetry (CV) experiments were conducted to assess electrocatalytic properties of Ni-sparked CFMEs compared to carbon-sparked CFMEs. Representative CVs of cetirizine on bare, carbon-sparked, and Ni-sparked CFMEs demonstrated the increased sensitivity of the Ni-sparked electrode. The modification of CFMEs with Ni-NPs led to improved electrocatalytic activity, resulting in sharper and higher cetirizine oxidation peaks.

HPLC separations employing electrochemical detection were optimized. Baseline separation of the studied piperazine antihistamines, including the internal standard, was achieved within 15 minutes. Hydrodynamic voltammetry measurements helped define the optimum working potential (+1500 mV) for the compounds of interest.

Calibration curves were plotted, and basic parameters for the partial validation of the method were calculated. LOD and LOQ values were determined using the signal-to-noise method, indicating good linearity for both intra- and interday assays. Interelectrode reproducibility was evaluated using four individual Ni-sparked CFMEs. The method demonstrated good reproducibility and sensitivity, with LOD values comparable to or better than those reported in the literature.

Conclusion

In conclusion, the study introduces an innovative HPLC method for simultaneously detecting piperazine antihistamine drugs using nickel spark-modified CFMEs for amperometric detection. This method demonstrates notable sensitivity and cost-effectiveness, with detection limits ranging from 3.8 nmol L^{-1} to 120 nmol L^{-1} and linear responses up to 5 $\mu mol \cdot L^{-1}$. Successfully applied to spiked plasma samples, this approach offers environmental benefits by minimizing the use of toxic chemicals and reducing solvent consumption. Overall, the study presents a promising advancement in pharmaceutical analysis, emphasizing both efficiency and eco-friendliness.

Acknowledgements

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Comparison of extraction efficiency of quercetin complex from onion peel using NADES and organic solvents

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Introduction

Deep eutectic solvents (DES) have emerged over the last two decades as nonconventional solvents. DES are attractive for their inexpensive and simple preparation (mixing and stirring), tunable properties, and simple biodegradability. DES are composed of a mixture of quaternary ammonium salts acting as hydrogen bond acceptor (HBA), and a counter compound, such as a sugar, performing as hydrogen bond donor (HBD) [1]. Properties of DES depend on the intermolecular interactions between their HBA and HBD components, with the nature of its hydrogen-bonding lowering its overall melting point, leading to a liquid eutectic solvent mixture without further processing or purification needed. As functional liquid media, natural deep eutectic solvent (NADES) species can dissolve natural or synthetic chemicals of low water solubility. Moreover, the special properties of NADES, such as biodegradability and biocompatibility, suggest that they are alternative candidates for concepts and applications involving some organic solvents and ionic liquids [2]. Enhanced efficiency of NADES in extraction of phenols and flavonoids over other organic solvents was reported in the literature, showing the efficiency of NADES constituted from natural and renewable non-toxic resources [3].

Experimental

Choline chloride – glucose NADES (1:2) was prepared by mixing 13.97 g of choline chloride (CC) with 36.03 g of glucose (Glu) and 50 g of deionized water (H_2O). The mixture was heated for 1 hour to 80 °C under constant stirring and kept in the dark for 24 hours to verify long-term stability of the product. 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 H_2O. Diluted Be-LA NADES (40%) was prepared from 15.8 g of betaine (Be), 24.2 g of 0.85% lactic acid (LA) and 60 g H_2O.

Dry ground onion peel sample weights varied in the range of 0.025 – 0.200 g. Samples were put into 20 ml glass vials, followed by 10 ml of NADES mixture and stirred manually to expose the whole active surface of the sample to the solvent. Extractions were carried out in laboratory sonic tank at 42 kHz and laboratory temperature for 15–50 minutes. The extracts were centrifuged at 13 000 rpm and analyzed by HPLC with DAD detection.

Results

Two most promising NADES combinations, i.e. CC-Glu and Be-LA, were selected. Experiments carried out using 0.2 g samples and 10 ml of NADES at laboratory temperature for 50 minutes showed Be-LA as more effective solvent. Pretreatment and matrix swelling

experiments were tried but proved to be unnecessary; 24-hour passive exposition to NADES or additional moisturizing before the extraction itself did not bring more favorable recoveries.
Extraction time was optimized thoroughly both from the viewpoint of the highest yield and due to the risk of analyte decomposition, as reported by Kumar et al. [4]. According to the comparison carried out at 15, 20, 25, 30, 35, 40, 45 and 50 minutes, the most favorable extraction time was 35 min for both Be-LA and 70% EtOH used as a comparative solvent.
Sample weight was tested in the next step in the range of 0.025–0.200 g per 10 mL of NADES. For Be-LA NADES (1:2), 50 mg sample weight showed the best results (166% recovery for quercetin), but only 113%, 115% and 113% recoveries were achieved at 100 mg, 150 mg and 200 mg sample weights, respectively, compared to reference 70% EtOH extracts.
An attempt was made to use a more diluted Be-LA mixture, but while the results were more balanced for the 40% solution, the recovery showed only 75–88% of quercetin compared to the reference EtOH extracts. The test for analyte stability was repeated for all sample weights. Only two cases of higher recovery were observed at 30 min. compared to 40 min., both without statistical significance.

Conclusion

According to the results, Be-LA NADES (1:2) shown excellent recovery under conditions of favorable s/l ratio (0.05 g of solid sample per 10 ml of NADES). At higher ratios (0.1 g to 0.2 g), the yield is not so great, but still exceeds the reference EtOH method. Thus, for analytical purposes, small-scale samples are strongly recommended, or bigger volumes of NADES to prevent higher RSDs caused by insufficient homogeneity of too small natural samples. For prospective industrial use, the s/l ratio should be finely tuned for maximum cost efficiency, because the raw material is cheap and widely available.
NADES, on the other hand, have higher purchase price compared to the reference EtOH solvent. This disadvantage is balanced by their health and environmental safety, zero costs caused by excise duties, regulation and waste disposal. The selected Be-LA solvent further supports the green approach; all of its components are made from renewable resources.

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Using mass spectrometry for study of salicylic acid metabolism in plants

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Introduction

Plant immunity is defined as the capacity of plants to prevent or ward off biological attacks by pathogens. It involves the recognition of the pathogen by specific receptors and the triggering of signalling pathways leading to processes that help plants to defend themselves. The defence signalling is mediated by cross-communication of groups of plant hormones. Salicylic acid (SA) is one of the most pronounced plant hormones involved in control of immunity and defence mechanisms in plants. Defence related SA accumulation comes from its biosynthesis, transport and possible release from some of its metabolites. The involvement of SA biosynthetic pathways as well as extend of SA metabolism in various plant species are still under investigation. Liquid chromatography tandem mass spectrometry (LC-MS/MS) is one of the most commonly used analytical technics in plant hormone quantification. The selectivity and high sensitivity enables to track typically low concentrations of phytohormones. The comprehensive multiclass phytohormone LC-MS/MS profiling methods usually include only SA (as the active compound) from the group of SAs. LC-MS/MS methods for determination of defence related phytohormones focus on SA, or some on its biosynthetic precursors or metabolites. In this study, we focus on identification of new SA metabolites and development of liquid chromatography mass spectrometry methods.

Experimental

The chromatographic separation was optimized in terms of separation selectivity, sensitivity, and peak shapes. Several chromatography columns were tested. LC-MS/MS conditions were tuned using authentic reference standards.

Conclusion

LC-MS/MS method optimisation as well as sample preparation protocol for determination of SA biosynthetic precursors and metabolites is in progress.

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CZE-MS/MS method development for simultaneous quantification of eight β-Lactam antibiotics and two β-Lactamase inhibitors in plasma samples

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Introduction

Monitoring plasma concentrations of β-lactam antibiotics is crucial, especially in critically ill patients, where variations in these concentrations can be significant, ranging from subtherapeutic to potentially toxic levels [1]. Low drug concentrations may cause treatment to fail, which could result in an extended hospital stay or possibly death. On the other hand, elevated levels of plasma concentration might lead to infrequent yet severe adverse reactions such nephrotoxicity and neurotoxicity. These reactions can be dependent on dosage and difficult to identify quickly in the intensive care unit [2]. Antimicrobial regimens for the "average patient" are frequently customized in clinical trials. But not everyone will benefit from the same therapeutic management that comes with such a standardized approach [3]. Antibiotic concentration prediction is difficult in certain patient categories, such as elderly, obese, or critically ill patients, who frequently show changed volume of distribution, protein binding, clearance, and other pathophysiological alterations [4]. Pharmacokinetic/pharmacodynamic (PK/PD) studies highlight the time-dependent antibacterial activity of these antibiotics, emphasizing the need for personalized dosing. Additionally, new information indicates a possible correlation between the clinical outcomes of critically ill patients and the serum concentrations of β-lactam antibiotics [5].

Experimental

All CZE-MS/MS experiments were conducted using an Agilent 7100 capillary electrophoresis system coupled with an Agilent 6410 Series Triple Quadrupole tandem mass spectrometer, featuring a commercial coaxial sheath liquid electrospray (ESI) interface. Separation occurred in a 90 cm × 50 μm inside diameter bare fused-silica capillary. Sample injection was performed hydrodynamically, lasting 10 s at 50 mbar. Subsequently, a short zone of BGE (20 mM NH_4HCO_3) was hydrodynamically injected for 2 s at 50 mbar pressure to enhance sample quantitative injection and reproducibility. Experiments were conducted under voltage of +20 kV and normal polarity, resulting in currents of 3–5 μA. The sheath liquid, consisting of IP and a 10 mM ammonium formate water solution (50/50, v/v), was delivered by an Agilent 1260 Infinity isocratic LC pump at a flow rate of 8 $\mu L \cdot min^{-1}$. The MS operated in positive-ion MRM mode, utilizing characteristic precursor ion–product ion mass transitions for each investigated substance. The dwell time was set at 100 ms. Additional MS parameters were configured as follows: capillary voltage – 4500 V, nebulizing gas (nitrogen) pressure – 8 psi, drying gas

(nitrogen) temperature – 300 °C, and drying gas (nitrogen) flow – 8 $L \cdot min^{-1}$.

Results

The developed CZE-MS/MS method for simultaneous determination of five penicillins (amoxicillin, ampicillin, flucloxacillin, oxacillin and piperacillin), two cephalosporins (cefotaxime, ceftazidime), one carbapenem (meropenem) and two β-lactamase inhibitors (sulbactam and tazobactam) in plasma matrix within a single run has a run time of 20 minutes. The method involves a simple sample pretreatment - precipitation with organic solvent (ACN). To ensure reproducibility, internal standards ^{13}C, $^{2}H_3$-cefotaxime (for cephalosporins), $^{2}H_6$-meropenem (for meropenem), $^{2}H_5$-piperacillin (for penicillins) and $^{13}C_2$, $^{15}N_3$-tazobactam (for β-lactamase inhibitors) were used in the analyses. Method validation according to the Food and Drug Administration (FDA) guideline for development of bioanalytical methods demonstrated satisfactory selectivity, linearity, recovery, robustness, and stability. The LLOQs were in the range from 0.48 $\mu g \cdot ml^{-1}$ to 1.00 $\mu g \cdot ml^{-1}$ and were adequate for quantification of selected analytes in their therapeutic ranges according to the publication from Schulz (6). The method's practicality was evaluated using the Blue Applicability Grade Index (BAGI), yielding a score of 77.5, which indicates a good suitability of the method for the clinical practice. Moreover, the greenness of the proposed method was evaluated by two commonly used metric tools – Analytical GREEnness (AGREE) and Green Analytical Procedure Index (GAPI).

Conclusion

First CZE-MS/MS method for quantification of eight β-lactam antibiotics and two β-lactamase inhibitors in human plasma was developed here. Complex optimization procedure and validation according to FDA guidelines was provided. The applicability of the method to clinical practice was verified using BAGI metrics.

Acknowledgements

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Development and validation of microchip isotachophoresis method for the analysis of cardiovascular drugs

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Introduction

Pharmaceutical analysis is strictly regulated on a global scale as it ensures the safety and efficacy of drug products for patients and clinical trial participants [1]. This includes close monitoring of process-related impurities, degradation products, and active pharmaceutical ingredients (API). Based on the objective of pharmaceutical analysis, it is necessary to choose a suitable analytical method and the analytical procedure must be validated. Validation of the analytical method used in the assay test needs to meet the criteria of the ICH guidelines, i.e., it has to provide information on accuracy, precision, specificity, linearity and concentration range [2]. In the last decade, emphasis has also been placed on the development of the methods that meet the principles of green analytical chemistry [3]. Microchip electrophoresis (MCE) is a green analytical technique characterized by short analysis time, low sample and reagent consumption, and low waste production. MCE allows easy implementation of various electrophoretic techniques using suitable electrolyte(s).

This study focused on the use of microchip isotachophoresis (μITP), as one of the MCE techniques, for the analysis of macrocomponents (API and counterion) present in cardiovascular drugs marketed in salt form, amlodipine besylate and perindopril erbumine. Amlodipine besylate consists of a positively charged API, amlodipine, and a negatively charged counterion, besylate. On the other hand, perindopril erbumine contains a negatively charged API, perindopril, and a positively charged counterion, erbumine.

Experimental

The μITP separations were performed on a poly(methyl methacrylate) microchip with coupled separation channels (IonChip^{TM} 3.0; Merck, Darmstadt, Germany). The microchip has integrated conductivity sensors for real-time monitoring of the μITP separations. Separations were carried out in a hydrodynamically closed separation system, and electroosmotic flow on the microchip was suppressed by adding 0.1% (w/v) methylhydroxyethylcellulose to the electrolyte solutions. Both, cationic and anionic APIs and counterions were analysed and determined in cardiovascular drugs. In the cationic mode, the separations were performed at pH 4.7 and in the anionic mode, the separations were performed at pH 5.0. During the separations, the current was stabilized at 40 μA in the cationic mode and 30 μA in the anionic mode. Just before the detection of the sample components, the current was changed to 10 μA in both separation modes.

Results

The purpose of this study was to develop μITP methods for the analysis of various macrocomponents, i.e., APIs (amlodipine, perindopril) and counterions (besylate, erbumine). An internal standard method was used for quantification, which required the addition of an internal standard to the sample solutions. This approach aims to increase the precision of

quantitative analysis by compensating the variations in injection volume and driving current. The developed μITP methods were evaluated using AGREE, an analytical greenness metric system [4], based on the twelve principles of green analytical chemistry. The AGREE software converts the twelve principles into an overall numerical score of the method ranging from 0.00 to 1.00, with 1.00 indicating full compliance with the green analytical chemistry criteria. According to this evaluation, the developed μITP methods achieved a score of 0.81.

The methods were validated in the range of 70 to 130% of the nominal concentration of amlodipine, erbumine, perindopril and besylate. Linearity was assessed in the presence of matrix and in the absence of matrix, and the ratio of the slopes of the calibration curves was in the range of 98.8 to 100.9% for all analytes. All calibration curves exhibited good linearity with a correlation coefficient of at least 0.9993. The intra-day and inter-day precision was expressed as the relative standard deviation (RSD) of the response factor of the analytes. For one-day analyses, the RSD of the response factor was below 0.9%, while for multi-day analyses it ranged from 0.6 to 0.8%. Accuracy of the analytical method was evaluated by analyzing standard samples prepared in the presence of matrix at three concentration levels (70%, 100% and 130% of the nominal concentration). The recoveries for cationic analytes ranged from 98.8 to 101.6% and for anionic analytes from 98.3 to 100.8%.

The suitability of the proposed μITP methods for the determining macrocomponents in cardiovascular drug products was confirmed by analysing three commercially available formulations containing amlodipine besylate and three formulations containing perindopril erbumine. The content of macrocomponents in tablet formulations was expressed in milligrams per tablet, with a relative error below 1.8%.

Conclusion

In this work, novel green μITP methods, developed for the determination of cationic and anionic macrocomponents in cardiovascular drugs, are presented. The developed methods met all the requirements of the validation process according to the ICH guidelines. The methodology presented in this work has the potential to be applied in drug quality control and meets most of the requirements of green analytical chemistry.

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RP-HPLC separation of amino sugars in blood serum samples

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Introduction

Amino sugars (aminosaccharides, hexosamines) represent an important group of compounds that are present in all living organisms as part of the structural units of some biopolymers and are also found in dead organic residues that pass into the structure of humic substances due to humification processes [1].

Structurally, amino sugars are derivatives of monosaccharides where one hydroxyl group (-OH) is substituted for an amino group (-NH2). Like sugars, amino sugars are chiral compounds, free of chromophores and fluorophores. By comparing their structures, they represent stereoisomers, in particularly epimers, among themselves. Epimers are stereoisomers differing in configuration at one stereogenic center but in a different than the last asymmetric carbon atom and the anomeric carbon atom. It follows that the amino sugars glucosamine (GlcN) and galactosamine (GalN), differing in the orientation of the bond on the fourth carbon, are C-4 epimers and glucosamine with mannosamine (ManN) represent C-2 epimers. In addition to simple forms of amino sugars, N-acetylamino sugars are also present in most biopolymers, which are formed by acetylation of the primary amino group [2,3].

Amino sugars are also found in tissues and body fluids of higher organisms, including humans. Their presence in synovial fluids suggests that amino sugars (especially GlcN) may be used in the symptomatic treatment of osteoarthritis [4], manifested by gradual degradation of the cartilage in the joints, possibly leading to complete cartilage loss. Given the importance of amino sugars, not only in the understanding and treatment of osteoarthritis, there is a need to develop a sensitive and reliable analytical method for their determination in clinical samples. The main problem in the development of this method is the character of amino sugars, namely their high polarity and low concentrations (the content of GlcN in the blood is 60 $ng.mL^{-1}$) [5,6]. The presented work was focused on separation of three amino sugars (GlcN, GalN, ManN) by RP-HPLC method with pre-column derivatization of analytes using diethylethoxymethylenemalonate reagent (DEEMM). The developed method was subsequently applied for the determination of analytes in a blood serum sample.

Results

Due to the natural occurrence of amino sugars in two anomeric forms (α- and β-anomer), it can be assumed that both isomers are also present in aqueous solutions. Based on the chromatographic record of separation of the mixture of amino sugar standards (Figure 1), we can confirm that the assumption of both forms of amino sugars in the solution has been confirmed. The blood serum sample supplied by the Faculty of Medicine of Comenius University in Bratislava after derivatization by DEEM reagent was analysed by RP-HPLC method (Figure 2). In the blood serum sample, we determined two amino sugars, glucosamine and galactosamine, using the standard addition method, at concentration levels of 1.034-3.343 $mg.L^{-1}$. From the calibration data, we calculated the detection limit (LOD), defined as the signal-to-noise ratio (S/N = 3:1) and the determination limit (LOQ) (S/N = 10:1) of the individual amino sugar anomers (Table 1).

Conclusion

The work deals with separation of the three most important amino sugars (glucosamine, galactosamine, mannosamine) by RP-HPLC method. The developed method was applied for the determination of analytes in a blood serum sample in which we determined the anomers of two selected amino sugars at concentration levels of 1.034 - 3.343 $mg.L^{-1}$. The presented RP-HPLC method can be applied to other clinical samples (synovial fluid, cerebrospinal fluid, blood plasma) as well as environmental samples (water, soil, humic acid degradation products).

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Figure 1. Chromatographic record of separation of mixture of amino sugars standards at 280 nm.

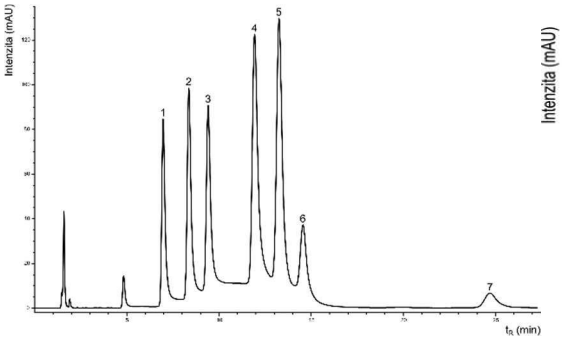

Figure 2. Chromatographic record of blood serum sample at 280 nm (1 - ManN 1, 2 - GalN 1, 3 - GlcN 1, 4 - GalN 2, 5 - GlcN 2, 6 - M anN 2, 7 – DEEMM).

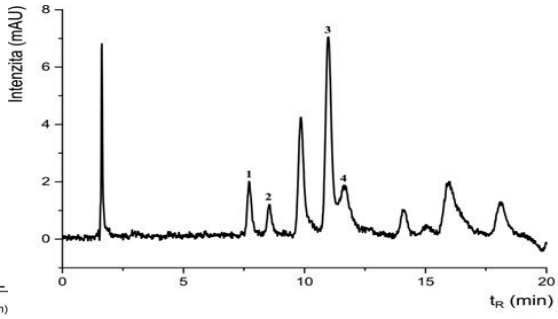

Table 1. Calculated detection and quantification limits for individual amino sugars

Amino sugar	LOD ($mg.L^{-1}$)	LOQ ($mg.L^{-1}$)	LOD ($\mu mol.L^{-1}$)	LOQ ($\mu mol.L^{-1}$)
ManN 1	0,033	0,099	0,153	0,459
GalN 1	0,039	0,120	0,181	0,557
GlcN 1	0,039	0,120	0,181	0,557
GalN 2	0,032	0,097	0,148	0,450
GlcN 2	0,026	0,080	0,121	0,371
ManN 2	0,084	0,256	0,390	1,187

Optimized HPLC-guided separation and purification of 1,3,5-triazine derivatives containing amino acids with non-polar side chain as potential bioactive molecules

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Introduction

1,3,5-triazines and their derivatives exhibit various biological effects useful in pharmacy and medicine. Currently, benzenesulfonamide derivatives with modified triazine ring belong to the most studied hybrid molecules due to their promising anticancer activity.[1] The development of new bioactive compounds, such as 1,3,5-triazine derivatives with 4-amino(alkyl)benzenesulfonamide and amino acids in our research [2,3], requires effective synthetic as well as isolation and purification procedures to obtain desired derivative with high purity.

Experimental

In the synthetic step, the microwave-assisted synthesis was carried out using a microwave reactor with *in situ* Raman spectroscopy (Anton Paar, Austria). Semi-preparative HPLC was performed on LC instrument (Shimadzu, Japan) with various tested stationary phases such as 60-10 SIL Kromasil 250x10 mm (Nouryon, Netherlands); C18 Kromasil 250 × 10 mm (Nouryon, Netherlands); Nucleodur C18 Htec 250 × 10 mm and VP 10 × 8 mm guard column (Macherey-Nagel, Germany) and mobile phase consisting of ammonium bicarbonate aqueous solution (pH 8, 50/100 mM) as A, methanol or acetonitrile as B. IR spectra were recorded on Spectrum Two FT-IR spectrometer (PerkinElmer Ltd., UK). NMR spectra (^{1}H and ^{13}C) were recorded on MR400MHz spectrometer (Agilent Technologies, USA) in Central NMR laboratory of the Faculty. HPLC-DAD/MS analyses were performed in Toxicological and antidoping center of the Faculty on a LC system (Agilent Technologies, USA) with photodiode array detector Infinity 1290 DAD and a quadrupole time-of-flight mass spectrometer 6520 Accurate Mass Q-TOF LC/MS.

Results

In the synthetic step, a novel and simplified method using microwave (MW) irradiation was developed. The most significant benefit of MW syntheses was a very short reaction time. Under MW conditions, the obtained yields (over 75%) and purities (70.3–96.8%) of the products were higher or comparable to those obtained by our conventional reflux syntheses. Subsequently, if conventional isolation and purification methods such as crystallization, distillation, or extraction are not sufficient and/or selective enough, a highly efficient technique such as liquid chromatography can be employed. In this context, new optimized procedures using semi-preparative HPLC methods were applied for the isolation and purification of a series of 1,3,5-triazine derivatives incorporating 4-aminobenzenesulfonamide and amino acids Ala, Val, Leu,

Phe, Trp to obtain the purity over 97.3%. The reverse-phase C18 and C8 Nucleodur system with guard column was advantageously employed for the purification of majority of derivatives to achieve lower flow rates of mobile phase, satisfactory injected sample volume (up to 4000 μl), comparable or shortened analysis time and thus lower consumption of mobile phases compared to our previously described conditions [3].

Conclusion

New optimized procedures using semi-preparative HPLC methods were developed and applied for the isolation and purification of a series of 1,3,5-triazine derivatives incorporating 4-aminobenzenesulfonamide and amino acid moieties with non-polar side chain to obtain the purity over 97%. The designed procedures allowed to achieve the well-defined and efficient separation of the desired derivatives, and the highest possible purity of the products in satisfactory yields. The applied methods and instrumental equipment for the synthesis as well as purification contribute to the preservation of the green chemistry principles. The high degree of purity of the derivatives, as presented in this work, is a prerequisite for obtaining reliable results when testing their various potential biological activities.

Acknowledgements

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Middle-up quantification of a monoclonal antibody in pharmaceutical matrix by capillary electrophoresis-mass spectrometry

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Introduction

Infliximab (IFX) is a chimeric mouse-human therapeutic monoclonal antibody (mAb) against tumor necrosis factor (anti-TNFα), commonly used in treatment of inflammatory bowel disease. IFX is an IgG type immunoglobulin that is, structurally, a large protein of size approximately 150 kDa. Every mAb consists of two types of subunits – two heavy (~50 kDa) and two light (~25 kDa) chains connected by disulfide bonds. Typically, bottom-up approach is employed for mAb analysis, where mAbs are enzymatically digested into smaller peptides and subsequently analysed. This work makes use of an alternative, middle-up approach, where the mAb disulfide bonds are reduced by reducing agent into smaller (but still relatively large) subunits. Capillary electrophoresis (CE) is a separation technique that has recently emerged in the proteomics field and proved as suitable for analysis of proteins due to its high efficiency separation, no need for a stationary phase (when compared to liquid chromatography) and low sample and chemical consumption. CE coupled to mass spectrometry represents a powerful tool for analysis of proteins. The presented work aims to couple middle-up mAb sample preparation to CE-MS for quantitative analysis of mAb infliximab (IFX) in pharmaceutical matrices.

Experimental

Stock solution of IFX reference standard (Sigma-Aldrich, US) (1 mg/mL in water) was prepared. IFX disulfide bonds were reduced by tris(2-carboxyethyl)phosphine (TCEP) by adding TCEP directly to IFX solution and incubating the sample at 37 °C for 1 hour. The reduced IFX was subsequently analyzed by Agilent 7100 CE system coupled to an Agilent 6410 triple quadrupole mass spectrometer (Agilent, Santa Clara, US). A fused silica capillary of (i.d. 50 μm) of length 830 mm was used. Background electrolyte (BGE) systems containing formic and acetic acid were tested. Separation was performed in cationic separation regime with voltage range 18 - 25 kV. Analysis was carried out in Scan and SIM modes.

Results

Initially, the CE-MS analysis of the reduced IFX performed in Scan mode allowed partial separation of heavy and light chains. Four light chain and one heavy chain *m/z* were selected for further analysis in SIM mode. Subsequently, BGE systems containing 0,5 M and 1 M formic acid and acetic acid in the range 2 – 4 M were tested together with different separation voltages applied (range 18 – 25 kV). Results show that the optimal conditions were BGE containing 1 M formic acid at a selected separation voltage 25 kV. Lastly, various concentrations of IFX stock solution (0,01 – 0,5 mg/mL) were analyzed to plot a calibration curve.

Conclusion

The results show that the most suitable BGE for analysis of reduced mAb IFX is 1 M formic acid and the optimal separation voltage 25 kV. Furthermore, a calibration curve was plotted from the acquired data showing that the proposed proof-of-concept quantitative method requires further optimization.

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Methods for GC-MS analysis of pyrolytic compounds in different matrixes

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Introduction

Flavor plays a big role in today's food production. This work aims to develop method for analysis of two analytes, 2-acetylfuran and 2-acetylpyrrole, in different food matrixes, such as coffee, chocolate and distillates. 2-acetyfuran and 2-acetylpyrrole are compounds, that are created during Maillard reaction and play a role in formation of smell of product. Twenty three samples were used for analysis, including ten samples of coffee beans, eight samples of distillates and five samples of chocolate. Four of coffee samples were coffee in different degrees of roasting. Eight distillate samples were of different types (rum, cognac, armagnac). And chocolate samples contained different additives (including nuts, caramel, sweetener, and two samples without additives). One GC-MS analysis method along with different extraction methods for different matrixes were developed.

Experimental

This work covers extraction of 2-acetylfuran and 2-acetylpyrrole from three different matrixes: coffee beverage, chocolate and distillates. For quantification purposes, standard addition method was used.

Extraction from coffee beverage was by far the easiest. Coffee beverage was prepared by mixing 5 g of milled coffee beans with 50 ml of hot water. Then 1 ml of beverage was extracted by 1 ml of ethyl acetate. In order to speed up extraction, system was first shaken and then centrifugated, this process was repeated three times.

Extraction from distillates was somewhat more challenging due to the high amount of alcohol in samples. In order to reduce the concentration of ethanol, 1 ml of sample was firstly diluted by 1 ml of saturated NaCl solution. Resulting solution was then extracted by 1 ml of ethyl acetate.

Extraction from chocolate matrixes was by far the most labor intensive. Method from Suzuki et al [1] was adapted for this extraction. First, chocolate was grated, 1 g of chocolate was then extracted by 2 ml of hexane twice. Suspension was shaken and centrifugated in order to speed up extraction. After transfer of hexane to another vial, chocolate residue was then extracted by 2 ml of methanol twice. Hexane extract was then reextracted by methanol extract in order to separate polar and non-polar compounds. Methanol extract was then diluted by saturated NaCl solution to 30% methanol concentration and extracted by chloroform. Chloroform extract was then analyzed by GC-MS method.

Results

The extraction and analysis method has shown good results for chocolate and coffee matrixes but somewhat **mediocre** results for distillate matrix.

Chosen extraction method was nearly optimal for coffee beverage. Analysis had more or less good repeatability, it was easy to locate analyte peaks and there was no coelution with different compounds. Furthermore, method worked for both, relatively high, and relatively low

concentration of analytes. Analysis of coffee beans in different degrees of roasting proved, that our analytes are created during roasting process and are not present in raw coffee beans.
On the other hand, the same extraction method used for distillates was suboptimal and required further optimalization. Most likely it was due to high concentration of ethanol in the samples. In order to lower ethanol concentration during extraction, distillate samples were diluted by saturated NaCl solution 1:1. This way, method has started to provide acceptable results. Despite that, extraction method can be optimized further, since even improved method suffered from change of retention time in different types of distillates.
Method for extraction of analytes from chocolate was, while laborious, reliable as well. Method worked for five different chocolate samples, each sample was somewhat different (for example had different additives). Elution time was more or less the same as it was for coffee matrix. Analysis of different samples has shown that concentration of analytes is higher in samples with higher cocoa percentage as well as in samples which contained thermally processed additives.
Another point that is worth mentioning, is that optimalization of starting analysis temperature as well as usage of column with retention gap has significantly improved method, due to concentration of analytes at the start of column at the beginning of analysis.

Conclusion

Three methods were developed for extraction of pyrolysis products from different food matrixes. Methods for extraction from chocolate and coffee are reliable enough and can be used in analysis of these samples. While method for extraction of analytes from distillates still requires further optimalization and possibly adaptation for different types of distillates. Retention gap has improved analysis results as well. Thanks to optimalization of starting temperature of analysis peaks of better shapes were obtained and method itself was somewhat more sensitive.

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Chiral separations of new potential pharmacophores: *nido*-[7,8-$C_2B_9H_{12}$]$^-$ and [Co($C_2B_9H_{11}$)$_2$]$^-$ derivatives

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Introduction

Carboranes, a group of boron cluster compounds containing carbon, are currently under extensive investigation. The high variety of substituents and their various spatial arrangements result in a multitude of different molecules. The properties of these molecules, which arise from the combination of organic and inorganic chemistry, have led to their investigation in numerous research fields. These include catalysis, synthetic chemistry, analytical chemistry, medicinal chemistry, photochemistry, material chemistry, and electrochemistry. [1]

Anionic carboranes are also studied in medicinal chemistry due to their high overall stability, the solubility of their salts in water, and their low toxicity. Recently, two subgroups of anionic carboranes, namely *nido*-[7,8-$C_2B_9H_{12}$]$^-$ and [Co($C_2B_9H_{11}$)$_2$]$^-$ derivatives, have gained attention in pharmaceutical chemistry. [1] However, their chirality has not yet been properly investigated. This is evident from the lack of a unified classification system of their stereoisomers and the insufficient presence of chiral analytical methods.

The underdevelopment in this area hinders the progress of new potential pharmaceuticals with unique pharmacophores. These new molecules have the potential to address the problem of antibiotic resistance and enhance the activity of known drugs by simply substituting phenyl rings or other parts of the molecule (for example, tamoxifen becomes boroxifen, aspirin becomes asborin). [1]

Moreover, neglecting chirality can lead to substantial financial losses at best and, at worst, loss of human lives, as we have learned from the Thalidomide affair. Therefore, exploring the possibilities of chiral separation in chromatographic methods, with an emphasis on purity control and the isolation of pure enantiomers, is of paramount importance.

Experimental

In this work, the chromatographic/electrophoretic behavior of different structural motifs of *nido*-[7,8-$C_2B_9H_{12}$]$^-$ derivatives and [Co($C_2B_9H_{11}$)$_2$]$^-$ derivatives with respect to chiral separation in HPLC and SFC were investigated. We explored quinine/quinidine-, cyclodextrin-, polysaccharide-based, and Pirkle type chiral stationary phases in HPLC and SFC.

Results

The anionic *nido*-[7,8-$C_2B_9H_{12}$]$^-$ derivatives were not previously separated in HPLC. We explained these unsuccessful attempts by various factors including strong ionic interactions with metal impurities or repulsive interactions with deprotonated silanols. These unwanted

effects were mitigated by addition of counter-ions or chelating agent to the mobile phase resulting in chiral separation of anionic *nido*-[7,8-$C_2B_9H_{12}$]$^-$ derivatives on the bromated β-cyclodextrin-based CSP in HPLC. [2] Zwitterionic *nido*-[7,8-$C_2B_9H_{12}$]$^-$ derivatives were base-line separated on the same column and other CSPs without any evidence of strong ionic interactions.
After the initial experiments, we tested multiple chiral columns and chromatographic modes. In general, SFC methods using polysaccharide-based columns are preferred for zwitterionic and anionic [Co($C_2B_9H_{11}$)$_2$]$^-$ and zwitterionic *nido*-[7,8-$C_2B_9H_{12}$]$^-$ derivatives. [3] However, for anionic *nido*-[7,8-$C_2B_9H_{12}$]$^-$ derivatives, RPLC using native β-cyclodextrin is the method of choice. [2] RPLC employing 2-hydroxypropyl-β-cyclodextrin immobilized on silica SPP can be used as an alternative to SFC for fast enantioseparations of dihydroxyalkyl-, oxygen bridged hydroxyalkyl-, and bisphenylene bridged derivatives of [Co($C_2B_9H_{11}$)$_2$]$^-$. [4]

Conclusion

This work provides a foundation for chiral separations of [Co($C_2B_9H_{11}$)$_2$]$^-$ and *nido*-[7,8-$C_2B_9H_{12}$]$^-$ derivatives (Figure 1), essential for controlling enantiomeric purity, isolation of pure enantiomers, and developing chiral bioanalytical methods. This allows for the development of chiral pharmaceuticals based on carboranes and the synthesis of enantiomerically pure materials with unique chiroptical properties. Furthermore, these methods enable exploration of the impact of chirality in various scientific fields, such as enantioselective catalysts, chiral selectors, enantioselective electrodes, and supramolecular chemistry, where the potential of enantiopure compounds remains unexplored.

Acknowledgements

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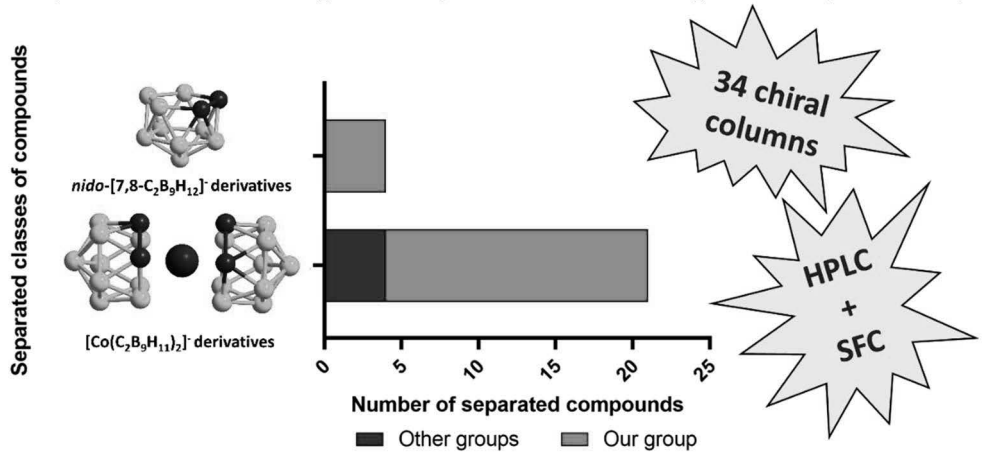
Figure 1. HPLC/SFC chiral separations of anionic compounds obtained by our group (red color) in the context with previously achieved chiral separations (blue color).



An insight into deschloroketamine metabolism – identification of minor hydroxy metabolites in human blood serum and urine

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Introduction

Deschloroketamine (DCK) is a designer drug from a group of new psychoactive substances. Chemically, it is an analogue of ketamine, differing only in the absence of chlorine on the phenyl ring, acting as a blocker of NMDA receptors (a type of glutamate receptor). It induces feelings of dissociation, depersonalization, hallucinations, disturbances of consciousness and coordination. Because DCK does not have a long history of human use, its toxicity and the consequences of long-term use have not been adequately studied. However, the prevalence and availability of this recreational drug on the illicit market are increasing. This work provides insight into the identification of its metabolites in blood serum and urine obtained from a psychonaut by LC-HRMS method.

Experimental

LC-MS analyses were performed by UHPLC Elute (Bruker) hyphenated with UHR-QTOF Maxis Impact II (Bruker). Chromatographic separations were performed at 40 °C on RP Intensity Solo HPLC Column, C18-2, 1.8 µm, 100 x 2.1 mm, Pore Size 90 Å (Bruker). The chromatographic conditions used were as follows: injection volume, 3 µL; flow rate, 0.2 mL/min; mobile phase solvents, (A) water–MeOH 99:1 (v/v) with 5 mM ammonium formate and 0.01 % formic acid (v/v) and (B) MeOH with 5 mM ammonium formate and 0.1 % formic acid (v/v). The gradient elution was as follows: 0 min 99 % A and 1% B, 0.1–2.5 min. 50 % A and 50 % B; 2.6–14.0 min. 1 % A and 99 % B; 14.1 min.–15.5 min 1 % A and 99 % B. The total run time was 20 min. A UHR–QTOF–MS Maxis Impact II equipped with a heated electrospray ionization source operated in positive-ion mode. The main source settings were as follows: capillary voltage, 2,500 V; nebulizer pressure, 3.0 bar; drying gas flow (N_2), 8 L/min; and drying gas temperature 200 °C. Patient blood serum and urine samples were prepared by protein precipitation and dilution procedure, respectively, by mixing 50 µL of blood serum spiked with lorazepam internal standard solution (100 mg/L) with 450 µL of MeOH and by dilution mixing 50 µL of urine with 450 µL of MeOH. Samples were vortexed and underwent

centrifugation (14,500 rpm, 3 min), 100 μL of supernatant was transferred into the clean vial with a glass insert, and 3 μL was injected into the UHPLC–UHR–QTOF MS system.

Results

LC-MS analysis of blood serum and urine of a psychonaut identified the presence of various psychoactive substances and their metabolites, including hallucinogenic LSD, new psychoactive substances – deschloroketamine and mitragynine, and additionally zolpidem, which is used as an insomnia drug. We focused on the identification and determination of the deschloroketamine and its major metabolite nordeschloroketamine (NDCK). We identified dihydro-NDCK and dihydro-DCK as other known metabolites of DCK in both blood serum and urine, respectively. Subsequently, we studied minor DCK hydroxylated metabolites using UHRMS, confirming their presence in urine and blood serum. Previously published work confirmed the presence of these minor metabolites in biological samples obtained from the experimental animals only.

Conclusion

As a result of our observation, we affirmed the presence of various drugs of abuse with hallucinogenic, psychostimulant and psycho-depressant effects in patient blood serum and urine samples. The presence of many known metabolites of these drugs has been also demonstrated, while this work also provides new insights into the metabolic pathway of DCK. The minor hydroxy metabolites of DCK were identified in blood serum and urine. To the best of our knowledge, the presence of hydroxy metabolites of DCK in human blood serum and urine has not been described yet.

Acknowledgements

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Separation and determination of carboxylic acids in complex samples by microchip electrophoresis coupled with ion mobility spectrometry

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Introduction

Microchip electrophoresis (MCE) is a miniaturized separation technique characterized by short analysis time, low sample and reagent consumption, and low waste production. MCE meets most of the requirements of green analytical chemistry [1]. One of the disadvantages of MCE is the need to implement a selective and/or sensitive detection technique for the analysis of complex samples. In addition to the most widely used detection techniques, e.g., laser induced fluorescence, mass spectrometry and conductivity detection, ion mobility spectrometry (IMS) also has great potential for use in combination with MCE [2,3]. IMS is a separation and powerful identification technique characterized by fast response and relatively low analysis costs. It has a limited separation capability and its coupling with some other separation techniques can be used to increase the peak capacity [4].

MCE and IMS are based on the similar separation principle, i.e., separation of ions based on their different mobility in the electric field. However, while MCE analysis is performed in the liquid phase, IMS analysis requires a sample in the gaseous phase. A key element in the development of the MCE-IMS coupling is therefore the development of an interface suitable for the transfer of separated sample components with minimal impact on the separation and resolution achieved in the MCE.

In this work, the development of the MCE-IMS coupling and its application to the analysis of complex liquid samples is shown.

Experimental

MCE separations were performed on a poly(methyl methacrylate) microchip with coupled separation channels and integrated conductivity sensors (Merck, Darmstadt, Germany) in a hydrodynamically closed separation system with suppressed electroosmotic flow. The electrophoretically separated sample components were transferred from the microchip using an auxiliary liquid that was introduced onto the microchip in the direction opposite to the electrophoretic migration of the components on the microchip. A thermal evaporation unit was used to vaporize the separated sample components and introduce them into the IMS analyzer. Carboxylic acids from the homologous series C_1-C_6 (formic acid, acetic acid, propionic acid, butyric acid, valeric acid and caproic acid) were analyzed in various food (apple vinegar, wine, fish sauce), pharmaceutical (ear drops), biological (saliva) and environmental (wastewater) samples.

Results

MCE allows simple implementation of different electrophoretic techniques by using the appropriate electrolytes. For its coupling with IMS two different electrophoretic technique were used: zone electrophoresis (ZE) and isotachophoresis (ITP). These techniques differ in the spatial arrangement of the sample components during the separation: in ZE, the sample

components are separated by the background electrolyte, and in ITP, the components form discrete zones with sharp boundaries between them.
Electrolytes used for the ZE and ITP separations, i.e., 50% (*v/v*) background electrolyte and 10% (*v/v*) terminating electrolyte, were found to be the most suitable auxiliary liquids, as they allowed a low dispersion transfer of the separated components from the microchip to the IMS analyzer and did not have a negative impact on the separation.
Various analytical parameters, such as sensitivity, linearity and precision, were evaluated for the developed ZE-IMS and ITP-IMS methods. Precision was expressed as the relative standard deviation (RSD) of the IMS qualitative parameter, i.e., reduced ion mobility (K_0). RSD of K_0 was less than 0.5% for the ZE-IMS method, and less than 0.3% for the ITP-IMS method for all studied carboxylic acids. Limits of detection, calculated as 3.3 times the standard deviation of the blank to the slope of the calibration curve, constructed from the peak height, were lower than 2.6 mg/L for the ZE-IMS method, and due to the preconcentration ability of the ITP, were lower than 0.3 mg/L for the ITP-IMS method.
The applicability of the developed methods was evaluated on the analysis of the wastewater sample using the ZE-IMS method and samples of apple vinegar, wine, fish sauce, saliva and ear drops using the ITP-IMS method. A standard addition method was used to study matrix effects. The K_0 values of the studied carboxylic acids in the real samples did not differ significantly from the values measured in the model samples.

Conclusion
Online two-dimensional MCE-IMS methods can be used for the separation and reliable determination of C_1-C_6 carboxylic acids in environmental, biological, food and pharmaceutical samples. In addition, the limitations of MCE and IMS as stand-alone techniques for analysis of complex samples can be solved by coupling MCE with IMS.

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Detailed LC-MS/MS analysis of sugars and their alditols for diagnosis of inherited metabolic disorders

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Inherited disorders of carbohydrate metabolism are characterized by abnormal levels of sugars and their alditols in body fluids, which form structural isomers, i.e. substances of the same molecular weight that differ in structure and are not easily distinguishable. As part of the routine examination of selected metabolites in urine, hydrophilic interaction chromatography with tandem mass spectrometry (HILIC-MS/MS) method is performed in the Laboratory for Inherited Metabolic Disorders, University Hospital Olomouc. This method allows the detection of >60 metabolites from the spectrum of purines, pyrimidines, *N*-acetylated amino acids, acylglycines, sugars, alditols and other diagnostically important markers. However, individual isomers of sugars and alditols cannot be distinguished by this method. If their differentiation is necessary, second-tier methods are applied. In our laboratory, gas chromatography-mass spectrometry (GC-MS) method is still mainly used for their differentiation, but it does not meet the urgent requirement due to the time-consuming sample preparation, analysis, and evaluation. In the most common case, hexitols (i.e. galactitol, mannitol and sorbitol) need to be distinguished. Galactitol is a key marker of galactosemia, which can progress to a life-threatening condition without immediate treatment, while mannitol/sorbitol accumulates in the urine due to its presence in many foods, dietary supplements, and drugs. Distinguishing galactitol from mannitol/sorbitol is therefore crucial in the diagnosis of galactosaemia. To speed up and simplify the diagnosis of disorders of carbohydrate metabolism, a liquid chromatography with tandem mass spectrometry (LC-MS/MS) method was developed that can reliably distinguish galactitol from other alditols using its labeled standard (galactitol-13C6) based on retention time. Overall, the new LC-MS/MS method allows simultaneous analysis of 19 sugars and 10 alditols in 17 min. It is diagnostic platform for >20 inherited metabolic disorders (of the pentose phosphate pathway, glucose transport, galactose, fructose, and glycogen metabolism), which has the potential to replace the GC-MS method in the future.

Acknowledgements
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The use of carbon-based columns in the analysis of structurally similar herbicides

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Introduction

Alachlor, acetochlor, and metolachlor are herbicides used for weed control. Their use has been banned within the European Union due to their potential carcinogenic effects and high persistence in the environment (including degradation products). For this reason, effective control methods are necessary to prevent their further use [1]. Liquid chromatography using conventional C18 columns is practically the only method used for these purposes [2, 3]. Because of the similar structure of these pesticides, the octadecyl-silica-based columns fail in their chromatographic separation, and therefore detection using tandem mass spectrometry (MS/MS) is necessary. Columns based on porous graphitic carbon can be used for the separation of isomers and structurally related substances. The advantage of these columns compared to silica-based columns is in their stability under extreme conditions, such as high temperature (up to 200 °C) and the entire pH range. The aim of this study was to find suitable conditions for the separation of alachlor, acetochlor, and metolachlor using carbon-based columns.

Experimental

Two graphitic carbon-based chromatographic columns were tested: Hypercarb (10 × 2.1 mm, 3 μm) and Discovery Zr-Carbon (7.5 × 2.1 mm, 3 μm). The analyses were performed using an Agilent 1260 Infinity II LC system (Agilent, Palo Alto, CA, USA) coupled with QTrap 4500 MS / MS detector (Sciex, Framingham, MA, USA). Using the Discovery Zr-Carbon column, the following optimal conditions for the separation of the parent compounds were found: gradient elution (A – H_2O + 5mM ammonium acetate and B – ACN), column temperature 135 °C, flow rate 0.4 mL/min and injection volume 5 μL. In the case of degradation products, the conditions were as follows: gradient elution (A – H_2O + 4 mM ammonium acetate + 0.065% triethylamine and B – ACN/H_2O (70:30) + 0.2% triethylamine), column temperature 135 °C, flow rate 0.4 mL/min and injection volume 10 μL.

Results

In the first step, the suitability of both selected columns to separate parent substances and degradation products was tested in the ACN / H_2O mobile phases. These experiments did not yield satisfactory results in terms of resolution or peak shape. Adding ammonium acetate (and triethylamine in the case of degradation products) as an additive to the aqueous mobile phase and increasing the separation temperature to 100 °C proved to be an effective way to influence the resolution and peak symmetry of the tested substances. Better results were achieved using the Discovery Zr-Carbon column, thus on this column the effect of the concentration of additives in the mobile phase and the column temperature was further determined. In the case of the parent compounds, a significant temperature effect was

found. Even at temperatures below 100 °C, acetochlor was separated from the other herbicides, and at the same time it was possible to observe two metolachlor peaks on the chromatogram, which were subsequently identified as R- and S-metolachlor. If the column temperature was increased to 135 °C, a satisfactory resolution of all parent substances occurred. For the separation of degradation products, a significant effect of the temperature and concentration of ammonium acetate and triethylamine in the mobile phase was found. The change in these parameters contributed to improvement or, on the contrary, to deterioration of the resolution of individual pairs of compounds. The conditions for the final method were thus achieved as a compromise in which all 8 degradation products were at least partially resolved.

Conclusion

The method of high-temperature liquid chromatography using carbon-based columns proved to be a suitable approach for the analysis of chloroacetanilide herbicides. Compared to conventional C18 columns, at least partial separation of all parent substances was achieved using the Discovery Zr-Carbon column. The column temperature and mobile phase additive concentration have been shown to be key parameters to improve resolution and peak shape.

Acknowledgements

The work was financially supported by the Czech Science Foundation, project No. 22-09556S.

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Chiral separation of ketamine and its metabolites from rat plasma samples using partial filling CE-ESI/MS

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Introduction

Ketamine is a chiral drug used for a nearly 60 years to induce and maintain anaesthesia. In addition, ketamine and especially its metabolites act as antidepressants. For that reason, the mechanisms of action of these species have been studied. Different effects of enantiomers of ketamine and its metabolites were observed. S-ketamine has about twice the analgesic potency than the clinically used racemic mixture [1] and is supposed to be responsible for high abuse liability of ketamine [2]. The metabolism of R-ketamine to 2R,6R-hydroxynorketamine is essential for its antidepressant effect [3]. Ketamine and its main metabolites were successfully separated by capillary zone electrophoresis (CZE) [4] but a complete separation of enantiomers of ketamine (Ket) and its main metabolites, norketamine (NK), dehydronorketamine (DHNK) and hydroxynorketamine (HNK), has not yet been achieved. The recently developed LC-MS method requires one achiral and two chiral columns for separation of six enantiomers [5]. Therefore, the aim of this work was to develop a new CZE method that could separate the enantiomers of all above species in a single run.

Experimental

The CZE analyses were performed in in-home built CE module online coupled with ESI/MS/MS detector (Orbitrap Exploris 240, ThermoFisher Scientific). The CE module was equipped with two Spellman HV power supplies and automatic carrousel with electrode vessel and sample vials. The 42.5 cm long separation capillary (150/25 μm O.D./I.D.) with negative 3% PAMAMPS coating [6] was placed into the lab-made nano-sheath liquid flow CE-ESI/MS interface [7]. A sheath liquid (SL) flow (≈ 70 nL/min) was generated by pressure (80 mbar) applied to the SL inlet vial. SL contained 10 mM NH_4OH and 104 mM AcOH in 1/1 (v/v) EtOH/H_2O mixture. The used background electrolyte (BGE) consisted of 10 mM NH_4OH, 104 mM AcOH, pH* 3.75 in 1/9 (v/v) EtOH/H_2O mixture. Separation voltage was 30 kV, and the spray voltage was 2 kV. Blood plasma samples were collected first after 3 min and then every 5 min (up to the 50 min) after ketamine administration (in dose 10 mg/kg). Two step microextraction with dichloromethane was used for sample preparation.

Results

The chiral separation of Ket, NK, HNK and DHNK enantiomers was achieved using partial filling CE-ESI/MS method. Based on the published works, highly sulfated β- and γ-cyclodextrins (CDs) were used as chiral selectors. It was found that both of them contribute to the chiral separation of all the analytes, but the S-β-CD separates mainly HNK and NK enantiomers, while the S-γ-CD separates mainly Ket and DHNK enantiomers. Because of the

more flexible optimization of the separation conditions, the CDs were injected into the separation capillary in two consecutive zones. The best separation (see Figure 1.) was achieved with 11% L_{tot} (total capillary length) zone of sulfated β-CD (30 mg/mL) and 12% L_{tot} of sulfated γ-CD (10 mg/mL). To prevent transfer of the analytes to the inlet vial by the selectors, BGE zone in length of 65% L_{tot} between the CDs and injected sample zone was used.
The method was applied for the study of metabolic pathway of racemic and S-ketamine. When focused on the HNK metabolites, the S-ketamine is predominantly metabolized to 2S,6S-HNK, the metabolism of R-ketamine is more complex. Beside the 2R,6R-HNK formation, other HNK metabolites with similar abundance were observed. However, due to the lack of the relevant standards, they could not be identified.

Conclusion

The baseline separation of Ket, NK and DHNK enantiomers and separation of nine HNK metabolites within the single run was achieved. The method was successfully applied for the comparative study of metabolic pathway of racemic ketamine and S-ketamine in rats.

Acknowledgements

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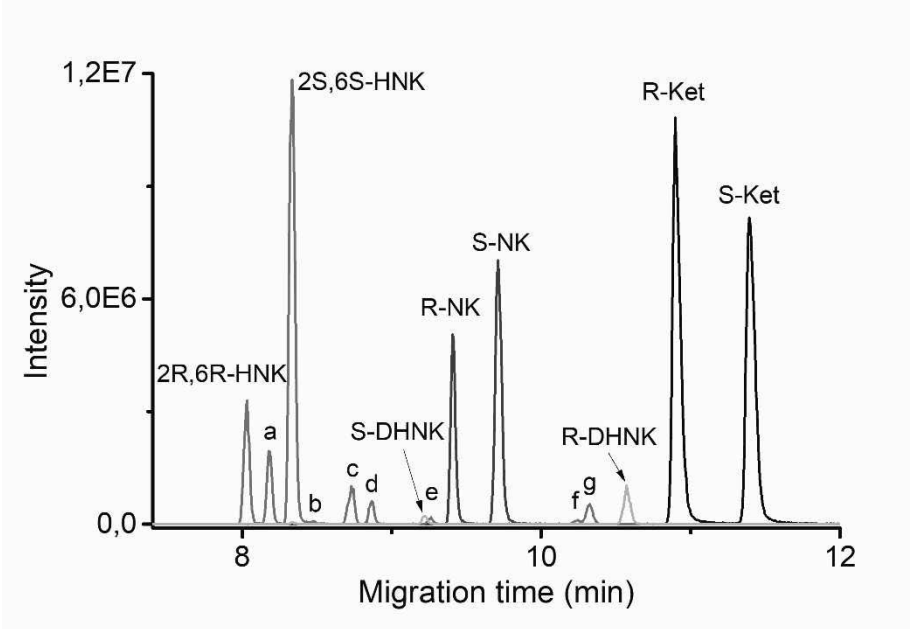
Figure 1. CE-ESI/MS separation of ketamine and its metabolites in rat plasma sample collected 45 min after racemic ketamine administration. Experimental conditions are described above. Peaks a–g corresponds to the unspecified HNKs.



Development of the metod for therapeutic drug monitoring of selected benzodiazepines by liquid chromatography

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Introduction

Benzodiazepines are among the most widely used psychopharmaceuticals today. In Czech Republic 48.84 defined drug doses per 1000 inhabitants were used according to research between 2008–2010 [1]. Nowadays, these substances are abused as illegal drug and are often used for suicide attempts [2]. For these reasons it is necessary to have sensitive and fast method for their determination in biological samples.

The aim of this work was to develop method, which would expand the existing method for determining clonazepam, diazepam and nordiazepam in serum at department of Clinical Pharmacology of University Hospital Ostrava and include new benzodiazepines using liquid chromatography. This task consisted of developing, validating and implementing a method for quantification of alprazolam, bromazepam, diazepam, nordiazepam, clobazam, norclobazam and clonazepam.

Experimental

HPLC/UV, UPLC/UV and UPLC/MS systems with various columns and mobile phases were tested to find the best separation and detection system for 7 benzodiazepines.

Apparatus

- Alliance liquid chromatograph (Waters 2695 Separations module) coupled with a spectrophotometric detector (Waters 2487 Dual λ Absorbance detector)
- Acquity UPLC system from Waters with PDA detection
- Acquity UPLC system from Waters with Acquity qDA detector

Columns

During the development of the method, four columns were tested: Phenomenex Kinetex C18 (100×2.1 mm, 5 μm), Arion® plus C18 (150×2.1 mm, 3 μm), Waters Nova-pak C18 (150×2.1 mm, 4 μm), and Acquity UPLC C18 (50×2.1 mm, 1.7 μm).

Mobile phases

7 different mobile phases were tested in either isocratic or gradient elution. Tested mobile phases were always composed of water, organic solvent (methanol or acetonitrile) and additives of different pH:

A. H_2O + 0.016 % triethylamine (TEA) (pH 4.2)
B. ACN + 0.016 % triethylamine (TEA) (pH 4.2)

C. MeOH + 0.016 % triethylamine (TEA) (pH 4.2)
D. 2mM ammonium acetate in 5% ACN + 0.1% HCOOH (pH 6.7)
E. 2mM ammonium acetate in 95% ACN + 0.1% HCOOH (pH 8.9)
F. 2mM ammonium acetate in 5% MeOH + 0.1% HCOOH
G. 2mM ammonium acetate in 5% MeOH + 0.1% HCOOH

Results

Three different HPLC and UPLC systems were tested. The systems which were using UV detection did not provide the necessary selectivity and sensitivity for the determination of chosen benzodiazepines which were structurally similar. For these reasons the final method was developed and validated on UPLC system with mass spectrometry detection (Acquity UPLC system from Waters with Acquity qDA detector).
The final analytical method was performed on an Acquity UPLC BEH C18 (50×2.1 mm column with a particle size of 1.7 µm from Waters. Mobile phase D:E was chosen as the mobile phase. The mobile phase flow rate was set at 0.5 ml/min in this gradient: 0 min, 20% E; 4.10 min, 20% E; 7 min, 50% E; 7.50 min, 60% E; 7.6 min, 100% E, 9 min, 100% E; 9.10 min, 20% E; 11 min, 20% E. The column thermostat temperature was 50 °C and the autosampler temperature was 10 °C. Injected sample volume was 10 µl.
This method was successfully applied to the determination of benzodiazepines in human serum after protein precipitation (established method at the department of clinical pharmacology). Resulting *LOQs* were 1 ng/ml for all benzodiazepines except the clobazam metabolite, which had *LOQ* = 5 ng/ml.

Conclusion

New UPLC/MS method was developed for the determination of seven benzodiazepines in human serum. The method was validated according to the biomedical method validation guidelines criteria of the U.S. Food and Drug Administration. This method is now routinely used at the department of clinical pharmacology at the University hospital Ostrava.

Acknowledgements

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In vitro pea seed feeding and analysis of labelled metabolites in monolignol pathway

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Introduction

Pea is an important agricultural crop with high content of secondary metabolites (SM). SM, such as phenolic and flavonoid metabolites, have a crucial role in seed protection against pathogens and abiotic external factors. Nevertheless, these compounds usually deteriorate the palatability and processing of the seeds. Presence of SM was reduced during seed domestication. Divergent gene expression (specifically oxidoreductase genes) and secondary metabolite profile in domesticated seeds was observed in our previous research [1,2]. Profiling of monolignol pathway metabolites and investigation of metabolite changes after seed feeding by labeled (D7) cinnamic acid (CA) by UHPLC/HRTMS was performed in this study.

Experimental

Pea seeds (wild – pigmented JI64, domesticated – pigmented ATC7025 and domesticated – non-pigmented Cameor) were *in vitro* incubated in Murashige-Skoog medium (5 mL) with/without substrate (1 mM, CA:CA-D7, 1:1) on Petri dish for 24- or 48-hours. After incubation, pea seed coats were manually isolated, frozen in liquid nitrogen and ground to powder using a ball mill. Powder sample (10 mg) was weighed into vials and extracted (1.5 mL, water:acetone, 30:70, v/v). Mixture was ultrasonicated for one hour and shaked overnight. Extracts were centrifugated (1400 rpm) for 2 minutes. Supernatants were dried by a fine stream of nitrogen and obtained residues were dissolved in mixture of mobile phases (200 µL, A:B, 1:1, v/v). Prepared samples were separated by UHPLC (ACQUITY, Waters) with MS detection (Select Series Cyclic IMS, Waters) in negative ionization mode. Separation of analytes was realized using ZORBAX Eclipse Plus C18 column (Agilent) thermostated at 30 °C. Mobile phase A consisted of water with 0.01% formic acid and mobile phase B consisted of acetonitrile with 0.01% formic acid. The flow rate of mobile phase was set to 0.500 mL/min, time of analysis 10 minutes and parameters of linear gradient elution were as follows: 0.1 % of mobile phase B in initial time and 100 % B in the time 9.00. Parameters of mass spectrometer were: spray voltage 2.5 kV, cone voltage 25 V, desolvation gas flow 600 L/hod, desolvation gas temperature 220°C.

Results

Presence of CA and labeled (D7) CA in pea seeds was confirmed by the presence of peak with retention time 3.77 minutes. Chromatograms of control seeds extracts (incubated in medium without CA) and seeds incubated with CA for 24- and 48-hours are presented in Figure 1. Relative contents, i.e. normalized intensities (NI) of other labeled metabolites from monolignol pathway compared among samples. Comparison of NI of newly synthesized coumaric, caffeic, ferulic acid and 5-hydroxyconiferol and their labeled forms in both studied feeding times for wild and domesticated peas is shown in Figure 2. Relative content of selected metabolites and their labeled forms was increased due to seed incubation with substrate. In the same time,

synthesis of 5- hydroxyconiferol was observed only in pigmented seeds with activated oxidoreductase genes. Significant differences in relative contents of these metabolites among wild and cultivated seeds were observed as well.

Conclusion

Increasing of several monolignol pathway metabolites content was observed after seeds incubation with mixture CA and labeled CA in comparison with control seeds. Different relative contents of synthesized labeled metabolites were observed in wild and domesticated seeds after 24- and 48-hours incubation. Further role of synthetized labeled structures in monolignol pathway will be studied in future using seed incubation for longer times with substrate. Moreover, different synthesis mechanism in monolignol pathway could be observed with respect to activated oxidoreductase genes.

Acknowledgements

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Figure 1. Chromatograms of control seed and seeds incubated with CA/CA (D7) for 24 and 48 hours (peak at 3.77 min corresponds with CA/CA (D7)).

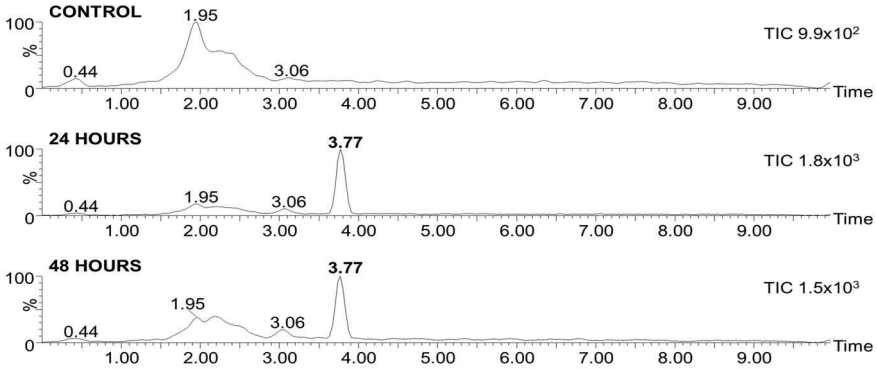
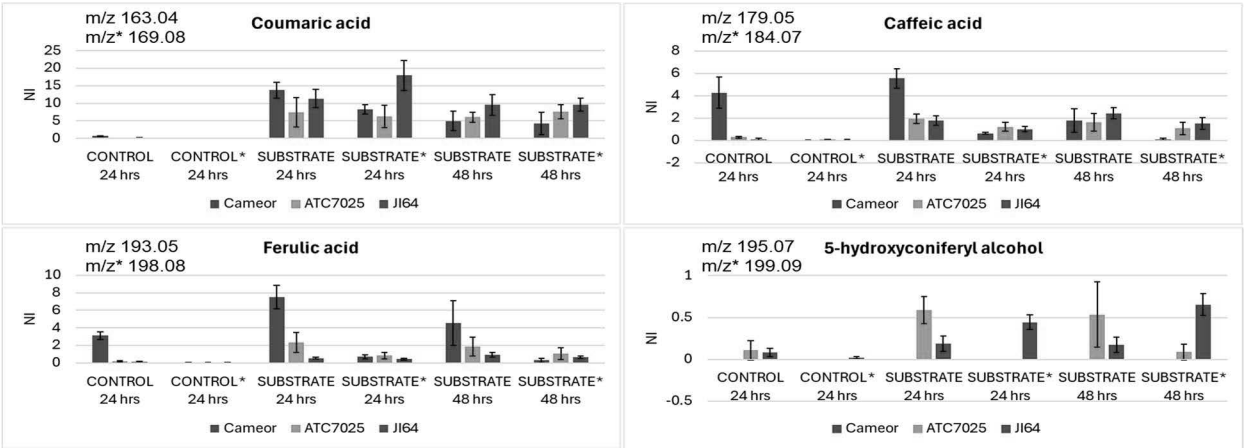


Figure 2. Relative content of coumaric acid, caffeic acid, ferulic acid and 5- hydroxyconiferol in studied samples. (* labeled form of metabolite)



Optimization of saliva sampling methods for the analysis of bile acids by HPLC-MS

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Introduction

Bile acids (BA) are natural compounds that play a critical role in digestion and absorption of dietary lipids in the intestine. BA were recognized to cause damage and irreversible changes to esophageal mucosa during reflux episodes in patients suffering from duodenogastroesophageal reflux (i.e., refluxate contains both the gastric acid and the bile acids). These changes may lead to the development of Barrett's esophagus (BE), a condition that can eventually progress to an esophageal carcinoma (EC). An early detection of susceptibility towards BE/EC is thus highly important and BA may play a crucial role. Saliva is a promising noninvasive sample that could be used for such diagnosis [1-2]. Saliva can be obtained by various sampling systems that are commercially available, but the simplest way is just spitting into a plastic container. In this work, we have systematically compared BA extraction and recoveries from three sampling systems, that is: simple spitting, Salivette and Salivette Cortisol.

Experimental

Saliva was collected by three different methods. Method 1 consisted of simple spitting into a plastic tube. Subjects were asked to simply spit into the 50 mL falcon tube for 5-10 min, until approximately 3-5 ml of saliva was collected. Method 2 and 3 consisted of using the Salivette® saliva collection system (Sarstedt, Nümbrecht, Germany). The sampling consisted of inserting a Salivette (Method 2) or Salivette Cortisol (Method 3) into the subject's mouth without chewing and keeping it there for 3 minutes to obtain a sufficient amount of saliva (at least 1 mL) for further processing. The swab was then placed into the insert of the tube and centrifuged for 5 min (3461 g). Bile acids were analyzed on the Agilent 1290 Infinity II UHPLC instrument coupled to the Agilent 6470 triple quadrupole mass spectrometer equipped with the Agilent Jet Stream electrospray ionization (ESI) source (Agilent Technologies). Chromatographic separations were performed on the Zorbax RRHD Eclipse Plus C18 column (3 × 50 mm, 1.8 µm, Agilent Technologies) maintained at 40°C with the 1290 Infinity II inline filter (0.3 µm, Agilent Technologies) in gradient elution of water (the mobile phase A) and methanol (the mobile phase B), both containing 0.1% formic acid.

Results

In this work we have optimized saliva sampling strategies for the analysis of bile acids. Three sampling methods were compared, i.e. sampling with Salivette sampler, Salivette Cortisol

sampler and simple spitting. The samples from 3 volunteers were sampled using the above methods and analyzed by HPLC-MS. The results showed a clear advantage of simple spitting compared to the Salivette samplers, as several bile acids could not be recovered from the Salivette sampler material. This was also verified on standard solution containing all bile acids at three concentration levels (0.5, 2 and 10 nmol/L) that were processed the same way as the saliva samples. We have also studied the stability of bile acids in saliva over a period of 14 days that is typical for sample collection and analysis. For this study a pooled saliva sample was obtained that contained all bile acids naturally. All studied bile acids were stable within the time period and the decrease in concentration of no more than 20% was observed which was within the RSD of the analysis and no decrease trend was observed.

Conclusion

There are different ways to collect saliva samples. Although it might seem intuitive to use the commercially available sampling systems, one must always be aware of the properties of the analytes and their compatibility with the sampling systems. For analysis of bile acids, the commercial Salivette samplers were deemed unsuitable, because several bile acids were retained on the sampler material and could not be recovered. Thus, simple spitting proved to be the most efficient way of saliva collection for our purpose, although it may not be the most comfortable collection approach for the patients.

Acknowledgements

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Ion-exchange chromatography of anions in environmental analysis

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Introduction

An ion-exchange chromatography (IEC) shares numerous common features with HPLC while possesses some new aspects such as separation principle and detection modes, mostly electrochemical. It is adapted to the separation of ions and ionizable polar molecules which is based on their affinity to the ion exchanger resin (stationary phase, SP) in presence of mobile phase (MP) consisting of an aqueous ionic medium. The separation of anions is taking place on positively charged anionic-exchanger phase where the process could be tuned by chemical composition and pH of mobile phase. This is its greatest utility for which there are no other rapid analytical methods. In this contribution, we will present the possibility of IEC application for development of new methods for anion analysis in environmental water samples.

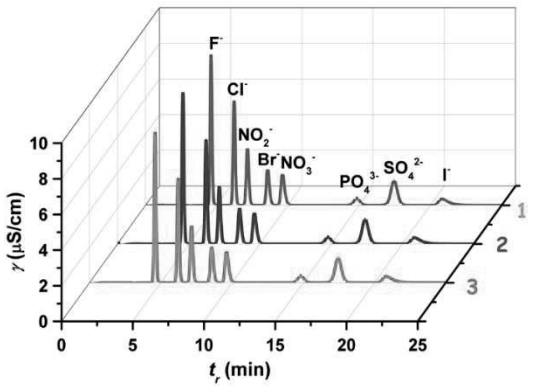
Experimental

The anion analysis was carried out on IEC instrumentation Metrohm 861 AdvancedCompact IC (Metrohm, Switzerland) using separation (Metrosep A Supp 5–250/4.0) and guard (Metrosep A Supp Guard/4.0) columns or Dionex ICS-5000+ (Thermo Fisher Scientific, USA) using Dionex IonPac (AG20, 2 × 50 mm, AS20 2 × 250 mm) columns. In both cases, the conductivity detector in suppressor mode was used. The pH of mobile phase was adjusted in region 11.0-13.5 by sodium/potassium hydroxide or 9-11 (bicarbonate/carbonate buffer).

Results

Firstly, the experimental conditions (pH, temperature, anion, OH^-/carbonate concentration in MP) for IEC separation of anions (example of chromatogram - see Fig. 1) have been optimized. The IEC analysis of anions is highly reproducible since their retention times are almost constant which ensures a high predictability of their retention behavior.

Figure 1. Chromatogram of sample containing some anions (c = 5 mg l^{-1}, c_{MF} = 3.5 mM, F = 1 ml min^{-1}, t = 22 ºC, pH = 10.22, t = 22 °C).



Anions could be divided into two groups. On contrary of the first group (fluoride, chloride, bromide, nitrate, nitrite), the retention behavior of the second group (sulphate, phosphate, iodide) is significantly dependent on experimental conditions and their optimization shortens the time of analysis which can be utilized for qualitative and quantitative analysis of some anionic pollutants, e.g. perchlorate, nitrate/nitrite, phosphate. The optimized experimental conditions were applied for anion IEC analysis of water samples (drinking/mineral water) when the concentrations of Ca(II) and Mg(II) ions present in mineral water could be simultaneously estimated after their transformation to negatively charged complexes. The analytical procedure could be also applied for solvent-extraction study of supramolecular complex anion@Dodecabenzylbambus[6]uril which selectively binds some anions, e.g. iodide, perchlorate, nitrate [2].

Conclusion

The IEC exhibits the possibility to detect and determine the concentrations of anions in mixture under experimental conditions which have been optimized to decrease the limit of detection (in micromolar scale) and the time of analysis. The developed procedure was then successfully applied to the analysis of real water samples containing common anions as well as pollutants (perchlorate, nitrate, phosphate). In addition, the solvent-extraction of new anion receptor based on bambus[6]uril could be studied by means of IEC.

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Enantioselective separation of piperazine derivatives via capillary electrophoresis with sulfated β-cyclodextrin in methanolic background electrolytes

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Introduction

Chiral drugs' enantiomers, while alike in physical and chemical properties within an achiral environment, can elicit vastly different biological responses. Thus, developing highly efficient separation techniques capable of discerning optical isomers is paramount in pharmaceutical chemistry. This becomes increasingly crucial given the growing abundance of chiral pharmaceutical products [1]. Charged cyclodextrins (CDs) modified with derivatized groups facilitate the separation of uncharged enantiomers by leveraging their inherent self-mobility. Furthermore, these derivatives offer advantages for charged analytes by forming robust ionic interactions with the modified CDs. Common derivatives such as sulfated, carboxymethylated, and sulfobutylated variants are readily accessible for use in such applications [2]. Organic modifiers play a crucial role in optimizing chiral resolution by affecting factors such as electroosmotic flow (EOF), the formation of diastereomeric complexes, and interactions with the capillary wall. Consequently, integrating additives with chiral selectors has garnered significant interest in numerous studies. However, implementing these systems often presents a challenge, requiring careful adjustment of experimental conditions to achieve the desired enantiomeric separation [3]. This research aims to develop a novel capillary electrophoresis (CE) method for effectively separating three distinct types of H1-antihistamines: chlorcyclizine (CCZ), norchlorcyclizine (NCCZ), and neobenodine (NEB).

Experimental

The background electrolyte (BGE) was formulated using a 100-mM phosphate solution, with pH adjustments made using 50% (w/w) NaOH in Milli-Q water. Sulfated β-cyclodextrin (S-β-CD) was dissolved and an organic modifier (v/v) was added in this solution to prepare the BGE. To avoid undesirable compositional changes, BGEs were prepared daily due to MeOH's low boiling point and CD's limited hydrolytic stability. All BGE solutions were vortexed for 2 minutes to ensure homogeneity. Racemic drugs (1.0 mg mL^{-1}) were dissolved in MeOH and diluted with water (50/50 v/v, 0.5 mg mL^{-1}) 5 minutes before each experiment. A new capillary was conditioned before use. It was treated with 0.1 M NaOH for 20 minutes, then rinsed with Milli-Q water for 10 minutes and MeOH for 5 minutes, all at 935 mbar pressure. At the start of each working day, the capillary was thoroughly flushed at 935 mbar with Milli-Q water, 0.1 M NaOH, Milli-Q water, 0.1 M HCl, Milli-Q water, phosphate buffer solution (pH 6.0), and MeOH for 5 minutes. Between runs, preconditioning steps included a 2-minute wash with Milli-Q water and 0.1 M NaOH, followed by a 3-minute cycle with MeOH/phosphate buffer (50/50 v/v). Electrophoretic conditions were consistent across all buffers: the temperature at 25°C, detection wavelength at 200 nm with a reference wavelength of 360 nm, an applied voltage of +8 kV, and a 10-second hydrodynamic injection at 20 mbar.

Results

In this study, we aimed to develop a novel CE method for the chiral separation of three H1-antihistamines (CCZ, NCCZ, and NEB) using S-β-CD as the chiral selector and an organic modifier in the BGE. We investigated the effect of buffer pH and S-β-CD concentration on enantiomeric resolution, finding that 100 mM phosphate buffer at pH 6.0 and 34 mg mL^{-1} S-β-CD provided optimal separation. By introducing MeOH into the buffer, we enhanced chiral resolution, with 40% (v/v) MeOH identified as the optimal concentration. Additionally, we explored the impact of voltage and buffer pH on resolution, determining that 8 kV and pH 6.0 were optimal, respectively. Lastly, we examined the effect of MeOH content in the sampling solution, finding that 50% (v/v) MeOH yielded the best resolution for all compounds. The optimized CE method was validated with 100 mM phosphate buffer (pH 6.0), 34 mg mL^{-1} S-β-CD, and 40% (v/v) MeOH, applying 8 kV voltage and analyte injection in a 50% (v/v) MeOH–water system. Calibration curves showed excellent linearity ($R^2 > 0.99$) across the 20-500 μmol L^{-1} concentration ranges for NCCZ and NEB and 10–500 μmol L^{-1} for CCZ. Intraday precision (0.38%–1.37% for migration time, 0.36%–2.79% for peak area) and inter-day precision (2.57%–6.60% for migration time, 2.42%–6.76% for peak area) demonstrated adequate repeatability. LODs (S/N = 3.3) and LOQs (S/N = 10) ranged from 5.9 to 11.4 μmol L^{-1} for LOD and 18 to 34.6 μmol L^{-1} for LOQ. At the same time, accuracy fell within the 93%-108% range across three concentration levels, meeting pharmaceutical quality control standards.

Conclusion

This study presents the development and validation of a CE method for separating three H1-antihistamine drugs: CCZ, NCCZ, and NEB. Optimal conditions, including a 100 mM phosphate buffer at pH 6.0 with 34 mg mL−1 S-β-CD and 40% (v/v) MeOH, yielded excellent enantioseparations. The method showed linearity in calibration curves, consistent intra- and inter-day precision, and suitable LODs and LOQs for pharmaceutical analysis.

Acknowledgments

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Desorption electrospray ionization coupled to cyclic ion mobility in analysis of new psychoactive substances

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Introduction

New psychoactive substances (NPS) are the wide group of abused drugs (e.g., synthetic cathinones, synthetic cannabinoids, piperazines, etc.).They are produced to mimic known narcotics such as LSD, cocaine, or cannabis. The potency of some analogs is much higher than the drugs they try to substitute[1]. Consequently, the production of new NPS increases the demand for fast, selective, sensitive, and user-friendly techniques. Despite their limitations, ambient ionization mass spectrometry (AIMS) techniques have such qualities. Coupling ion mobility-mass spectrometry separation (IM-MS) with desorption electrospray ionization increases the selectivity and applicability in separating isomers[2–4]. Nevertheless, there are limited applications of IM-MS and IM-IM coupled with AIMS in the analysis of NPS. In this work, we used DESI with cyclic traveling wave ion mobility-mass spectrometry (DESI-cIM-MS) to separate and determine the isomeric ratios of NPS in a mixture by utilizing the characteristic arrival time distribution (ATD) profiles.

Experimental

Stock and working solutions of individual analytes were prepared at the concentrations 1 mg/mL and 100 ng/mL, respectively, in methanol/water (50/50, v/v). Three mixtures of isomers with a total concentration of 100 ng/mL (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). Isomers were mixed in the ratios: 5:95, 10:90, 25:75, 40:60, 50:50, 60:40, 75:25, 90:10, 95:5. Three isomeric pairs were deposited onto the Omni Slide Hydrophobic Arrays at 3.5 ng/mm^2 (Prosolia, Waters Corp., Wilmslow, UK). Analysis was performed on Select Series Cyclic IMS equipped with a 2D-DESI ionization source (Prosolia, Waters Corporation, Wilmslow, UK)[5]. The original sprayer was replaced with a DESI XS sprayer (Waters Corporation, Wilmslow, UK). Methanol/water (80/20, v/v) was used as the spray liquid at a flow rate of 2.0 µL/min. The capillary voltage was maintained at 0.75 kV, nebulizing gas pressure 11 psi, source temperature 150 °C, cone voltage 10 V. The DESI geometry was as follows: spray impact angle ~75°, spray nozzle – inlet tube orifice ~4 mm, inlet tube orifice - sample surface ~0.5 mm, spray nozzle – sample surface ~2 mm**.** cTWIM parameters were as followed: 1 TOF push per bin, TW static height 15 V, array TW velocity 375 m/s, wave amplitude 15 V. Data were acquired, processed, and evaluated using Masslynx v.4.2 (Software Change Note 1016, Waters Corp., Wilmslow, UK), a modified version of Driftscope v.2.9 (Waters Corp.), and statistics software OriginPro 2020 (OriginLab, Northampton, USA).

Results

Before the DESI, all pairs were analyzed by electrospray ionization. In comparison, DESI generated protonated molecules with lower intensities, but produced the characteristic ATD profiles, although not exactly of the same shapes as ESI. While the profiles were similar for 3-FMC/4-FMC, they were different for 3-MMC/buphedrone. This might be due to the difference in ESI and DESI ionization processes. It was, therefore, not possible to use the ATD profiles obtained by ESI to evaluate the samples measured by DESI. Seven-pass experiments (686 cm) were used for 3-MMC/buphedrone and 10-pass ones (980 cm) for 3-FMC/4-FMC. Since the high resolving power of cIM-MS was insufficient for achieving the required separation of isomers in the mixture, we used characteristic ATD profiles of individual isomers to determine their ratios. We applied multiple linear regression (MLR), which is simpler than the previous fitting procedure using the Gaussian function[6]. Extracted ATD profiles of the pure isomers (intensities x_1 and x_2) were used as the input data (independent variables). The isomeric ratio was $a_1 : a_2$, where $y = a_0 + a_1x_1 + a_2x_2$. The relationship between the given and determined ratios showed good linearity for protonated ions of both pairs, 3-MMC/buphedrone and 3-FMC/4-FMC (COD = 0.9676 and 0.9911, respectively), although the lower coefficient of determination and higher variability of the slope and intercept were observed compared to ESI. The third pair, BDB/methedrone, showed significant differences in protonated molecules' intensities due to BDB fragmentation. Both isomers produced symmetrical overlapped mobility peaks. We proposed an alternative approach using ATD profiles of fragment ions. Protonated molecules were fragmented in the trap producing fragment ions at *m/z* 135.04 and for methedrone also at *m/z* 135.08. Both fragments were included in extracted ATD profiles that were characteristic of individual isomers. The relationship between the determined and given content of BDB showed good linearity with COD 0.9914.

Conclusion

The determination of isomeric ratios in solid samples of NPS was performed by DESI-cIM-MS. Although the ATD profiles of the isomers overlapped significantly even at the higher resolving power of cTWIM, they were characteristic of individual isomers, as the profiles observed using ESI-cIM-MS. This work widens the application of DESI-cIM-MS in the analysis of NPS.

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Off-line microelution SPE as a sample pretreatment step in quantitation of intact proteins in biological fluids using CZE-MS

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Introduction

Protein analysis in biological samples is one of the key areas of biomedical research. To miniaturize the entire analytical process and lower its environmental impact, attention is currently being paid to the development of new greener approaches and techniques aimed at the targeted determination of intact proteins, in contrast to the traditionally used approaches for protein analysis. In this field, capillary electrophoresis is becoming more popular and meets the criteria for greener techniques [1,2]. When combined with mass spectrometry (MS), it can compete with established chromatographic techniques in terms of performance and meets the requirements to become a routine part of practice [3,4]. However, when it comes to the analysis of biological matrices, its reliable application requires a comprehensive optimization of the separation and detection conditions in addition to the implementation of effective preconcentration techniques and pretreatment procedures [5,6]. In this work, we focused on the development of an on-line hyphenated capillary zone electrophoresis-mass spectrometry method (CZE-MS) employing off-line microelution solid-phase extraction (μSPE) as a sample pretreatment step for the quantitation of multiple intact proteins (<20 kDa) in various biological fluids (human serum, plasma, urine, and saliva).

Experimental

All experiments were performed on a capillary electrophoresis instrument Agilent 7100 CE System coupled to mass spectrometer Agilent 6410 Series Triple Quadrupole using standard electrospray ionization interface (ESI). The analyses were carried out in uncoated bare fused silica capillary with 50 μm i.d. and 85 cm in total length. The separation voltage was set at 20 kV and as an optimum background electrolyte (BGE) was selected 500 mmol/L formic acid with an addition of 5% acetonitrile. The CZE-ESI-MS connection was established using coaxial sheath liquid interface. The sheath liquid was composed of 50/50 (v/v) MeOH/water with an addition of 0.1% formic acid and delivered at the flow rate of 8 μL/min. The pressure of the nebulizing gas (nitrogen) was kept on 10 psi. Drying gas was delivered at the flow rate of 10 L/min and its temperature was 300°C. The voltage on the ESI tip was 4500 V.

In-capillary preconcentration based on transient isotachophoresis (tITP) was performed using 200 mM ammonium formate (pH 4.0) added to the sample matrix as leading electrolyte and optimized BGE as terminating electrolyte. Sample was introduced to the capillary using hydrodynamic injection for 250s.

The Oasis Hydrophilic Lipophilic Balance® (HLB) μElution 96-well sample plate and a 96-well plate manifold (Waters Corporation, Milford, MA, USA) were used for μSPE extraction.

Results

19- to 127-fold increase in signal intensity was achieved by employing transient isotachophoresis (tITP) as an in-capillary preconcentration method. Off-line µSPE with various eluate treatment procedures was evaluated to ensure the compatibility of the sample pretreatment method with the selected in-capillary preconcentration, separation, and detection process. Achieved extraction recoveries of spiked proteins were in the range of 76–100 % for urine, 12–54 % for serum, 21–106 % for plasma, and 25–98 % for saliva when the eluate was evaporated and reconstituted into the solution of the leading electrolyte to achieve the tITP process. Simple dilution of the eluate and the lyophilization proved not to be suitable for the eluate treatment. Validation of the optimized pretreatment protocol was performed on spiked pooled biological matrices (human serum, plasma, urine, and saliva) offering good linearity (r^2 0.9802–0.9996), precision and accuracy. In all biological matrices, the accuracy ranged from –22.9 % to +27.4 %. The data also showed a rather high degree of precision with %RSD ranging from 0.37 % to 22.0 %. For some proteins, precision and accuracy data were slightly higher than the required <15 % for %RSD and %RE as stated in validation guidelines [7]. However, those could be improved in future studies by using appropriate internal standards.

Conclusion

The results showed the potential to use the developed tITP-CZE-ESI-MS method for the quantitation of small molecular mass proteins in less complex biological matrices, such as saliva and urine. More complex biological matrices, such as serum and plasma, would need a more selective sample pretreatment protocol, e.g., based on (immuno)affinity depletion of high abundance proteins. The limits of quantitation of the developed method were in hundreds of ng/mL and therefore, the developed method can be employed for targeting intact proteins present at elevated concentration levels.

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Quantification study of selected polyphenols during pea seed maturation

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Introduction

Pea is an important agricultural crop that has been cultivated by humans for centuries. Various pea seeds differ in pigmentation of seed coat which is closely related to seed dormancy. In the population of dormant seeds only few individuals germinate under suitable conditions (humidity, temperature etc.). Polyphenols (e.g. flavones and anthocyanins) are common natural dyes and their representation has effect on pigmentation. In this study, quantification of selected polyphenols was realized (apigenin, delphinidin, delphinidin-3-galactoside, cyanidin and pelargonidin) in three developmental stages, DS, (17 DAP*, 23 DAP* and mature) of various pea seeds. Study of polyphenol content and its changes during seed maturation could provide new knowledge of seed coat development, seed maturation and pigmentation. Ultra-High Performance Liquid Chromatography / Mass Spectrometry (UHPLC/MS) was used for polyphenol quantification. Presented study follows our previous research of pea seed dormancy and pea seed coat composition (metabolite profiling) [1–3].
*DAP – days after pollination

Experimental

Four different genotypes of pea seeds were chosen for study (dormant - pigmented JI64, non-dormant - pigmented JI92 and JI1794 and non-dormant - non-pigmented Cameor). Pea seed coats were manually isolated and were ground to powder using a ball mill. Powder sample (5 mg) was weighed into a plastic microtube and extraction solution was added (1 mL, water:methanol:ethanol:acetone, 5:2:2:10, v/v/v/v). Mixture was placed in ultrasonic bath for one hour. Extracts were purified using Strata SDB-L columns (Phenomenex) after sonication. Purified extracts were dried by a fine stream of nitrogen and residues were dissolved in a mixture of mobile phases (200 µL, A:B, 1:1, v/v). Prepared samples were analyzed by UHPLC (ACQUITY, Waters) / electrospray ionization MS (Select Series Cyclic IMS, Waters). Separation of analytes was performed using Raptor ARC-18 2.7 µm column (Restek) tempered at 30 °C. Mobile phase A consisted of water with 0.1% formic acid (v/v) and mobile phase B consisted of methanol with 0.1% formic acid (v/v). The flow rate of mobile phase was set to 0.200 mL/min and parameters of gradient elution are described in Table 1.

Results

Quantification of selected polyphenols was realized using calibration curves of polyphenols standards. Comparison of the content of studied polyphenols in different DS of studied pea genotypes is shown on Figure 1. In general, the studied compounds were the most represented in developmental stage 23 DAP and least in the stage 17 DAP. Genotype Cameor had the lowest content of polyphenols which is related to its minimal pigmentation and non-dormant character.

Delphinidin was the most represented polyphenolic compound in all studied samples while its glycoside was represented the least and was not present in any stage 17 DAP. At the same time, decrease of delphinidin and increase of its glycoside were observed during maturation of non-dormant seeds while presence of delphinidin-3-galactoside was not confirmed for dormant JI64 genotype.

Conclusion

Quantification of selected polyphenols in various pea seed coat extracts was realized by UHPLC/MS technique. Differences in polyphenol content were studied during pea seed maturation and among pea genotypes. Biosynthesis of polyphenol glycosides were observed during seed maturation. Studied polyphenols clearly contribute to seed coat pigmentation and their connection to seed dormancy is the subject of present research.

Acknowledgements

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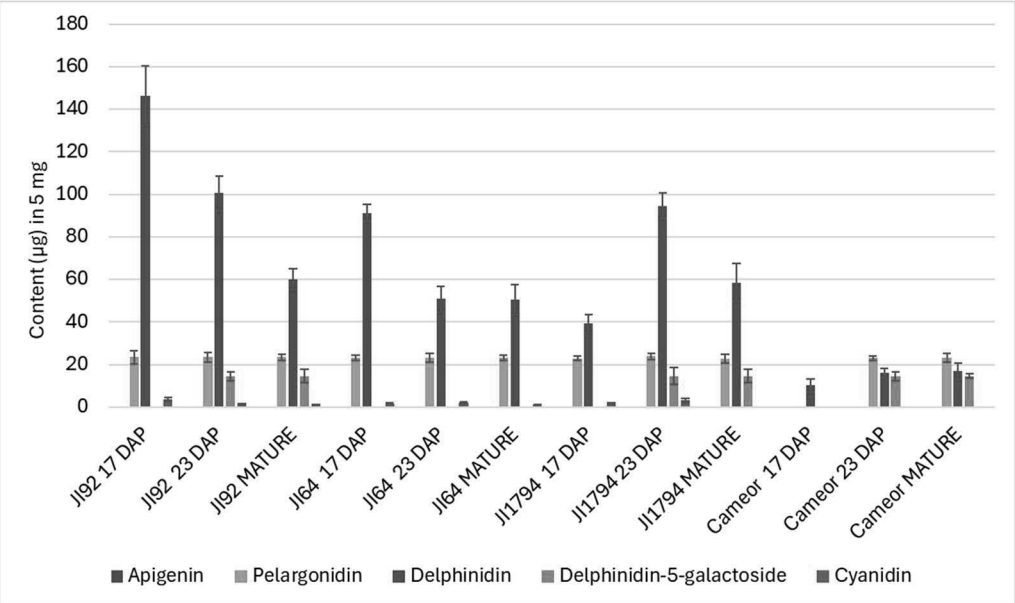
Table 1. Parameters of gradient elution for UHPLC/MS method.

Time (min)	Mobile phase A (%)	Mobile phase B (%)
Initial	99	1
14.00	0	100
14.05	99	1

Figure 1. Content of selected polyphenols in developmental stages of studied pea genotypes.



Cardiovascular event risk assessment using the CERT score

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Lipids are generally associated with the development and pathology of atherosclerotic cardiovascular disease. Based on the 2019 recommendations of the European Society of Cardiology and the European Atherosclerosis Society, the SCORE (Systematic Coronary Risk Evaluation) metric is recommended as a baseline assessment of these diseases. SCORE is based on a combination of factors such as age, sex, smoking, blood pressure and total cholesterol. However, not only cholesterol and triacylglycerols, but also ceramides and phosphatidylcholines, which are associated with myocardial infarctions, ischaemic heart disease, stroke and increased mortality in general, play a major role in these inflammatory diseases.

To create a simple score for predicting these cardiovascular events, measured ceramide and phosphatidylcholine concentrations were combined with advanced statistical models. Among these developed scores, CERAM (Mayo Clinic, USA), CERT1 and CERT2 (Zora Biosciences, Finland) have great potential. CERAM and CERT1 include the calculation of this score only in the context of ceramide concentration, whereas CERT2 also takes into account phosphatidylcholine concentration. In the present study, serum concentrations of selected ceramides and phosphatidylcholines were measured in a cohort of patients from the 1st Internal Medicine Clinic, University Hospital Olomouc.

For this purpose, the LC-MS/MS method was optimized for the analysis of ceramides: Cer 18:1/16:0, Cer 18:1/18:0, Cer 18:1/24:0, Cer 18:1/24:1, and phosphatidylcholines: PC 16:0/16:0, PC 14:0/22:6, PC 16:0/22:5. Analysis was performed using an Acquity BEH C18 2.1 × 75 mm × 1.7 µm column (Waters), an Exion LC HPLC instrument (SCIEX), and a QTRAP 6500+ mass spectrometer (SCIEX). Mobile phase A contained 10 mM ammonium acetate + 0.1% formic acid and mobile phase B contained 10 mM ammonium acetate in acetonitrile:2-propanol (4:3, v/v) + 0.1% formic acid. The column heating was set at 60 °C, the flow rate at 0.5 ml/min, sample injection at 0.5 µl and analysis time at 5 min. Concentrations of analytes were calculated based on their respective stable isotope-labeled internal standards. Subsequently, the CERT score and its quartiles were calculated, and risk groups were determined according to these results. In addition, these results were subjected to correlation analysis with routine biochemical parameters.

The results show that this rapid quantitative analysis and the subsequent calculation of the CERT score have great potential for cardiovascular event prediction and prevention.

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Verification of the microfluidic device design for single cell detection and isolation using FEM and SPICE analysis

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Introduction

Despite the enormous activity in the fields of microfluidics-based cytometric and cell sorting equipment, there remains a pressing need for a budget-saving accessory instrument that can be attached to commercial mass spectrometers. In response to this need, a prototype of the aforementioned instrument is being developed with a microfluidic device (MFD) as the core component.

The presented results demonstrate the outcomes of *in silico* verification of the MFD, comprising features of 2D flow focusing, single-cell detection, impedance characterization, and diverting a cell into the sample loop (see Fig. 1). Extensive simulations employing the Finite Element Method (FEM) and the Simulation Program with Integrated Circuit Emphasis (SPICE) were undertaken utilizing Comsol Multiphysics 5.3 and LTSpice XVII software, respectively. These simulations were aimed to: (1) facilitate the adaptation of microfluidic components design to specific applications and anticipated manufacturing technology; (2) empower the design of optimal configurations of electrodes for source-differential flow cytometry (SDFC) [1]; (3) verify the functionality of optimized components within the MFD, thereby ensuring its anticipated operation under real-world conditions.

Experimental

Two initial designs of microfluidic components (2D focuser [2] and hydrodynamic sorter [3]) were taken from the literature, while the others (interior of flanged membrane valve, electrode assemblies, channel width reducer, channel elbow) were developed by the authors. All components and electrode assemblies were designed in SolidWorks 18 CAD software, with the following import of 3D models into the Comsol project. Afterward steps involved the use of the *creeping flow interface (fpt)*, and the particle tracing for *fluid flow interface (ptt).* As the *Euglena spp.* are foreseen as single-cell organisms for proof-of-concept experiments, physical parameters of the modified Watanabe medium [4] were used in the simulations. Similarly, the average diameter and density of the *Euglena viridis* cell were used as the particle parameters in the particle tracing simulations. An electrical simulation of detection and SDFC electrode assemblies was conducted using the *electric currents interface (ec)*. The electric parameters of the electrode-facing materials (SU-8 and FR-4) were obtained from the COMSOL material library. A simulation of the equivalent circuit, comprising the SDFC electrode assembly, differential voltage source, and electrode-liquid-cell interface, was conducted using the *.tran()* and *.ac lin()* directives of the LTSpice software. The reference parameters for resistance and capacitance were taken from [1].

Results

Three datasets have been obtained for each microfluidic component, each comprising maps of Reinold's number, velocity, and pressure profiles of the liquid flow within the component,

along with particle trajectories, velocities, and transfer probability. The first dataset refers to the initial design, providing conclusions on component operability, and its performance in a variety of regimes. Furthermore, this dataset was employed as the basis for subsequent optimization. The second dataset comprises simulation outcomes of the application-specific component, with an optimized 3D model and operation regime foreseen in a real MFD. The third set of data represents an optimized component for the glass-on-PCB manufacturing technology (a modified version of microfluidics-on-PCB technology), specifically: (1) a channel width of 70 um, (2) its height equal to 50 um; (3) electrode width and interelectrode gap both equal to 70 um. Simulations conducted with the *ec* interface yielded insights into the electric field strength and current density within the interelectrode microfluidic channel, as well as the total current flowing through the current sensing output of each assembly under conditions of particle presence/absence in the channel. Finally, the equivalent circuit SPICE analysis has yielded a frequency characteristic up to 200 kHz, which was later employed as an input for the design of excitation and current acquisition circuits.

Conclusion

The utilization of Comsol Multiphysics and LTSpice software for the simulation of microfluidic flow dynamics, particle behavior, and electrical characteristics, respectively, provided invaluable insights into the performance of the developed MFD. These simulations served as a foundation for the subsequent refinement and pre-manufacture verification of its capability in single-cell detection, characterization, and isolation. This eliminated the probability of additional time being spent on debugging the design and mitigated the expenses associated with possible repeated manufacturing of the MFD.

Acknowledgements

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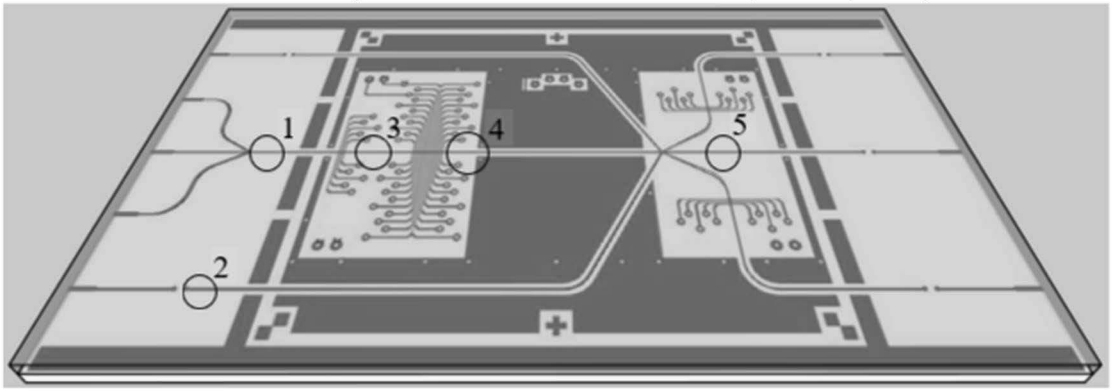
Figure 1. A simplified 3D model of the MFD (not to scale, original size 40 × 75 mm). The key components are labeled: 1 – 2D flow focuser; 2 – microfluidic valve connection port; 3 – detection electrode assembly; 4 – SDFC electrode assembly; 5 – hydrodynamic sorter.



GC/MS analysis of binders in a single microsample from František Emler painting

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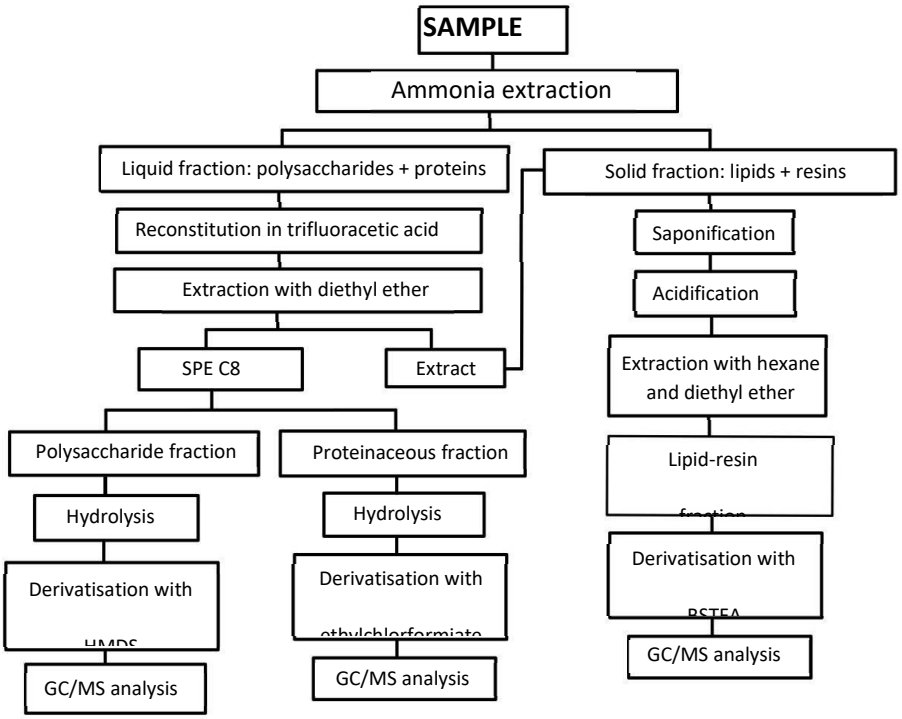
Introduction

The identification of binders in painting samples is an integral part of the analysis of objects of cultural heritage. Invasive methods provide reliable results, so a method was developed that uses gas chromatography with mass spectrometry for analysis of organic binders. The sample preparation procedure was inspired by M. P. Colombini [1,2], who uses one microsample to identify all components of organic binders. The principle of the method consists of sample preparation, which leads to the separation of three separate fractions for analysis by GC/MS. In this work, the procedure was adapted and applied for the detection of the saccharides, proteins, lipids and resins in sample from František Emler painting.

Experimental

Figure 1. Scheme of sample preparation procedure (modified from [1,2]).



The sample was processed for GC/MS analysis according to the procedure shown in Figure 1. A 0.2 mg sample was first processed by ammonia extraction and afterwards was separated into two fractions, liquid and solid. The liquid fraction which contained polysaccharides and proteins was evaporated, dissolved with 1% TFA and then extracted with diethyl ether. The

ether phase after extraction was added to the solid fraction containing lipids and resins after separation by ammonia extraction. The remaining solution in the vial was extracted by in-lab pipette tips with C8 sorbent. This step separated the polysaccharides from the proteins. Hydrolysis of the protein fraction was carried out with 6 M HCl at 120°C for 120 min, whereas hydrolysis of the polysaccharide fraction was carried out in the presence of 2 M TFA for only 20 min. After hydrolysis of both fractions, derivatization was performed. The carbohydrate fraction was derivatized with HMDS reagent, while the protein fraction was derivatized with ethyl chloroformate before injection. The lipid-resin fraction underwent several steps of treatment (saponification, acidification and extraction with hexane and diethyl ether), then was derivatized with BSTFA and after dilution with hexane applied to the GC/MS system for analysis.

Results

Changes in the procedure used by M. P. Colombini consist of several steps, namely (i) replacing the commercial OMIX C4 SPE pipette tips with "in-lab" filled C8 pipette tips, (ii) replacing the silanization of the protein fraction by derivatization with ethyl chloroformate, (iii) substitution of BSTFA with hexamethyldisilazane to increase the yield of the saccharides derivatization, (iv) addition of a second silanization step to increase the yield of the lipid-resin fraction derivatization, and (v) addition of a base catalyst in the second derivatization step of the lipid-resin fraction.

As a part of the development and optimization of the method, samples of two different paintings by unknown artist from the second part of the 20^{th} century were first analysed. The hypothesis, in this case, was that they were an oil painting and a watercolour. The primary purpose of the analysis was to confirm the effectiveness of the procedure for processing a real sample for analysis by GC/MS, namely the efficiency of SPE extraction for separation of polysaccharide and protein fractions.

After this successful analysis, the sample of the painting by František Emler was processed in the same way. The hypothesis for this sample was that the artist used oil tempera for his work. Thus, the detection of primarily saccharides and oil components was expected. Saccharides (ribitol, sorbophuranose, thalose, sorbose and dulcitol) and lipid components (behenic acid, stearic acid, linoleic acid and oleic acid) were readily detected in the sample of František Emler painting. The proteinaceous components of the organic binder were not detected in the sample. The findings confirm the hypothesis that the author of the painting used oil tempera for his work.

Conclusion

The above method for the separation and detection of organic binder components proved to be effective and confirmed the hypothesis that the author of the painting used oil tempera for his work. This approach highlights the strength of interdisciplinary collaboration between scientific disciplines and art experts, which is key to the effective utilization of analytical procedures for understanding and preserving cultural heritage for future generations.

Acknowledgements

Authors gratefully acknowledge the financial support from the UP Internal Grant Agency (projects IGA_PrF_2023_027, IGA_PrF_2024_026).

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Bridging microsampling with microextraction for doxorubicin and doxorubicinol determination

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Introduction

Anthracyclines (ANT), including doxorubicin, are potent cytostatic drugs that are utilised in the treatment of various cancers. Doxorubicin stands out as the most commonly used ANT in clinical settings and is also a typical choice for preclinical cancer studies. Among the most reliable *in vivo* models for cancer research are athymic nude mice, which allow visual evaluation of tumours [1].

Preclinical studies are increasingly prioritizing adherence to the 3R principles (refine, reduce, replace) [2]. Microsampling has emerged as a practical approach to significantly reduce the volume of blood samples required for testing, enabling repeated sampling from individual animals, particularly smaller rodents with limited blood volume. This reduction in sample volume translates to fewer animals being needed for studies. Furthermore, when microextraction techniques are employed, the entire analytical process becomes more environmentally friendly.

Volumetric absorptive microsampling (VAMS), an alternative to DBS less dependent on haematocrit, represents one of such approaches, requiring 10–30 µL of sample [3]. When coupled with electromembrane extraction (EME), it enables simultaneous desorption from the VAMS tip and extraction in a single step using only 3 µL of organic solvent, offering not only environmental benefits, but also accelerated and efficient analysis.

This work aimed to develop a method for the determination of doxorubicin (DOX) and its metabolite doxorubicinol (DOXol) in blood adsorbed on VAMS tips using EME or conventional extraction method. Compare both methods and validate the most effective one. Subsequently, the validated method was applied for the analysis of blood samples from *in vivo* studies on nude mice, collected using VAMS tips after DOX administration.

Experimental

In this study, 10 µL VAMS tips (Mitra®, Neoteryx) were employed. The development of the EME procedure involved optimalization of the composition and volume of donor and acceptor solutions, the supported liquid membrane (SLM), the voltage, extraction time, and agitation speed. Subsequently, we developed a conventional extraction method, exploring protocols for direct desorption of analytes followed by protein precipitation. A comprehensive comparison between the two methods was conducted, evaluating factors such as recovery, matrix effects, extraction time, and environmental impact.

Analysis was conducted using a UHPLC-MS/MS system, Agilent 1290 Infinity LC with Triple Quad LC/MS (6400 series), equipped with Jet Stream Electrospray and Mass Hunter software

(Agilent, Santa Clara, CA, USA). We utilized a Kinetex C18 column (100 × 2.1 mm, 1.7 µm, Phenomenex, Torrance, CA, USA), safeguarded with a precolumn. The mobile phase consisted of 0.0025% formic acid and acetonitrile with 0.0025% formic acid in a gradient profile was employed for the analysis. Isotopically labelled internal standards were used for both analytes. Before validation, essential aspects related to whole blood analysis were examined, including the impact of haematocrit, the formation of ANT-Fe complexes, and the influence of anticoagulants. Our method underwent full validation across a concentration range of 3.5–8600 nM for DOX and DOXol adhering to EMA guidelines. Subsequently, it was used for the analysis of samples from *in vivo* studies following DOX administration (5 mg/kg, i.v.) in mice.

Results

We have successfully developed an EME method coupled with UHPLC-MS/MS for the quantification of DOX and DOXol from blood samples dried on 10 µL VAMS tips. The following conditions were selected as optimal for the extraction of analytes: a donor solution of 205 µL of 0.2M formic acid, an acceptor solution of 75 µL of 0.5M acetic acid, and a supported liquid membrane (SLM) consisted of 3 µL of a 1:1 mixture of 1-undecanol and 1-ethyl-2-nitrobenzene (v:v). The extraction was carried out for 25 minutes at an agitation speed of 850 RPM, with the voltage starting at 20 V and increasing to 30 V after 1 minute. Subsequently, the conventional extraction method was developed in which VAMS tips were first extracted in 50 µL of 0.1% of formic acid in water and then the extract was precipitated with 200 µL of acetonitrile, centrifuged, evaporated, reconstituted in 50% methanol, filtrated and analysed.

Both protocols were compared across various parameters, including recovery, matrix effect, time efficiency, and environmental impact. EME stood out as the preferred option, given its notable environmental friendliness and time-saving efficiency. Pre-validation experiments confirmed that none of the tested parameters (haematocrit, formation of ANT-Fe complexes, influence of anticoagulants) significantly affected the assay. Consequently, EME was validated, meeting all the defined limits for the validation parameters. Finally, it was employed for the determination of the pharmacokinetic profile of DOX and DOXol in mice from blood dried at VAMS tips and for determination of blood/plasma ratio, demonstrating the applicability of the developed method.

Conclusion

This study effectively integrated VAMS with electromembrane microextraction. Compared to conventional extraction methods, EME demonstrated substantial time savings and environmental benefits. Through comprehensive validation and application to the analysis of *in vivo* samples, treating blood on VAMS tips with EME showed promising potential for the analysis of ANT in preclinical experiments with nude mice.

Acknowledgements

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Separation of selected catecholamines and determination of binding constants of their complexes with HS-β-CD by capillary electrophoresis

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Introduction

Catecholamines play an important role in carbohydrate and fat metabolism, cardiovascular system regulation, unstriped musculature function, blood coagulation, and the regulation of the acute adaptive reactions of the body. Analyses of the most important neurotransmitters such as dopamine, adrenaline and noradrenaline are of great significance for diagnostics and treatment of various brain diseases. The main path of formation of catecholamines in the body is: tyrosine - dihydroxyphenylalanine (DOPA) - dopamine - noradrenaline - adrenaline.
L-DOPA (levodopa) is a chiral drug used in the treatment of Parkinson's disease, which is related to the depletion of the dopamine in the brain. Only this enantiomer is converted to dopamine while D-DOPA may cause side effects. Hence, control of enantiomeric purity of L-DOPA is necessary. Capillary electrophoresis (CE) using chiral selectors, among them especially cyclodextrins, is powerful tool for separation of enantiomers [1].

Experimental

Tyramine, dopamine, noradrenaline, and adrenaline were separated as cations in several aqueous background electrolytes (BGEs), pH 2.00–8.10, in bare fused silica capillary using Beckman-Coulter MDQ analyzer equipped with diode array UV-absorption detector set at 200 nm. L-DOPA and D-DOPA enantiomers were separated in BGE composed of 22/35 mM $NaOH/H_3PO_4$, pH 2.50, containing 0–6 mM highly sulfated β-cyclodextrin (HS-β-CD) as chiral selector.

Theory

For the analyte-cyclodextrin (CD) complex with 1:1 stoichiometry ratio, the dependence of the effective mobility of analyte A, $m_{A,eff}$, on the concentration of the CD in the BGE is described [2] by the equation:

$$m_{\mathrm{A,eff}} = \frac{1}{1 + K_\mathrm{b} c_\mathrm{CD}} m^0_{\mathrm{A,eff}} + \frac{K_\mathrm{b} c_\mathrm{CD}}{1 + K_\mathrm{b} c_\mathrm{CD}} m_{\mathrm{A,CD}}$$

where $m^0_{A,eff}$ is the effective mobility of a free (non-complexed) analyte (i.e. analyte mobility in the BGE free of the CD), $m_{A,CD}$ is the ionic mobility of analyte-CD complex, A,CD. K_b is the apparent binding constant of the A,CD complex, and c_{CD} is the equilibrium concentration of the CD in the BGE. The apparent binding constant, K_b, and the ionic mobilities, $m_{A,CD}$ of the analyte-CD complexes were determined by non-linear regression analysis according to the upper equation using program OriginPro 8.5.

Results

Best resolutions between tyramine and dopamine, dopamine and noradrenaline, and noradrenaline and adrenaline peaks were achieved in BGE composed of 44/70 mM $NaOH/H_3PO_4$, pH 2.50 (see Fig. 1). From dependence of the effective electrophoretic mobilities

of L-DOPA and D-DOPA enantiomers (determined by CE at 25°C) on the concentration of HS- β-CD in BGE (see Fig. 2) the average apparent binding constants of L-DOPA and D-DOPA complexes with HS-β-CD were calculated. These complexes were found to be relatively weak with the binding constants equal to 292 L mol^{-1} and 276 L mol^{-1}, respectively.

Conclusion

Capillary electrophoresis proved to be a suitable method for achiral and chiral separations of catecholamines. The interactions of D-DOPA and L-DOPA enantiomers with HS-β-CD are relatively weak, but highly enantioselective.

Acknowledgements

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Figure 1. CE analysis of the mixture of analytes, 1-tyramine, 2-dopamine, 3-noradrenaline, 4-adrenaline, in the optimized BGE (44/70 mM $NaOH/H_3PO_4$, pH 2.50).

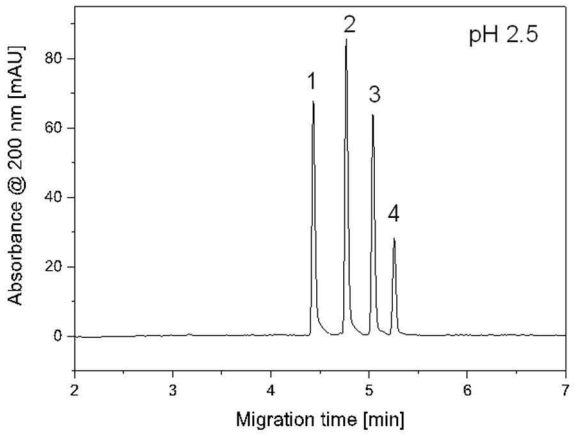

Figure 2. Dependence of the effective electrophoretic mobility, $m_{A,eff}$, of L-DOPA and D-DOPA enantiomers on the concentration of HS-β-CD, c_{CD}, in 22/35 mM $NaOH/H_3PO_4$, pH = 2.5.

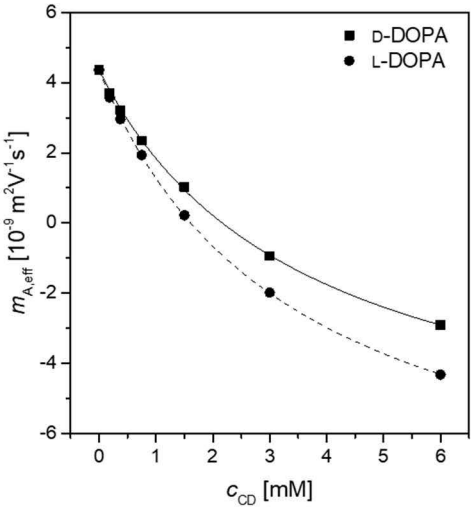

Table 1. The calculated average apparent binding constants, K_b, and the average ionic mobilities, $m_{A,CD}$, of the complexes of L-DOPA and D-DOPA enantiomers with HS-β-CD; electrophoretic mobilities of the enantiomers in the BGE free of HS-β-CD, $m^0_{A,eff}$; coefficient of determination, R^2.

Enantiomer	$K_b \pm$ SD [L mol^{-1}]	$m_{A,CD} \pm$ SD [10^{-9} m^2 V^{-1} s^{-1}]	$m^0_{A,eff} \pm$ SD [10^{-9} m^2 V^{-1} s^{-1}]	R^2
D-DOPA	276.1 ± 11.4	-7.29 ± 0.24	4.36 ± 0.12	0.999
L-DOPA	292.9 ± 7.3	-9.24 ± 0.17	4.36 ± 0.12	0.999

Application of the multi-component integrated calibration method to study the origin and concentration of polycyclic aromatic hydrocarbons in the Katowice (Poland)

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Introduction

Polycyclic aromatic hydrocarbons (PAHs) are a group of hazardous substances that are susceptible to bioaccumulation and have a long half-life. These compounds are a huge group, including several hundred PAHs. Due to metallurgical and technological development, significant increase of their presence in the environment has been observed. As a consequence, PAHs are present in various food sources and negatively impact living organisms. Study shows that these compounds have mutagenic, carcinogenic, and genotoxic properties. Additionally, it was noticed that these compounds are phototoxic. PAHs are most often determined using chromatographic techniques, such as gas chromatography (GC) or high-performance liquid chromatography (HPLC), with various detection methods. During analyses various types of interference effects may occur due to the coelution of other substances [1]. Therefore, it is necessary to use appropriate methodological approaches based on analytical calibration to improve the quality of analytical results [2,3]. The aim of this study was to use appropriate diagnostic coefficients to determine the sources of PAHs in the Katowice region (Poland) based on results with increased accuracy [3,4].

Experimental

This research focused on 16 PAH compounds identified by the US Environmental Protection Agency (EPA) as priority pollutants. High-performance liquid chromatography (HPLC) with fluorescence detection (FLD) was used to determine PAHs. The optimized method has been subjected to a validation process. This method used an appropriate gradient consisting of water and acetonitrile and a suitable program for changing the excitation and emission wavelengths. The time of a single chromatogram was 35 min. Research on the determination of PAHs was carried out for several types of samples: air, dust, river water, tap water, river sediment, and snow collected in the city of Katowice. An extraction method using acetonitrile and ultrasound (1 hour) was used and followed by samples filtration.

Results

The research used an advanced methodological approach: a multi-component integrated calibration method (MC-ICM) to improve the quality of analytical results. The method involves preparing a series of calibration solutions (Figure 1a) consisting of a sample (S), standard mix (ST_{mix}), and diluent (D) in two different volumes, p and q. After measuring the analytical signals (R_0-R_3), two calibration curves are obtained (Figure 1b), based on which estimations of the analytical result are calculated by interpolation (c_1) and extrapolation (c_2). Based on the result obtained by extrapolation, results are obtained free of multiplicative interference effects (resulting from the coelution of other substances).

Figure 1c shows an exemplary chromatogram using fluorescence detection. First, the

effectiveness of the MC-ICM method was confirmed by analyzing a synthetic sample with a known concentration. Next, measurements were made for natural samples, and accurate results were obtained based on extrapolative estimations. After determining the concentrations of individual substances from the PAH group, an analysis was carried out, considering the sum of compounds depending on the number of rings. The type of individual compounds was similar among individual sample types. Next, appropriate diagnostic coefficients (Figure 1d) were calculated, which allowed determining the origin of PAHs in individual samples, focusing on petrogenic or pyrogenic origin, as well as determining the source of origin more precisely (petroleum, grass, wood, coal, or mix sources). The obtained results were consistent with conclusions drawn on the basis of principal component analysis (PCA). The developed method was evaluated using the RGB model and the AGREE approach (Figure 1e).

Conclusions

A method has been developed to determine the origin of PAHs with increased accuracy. Both the chromatographic method and the methodological approach have been classified into the White group – the method is complete and consistent.

Acknowledgments

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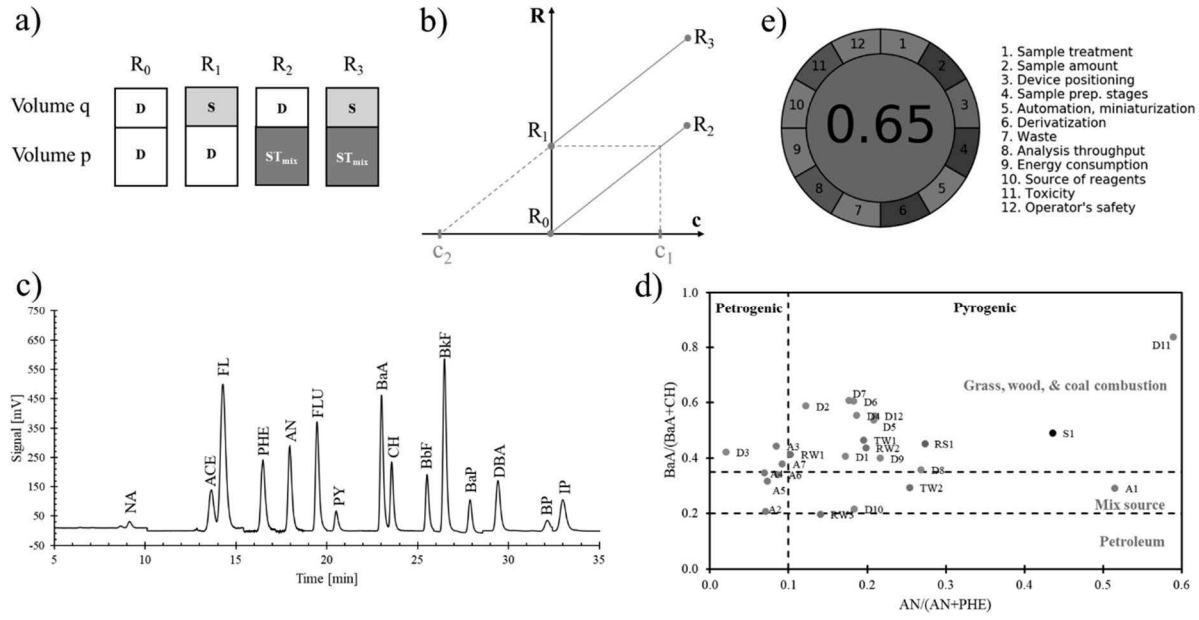
Figure 1. a) diagram of calibration solutions according to the integrated calibration method (ICM), b) exemplary calibration curves compatible with the ICM method for a single analyte, c) exemplary chromatogram with fluorescence detection, d) exemplary diagnostic ratios and possible sources in tested samples, e) analytical method assessment with the use of AGREE – Analytical GREEnness Metric Approach.



Analyses and physico-chemical characterization of peptides and lipopeptides regulating food intake by capillary electrophoresis and isotachophoresis

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Introduction

New lipidized analogs of peptides (ghrelin, prolactin-releasing peptide (PrRP) and cocaine- and amphetamine-regulated transkript peptide (CARTP)) are studied for regulation of food intake. Ghrelin (28 amino acids) is native peptide hormone, which is secreted in the stomach and acts both peripherally and centrally [1]. PrRP (31 amino acids) was discovered as a ligand for an orphan G-protein coupled receptor GPR 10 and as a regulator of prolactin release [2]. The CARTP (42 amino acids) is a brain-born and brain-acting neuropeptide with anorexigenic properties regulating hormone leptin [3]. The inability of these peptides to cross blood-brain barrier to their target receptors may be overcome by their lipidization at biologically inactive sites of their molecules. The set of ten (lipo)peptides was synthesized to be tested as potential drugs. Prior their application, they have to be analyzed and characterized. For that reason, the aim of this work was to check their purity and to characterize their acid-base properties by capillary zone electrophoresis (CZE) and capillary isotachophoresis (CITP).

Experimental

CZE and CITP analyses were performed in CE 7100 analyzer (Agilent, Waldbronn, Germany) equipped with UV-vis/DAD (200 nm) and/or contactless conductivity detector or P/ACE MDQ DNA System (Beckman–Coulter, Fullerton, CA, USA) with UV-vis/DAD detector. Hydroxypropyl cellulose or polyacrylamide coated capillary ID/OD was 50(100)/375 μm and its total/effective length was 395-500(600)/294-415(515) mm for CZE (CITP). Peptides were introduced hydrodynamically (10–35 mbar × 10–20 s).

Results

Ten newly synthesized (lipo)peptides (3.3-4.7 kDa) containing 7-9 basic groups (His, α-NH_3^+, Lys, Arg) and 3-5 acidic groups (α-COOH, Asp, Glu, Tyr) at variable positions of peptide chain with/without attached fatty acid (octanoic, myristic or palmitic acid) were analyzed by CZE in acidic background electrolytes (BGEs) and in some of the (lipo)peptides, a few unidentified admixtures were found, see Fig. 1A. In general, the purity degree of (lipo)peptides was in the range 85–100%.

CZE was employed for the determination of thermodynamic acidity constants (pK_a) and actual ionic mobilities of the above ten (lipo)peptides. Their effective electrophoretic mobilities were measured by CZE in a series of the BGEs within a wide pH range (2.0–10.5), at constant ionic strength (25 mM) and constant temperature (25 °C). Mixed acidity constants, p$K_{a,i}^{mix}$, and actual ionic mobilities, m_i, of (lipo)peptides were determined by the nonlinear regression analysis of pH dependence of their effective mobilities, see Fig. 1B [4]. The p$K_{a,i}^{mix}$ values were recalculated to thermodynamic pK_as using the Debye-Hückel theory. Thermodynamic pK_a of acidic group such as α-COOH was in the range 1.94–2.07, pK_a of carboxyl group of Asp was

in the range 1.96–3.67, pK_a of carboxyl group of Glu was in the range 3.72–5.78, and pK_a of basic group as imidazolium group of histidine residues was in the range 5.83–7.23, pK_a of α-NH_3^+ group was in the range 7.04–10.34, and pK_a of ε-NH_3^+ group of lysine spanned the interval 9.87–10.52, depending on the particular amino acid sequence of the peptides and fatty acid attached to peptide chain.
CITP and CZE were applied for the determination of effective charges and ionic mobilities of (lipo)peptides. Effective charges of the (lipo)peptides were determined from the lengths of their ITP zones, ionic mobilities, and molar concentrations, and from the same parameters of the reference compound [5]. Lengths of the ITP zones of peptides and reference compounds were obtained from their CITP analyses in cationic mode using leading electrolyte (LE) composed of 10 mM NH_4OH, 40 mM AcOH, pH 4.0, and terminating electrolyte containing 40 mM AcOH, pH 2.9. Ionic mobilities of peptides and singly charged reference compound (sodium cation) were determined by their CZE analyses in the BGE of the same composition as the LE. The effective charge numbers of (lipo)peptides were found to be in the range 1.37–5.11, i.e. reduced as compared to the theoretical charge numbers by 34–66%. Ionic mobilities of (lipo)peptides achieved values (13.8–23.2) × 10^{-9} $m^2 \cdot V^{-1} \cdot s^{-1}$.

Conclusion

CZE and CITP have been shown as suitable tools for qualitative and quantitative analysis and determination of the mixed and thermodynamic acidity constants, effective charges and ionic mobilities of highly charged peptides and their lipidized analogs.

Acknowledgements

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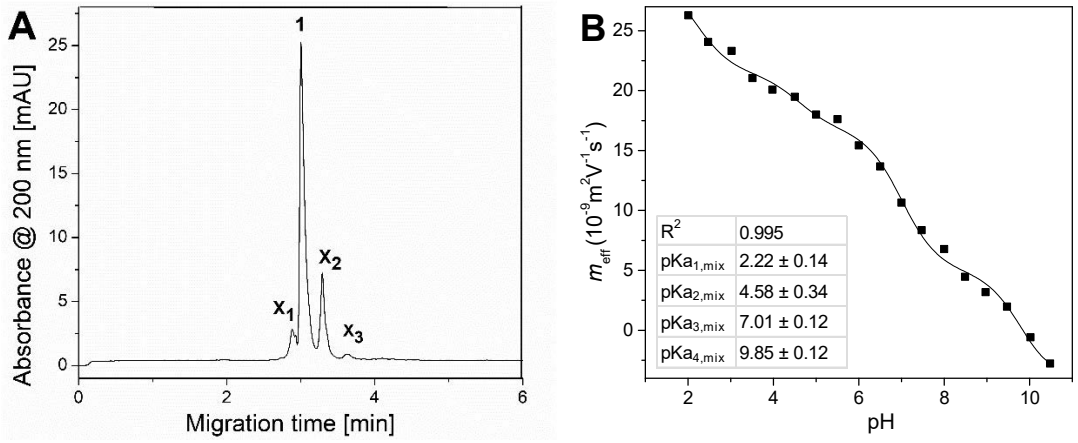
Figure 1. A) CZE analysis of prolactine releasing peptide with attached octanoic acid (PrRP-oct, peak 1) in 2 M CH_3COOH, pH 2.5; x_i, unidentified admixtures. B) pH dependence of the effective mobility, m_{eff}, of PrRP-oct and the calculated mixed acidity constants of its ionogenic groups.



Quantitative analysis of two therapeutic peptides using multiple sample injection in a hydrodynamically closed system capillary electrophoresis

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Introduction

Triptorelin and lanreotide, both short synthetic peptides, serve significant therapeutic roles. Triptorelin, a synthetic decapeptide, functions as a potent agonist of the human gonadotropin-releasing hormone (GNRH). Due to the rapid degradation of endogenous GNRH, analogs like triptorelin with prolonged half-lives were synthesized. This synthetic peptide plays a crucial role in oncology, as well as in managing endometriosis, uterine fibroids, premature puberty, and hypersexuality through chemical castration [1].

Lanreotide, a synthetic cyclic octapeptide, serves as a long-acting analog of somatostatin. It boasts higher binding affinity to somatostatin receptors and a substantially extended half-life compared to somatostatin itself. Lanreotide effectively suppresses various hormones and neurotransmitters, making it valuable in controlling symptoms and growth of neuroendocrine tumors, as well as in treating conditions like acromegaly [2].

While liquid chromatography has traditionally been the method of choice for peptide analysis, capillary electrophoresis emerges as a promising alternative. Hydrodynamically closed system capillary electrophoresis (CSS) shows promise in overcoming one of the biggest challenges faced by commercial CE instrumentation – low sample capacity, thus achieving better limit of detection and quantification values [3]. Addressing low sample throughput, our research group has previously developed a repeated injection (RI) technique to enhance the sample throughput in CSS systems [4].

Experimental

Electrophoretic runs were performed using the experimental EA 102 apparatus in a single column setup. The separation column included a polytetrafluoroethylene capillary tube with an internal diameter of 300 µm and a total length of 90 mm. Electroosmotic flow was mitigated by adding 0.05% m-HEC to the background electrolyte (BGE).

The detection of analytes was provided by a UV detector, ECD 2600 UV-VIS, at a wavelength of 214 nm.

Before each electrophoretic run, the capillary was replenished with a new BGE. Sample injection was facilitated through a 200 nL internal sample loop of the CE apparatus. The

repeated injection (RI) procedure was implemented following specific time intervals between successive sample injections. For both therapeutic peptides, three injections of the respective samples were realized in a single electrophoretic run.

Results

Firstly, we optimized the separation parameters of CE. We tested various BGEs composed of formic acid (HFo) and acetic acid. HFo buffer solutions with high concentrations demonstrated higher separation efficiencies, therefore we determined, that 50 mM HFo solution will be the optimal BGE. We then proceeded to optimize repeated injection (RI) procedure. For triptorelin, a time interval of 100 s and for lanreotide, a time interval of 80s achieved high resolution values of the three peaks analyzed in a single run.

Extensive validation protocol produced favorable validation parameters: low limits of detection (LODs) in aqueous matrix – 0.25 μg/mL and in synthetic urine – 0.5 μg/mL, coefficient of determination exceeding 0.99 in a concentration range of 1–50 μg/mL for triptorelin and 0.5–50 μg/mL for lanreotide, satisfactory precision (relative standard deviation ranging from 5.2% to 14.9%) and accuracy (relative errors in the range of 91.1–107.8%).

The method developed for triptorelin determination was then applied for triptorelin quantification in a commercial drug (powder for injection) and in spiked synthetic urine samples. Applicability of the methods was evaluated using the novel Blue Applicability Grade Index (BAGI) confirming their superior practicality.

Conclusion

Our study presents innovative analytical methods for the quantification of both triptorelin and lanreotide in aqueous and synthetic urine matrices using hydrodynamically closed CE systems (CSS) and repeated injection (RI) strategy.

RI increased sample throughput three times compared to conventional, single injection approach. Extensive validation resulted in favorable performance and validation parameters. Excellent triptorelin and lanreotide LOD concentration levels were attributed to the increased sample loading capacity of the CSS system. Developed methods were then applied to pharmaceutical and synthetic urine matrices with implications for use in contemporary quality control laboratories in pharmaceutical industry.

Acknowledgements

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Separation of cyclic diadenosine diphosphorothioate and the diastereomers of its difluorinated derivative and estimation of binding constants of their complexes with 2-hydroxypropyl-β-cyclodextrin by affinity capillary electrophoresis

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Introduction

Cyclic dinucleotides (CDNs) are known to activate the stimulator of interferon genes (STING), a protein that is a member of the cyclic-guanosine-adenosine synthase-STING signaling pathway and that is important for the innate immune response in eukaryotic cells. CDNs contain two ribonucleoside monophosphates linked via 3′–5′ or 2′–5′ phosphodiester bonds. Many therapeutic oligonucleotides contain phosphorothioate modification.

The aim of this work was to develop a new affinity capillary electrophoresis (ACE) method for the separation of a potential anticancer drug, 2′,3′-cyclic diadenosine diphosphorothioate (R_p, R_p) (ADU-S100), and three recently synthesized diastereomers of its difluorinated derivative, 3′,3′-cyclic di(2′-fluoro,2′-deoxyadenosine phosphorothioate) [1] (see Fig. 1) using native (α-, β-, and γ-cyclodextrins (CDs)) or modified CDs (2-hydroxypropyl-β-CD (HP-β-CD) and 2-hydroxypropyl-γ-CD (HP-γ-CD)) as chiral selectors. In addition, the background electrolyte (BGE) composition and the concentration of the suitable CD should be optimized. Moreover, ACE should be applied for the estimation of the average apparent binding constants of the complexes of the analyzed CDNs with the chiral selector providing best separation.

Experimental

ACE experiments were carried out on the P/ACE MDQ system (Beckman-Coulter). Data acquisition and evaluation were performed using the software P/ACE System MDQ, version Karat (Beckman), the Clarity station (DataApex) and Origin Pro 9.1 (OriginLab), respectively. Internally uncoated fused silica capillaries with the total/effective length of 407/304 mm, and the ID/OD of 50/375 μm were used. The analytes dissolved in deionized water at concentrations 0.2-0.3 mM were detected by a UV–Vis absorption spectrophotometric photodiode array detector set at 200 nm. The electrophoretic mobilities were measured at 25°C.

Results

The experimental conditions for the separation of CDNs were optimized based on their acid-base and electromigration properties. The analyzed CDNs are amphoteric compounds possessing two basic groups (N1 atoms of the two adenine bases) and two acidic phosphorothioate groups. The negative effective charge of these compounds is the highest at pH ≥ 6, at which the N1 atom at both adenine bases is deprotonated [2]. Therefore, CDNs were analyzed as anions in different BGEs at pH values 5–10; the best separation was achieved at pH 8.0.

From the various CDs, relatively good separations were obtained with BGEs containing β-CD, γ-CD or HP-γ-CD, nevertheless, only with HP-β-CD baseline separation was achieved. Then, the BGE composition and HP-β-CD concentration were optimized. Finally, a potential

anticancer drug, 2′,3′-cyclic diadenosine diphosphorothioate (R_p, R_p) (ADU-S100), and three diastereomers of its difluorinated derivative, 3′,3′-cyclic di(2′-fluoro, 2′-deoxyadenosine phosphorothioate), were baseline separated within 4 min in the BGE composed of 40 mM Tris, 40 mM tricine, pH 8.1 containing 43.5 mM HP-β-CD (see Fig. 2).
Besides, the average apparent binding constants of the CDNs-HP-β-CD complexes were estimated by ACE. The method involved the measurement of the dependence of effective electrophoretic mobility of the analytes on the concentration of chiral selector in the BGE. The mobilities were corrected for viscosity change caused by the addition of CD to the BGE [3]. The average apparent binding constants of the analyte-HP-β-CD complexes were obtained from the above-mentioned dependence using non-linear regression analysis. These complexes were found to be relatively weak with the binding constants in the range of 12.2–94.1 L/mol.

Conclusion

ACE using HP-β-CD as chiral selector enabled rapid and highly efficient separation of structurally similar CDNs. The developed method can be applied for the separation, analysis and characterization of the currently investigated CDNs as well as for other, similar CDNs. It is advantageous that it requires only very small amounts of CDNs.

Acknowledgements

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Figure 1. Molecular structures of the studied 2′,3′-cyclic diadenosine diphosphorothioate (ADU-S100), compound **1**, and the diastereomers of its difluorinated derivative, 3′,3′-cyclic di(2′-fluoro,2′-deoxyadenosine phosphorothioate), compounds **2-4**.

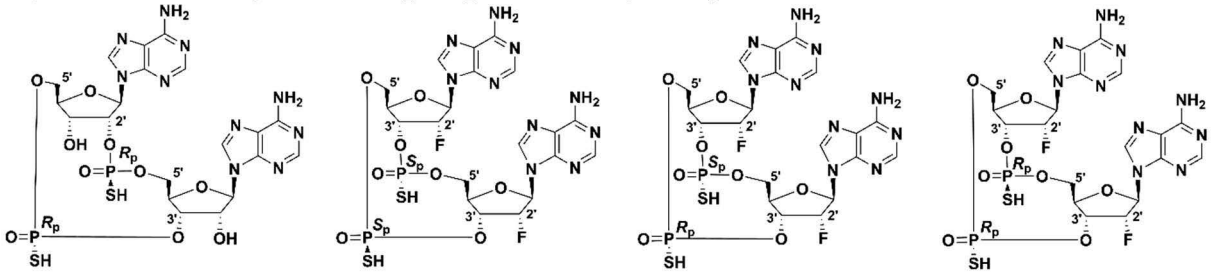
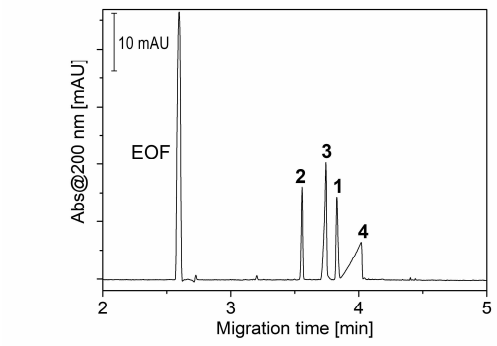


Figure 2. The best separation of CDNs **1-4**. The experimental conditions are given in the text.



Development of simple and rapid CE-UV method for analysis of salivary lysozyme

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Introduction

In modern medicine, biomarkers have a fundamental importance. Their discoveries bring new possibilities for simple screening, diagnosis, and monitoring of diseases or prediction of treatment. Saliva is a good source of biomarkers mainly due to its ease of collection and non-invasiveness. A potential biomarker in saliva is lysozyme, one of the most important antimicrobial proteins in the human body. The level of salivary lysozyme is closely related to several diseases such as cancer, periodontal diseases, intestinal inflammatory diseases, psoriasis, type 2 diabetes, COVID-19, Sjögren's syndrome, ischemic heart disease or hypertension.

Capillary electrophoresis (CE) is a high-throughput analytical method with high separation efficiency for analyzing both small and large ions in complex matrices. In combination with UV detection, it provides robust, simple, and fast analytical instrumentation suitable for detection and quantification of lysozyme in saliva. The aim of this work is the development of a CE method for the quantitative analysis of salivary lysozyme using simple UV detection.

Experimental

Salivary lysozyme analyses were performed by capillary zone electrophoresis (CZE) using transient isotachophoresis (tITP) as a preconcentration step. The CZE was carried out in a 75 μm I.D. uncoated fused silica capillary of 52 cm effective length. The samples were hydrodynamically injected by applying a pressure of 50 mbar for 100 s, which was possible due to the addition of a leading electrolyte to the sample for tITP. The cationic regime of the separation was used, and a separation voltage of +15 kV was applied. UV detection was provided by a photodiode array detector, while the highest intensity was achieved at a wavelength of 194 nm, but the most selective detection was at 280 nm.

Results

In this work, we optimized the parameters of CZE-UV for analysis of salivary lysozyme. During the optimization, we made sure that the method could be used with mass spectrometry (MS) detection at any time, so we chose the BGE composition regarding volatility and compatibility with MS. High intensity, narrow peak shape and good separation were achieved using BGE containing 1 M formic acid with 10% isopropanol addition. The optimization of injecting consisted in the comparison of hydrodynamic (HDI) and electrokinetic injection in combination with and without preconcentration step. Online tITP using HDI provided the highest intensity and excellent separation efficiency with an acceptable migration time (<15 min). We prevented

the adsorption of the lysozyme onto the insert surface thereby enhancing the repeatability of peak areas, especially at low concentrations, by pretreating the insert with a carrier protein before loading the sample. We also tried various sample treatment approaches including centrifugation and simple dilution, filtration and solid-phase extraction.

Conclusion

The optimized tITP-CZE-UV method for the determination of lysozyme in saliva is sufficiently robust and selective to reproducibly analyze the salivary matrix without extensive pretreatment. The preliminary LOD of lysozyme in water and artificial saliva is 1.5 μg/ml, and it is sufficient for the intended application, as the concentration of salivary lysozyme in healthy people ranges from 10 to 70 μg/ml[1].

Acknowledgments

This work was supported by the grants UK/3181/2024, VEGA 1/0514/22 and carried out in the Toxicological and Antidoping Center at the Faculty of Pharmacy, Comenius University in Bratislava.

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Characterization of retention with ternary mobile phases in HILIC chromatography

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Introduction

Recently, the interest in Hydrophilic Interaction Liquid Chromatography (HILIC) applications has been growing significantly. It is caused by the increased need for the separation of highly polar and ionized substances, which are difficult to achieve in other modes of liquid chromatography. HILIC is popularly used for the separation of polar compounds and is widely applied in the fields of pharmacy, proteomics, metabolomics, food analysis, bioanalysis or environmental analysis. The aim of this work was to evaluate the potential influence of ternary mobile phases on the retention and selectivity of selected polar substances. The combination of acetonitrile, methanol, and aqueous buffer can influence the character of the diffuse aqueous layer on the surface of the HILIC stationary phases and affect the quality of the separation.

Experimental

A liquid chromatograph with UV detection Agilent Technologies (USA) was used and five commercial columns were tested: Ascentis® Express HILIC (3 × 150 mm, 2.7 µm, Supelco, USA); InfinityLab Poroshell 120 HILIC-Z (2.1 × 150 mm, 2.7 µm, Agilent Technologies, USA); Luna® Omega SUGAR (3) 150 mm, 3 µm, Phenomenex, USA); SeQuant® ZIC-HILIC (2.1 × 150 mm, 3.5 µm, Supelco, USA); YMC-Triart Diol HILIC (2.0 × 150 mm, 5 µm, YMC, Japan). The column temperature was set at 25 °C, and a constant linear flow rate of 5.67 cm/min was preserved. Water, 98% acetonitrile and 98% methanol were used as the mobile phase A, B, and C in gradient chromatography with ionic additive 5 mM ammonium acetate. Nucleotide bases, nucleosides, and sulfonamides with a concentration of 100 µl/ml, which belong to the group of polar substances suitable for separation, were chosen as model analytes. Sample volumes of 1 µl were injected in all experiments. The detection wavelength was chosen according to the type of substance being measured (254 and 284 nm).

Results

Using gradient elution with a mobile phase containing acetonitrile, the retention behaviour of selected substances was measured in several gradients with different steepness. The columns used were compared in terms of retention and selectivity of substances. The ZIC-HILIC column was chosen as the most appropriate, with the best separation of standards (highest resolution) and the highest efficiency recorded within the gradients used. Thanks to its ability to retain polar compounds, it enables efficient separation, which leads to a higher probability of separation of mixtures of analytes in real matrices and to better quantitative analysis parameters. To compare the influence of the organic solvent, the same series of experiments were also performed for methanol. In the case of using a mobile phase containing methanol, the retention of substances is significantly lower than in the case of a mobile phase containing acetonitrile, due to the differences in solvation strength of both solvents. Furthermore, it was shown that the retention is in tested range of gradient profiles practically independent of the gradient conditions

used. Subsequent analysis of the standards using ternary mobile phases showed that the addition of methanol to the commonly used acetonitrile/water mobile phase combination had an effect on selectivity and separation. The gradient profiles were chosen to start with 100% acetonitrile and differ in the final acetonitrile/methanol concentration ratio. Using the acetonitrile/methanol concentration ratio during the gradient, the separation, especially of polar substances, can be influenced.

Conclusion

Analysis of experiments on different columns confirmed that the ZIC-HILIC column is the most suitable for the separation of polar compounds due to its ability to retain these substances and thus provide higher separation efficiency. Subsequent testing of the gradient with ternary mobile phases showed the possibility of using a combination of acetonitrile and methanol in the gradient for the targeted setting of separation selectivity when working with polar compounds.

Acknowledgements

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Effect of degradation processes on polyphenol content in wine

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Introduction

Wine is an ancient drink with a high presence of polyphenols which are responsible for characteristic attributes of wine. Polyphenols are also important antioxidants with positive effect on human health. Anthocyanin dyes are belonged among polyphenols responsible for color of red wines [1,2]. Study of the effect of UV radiation on polyphenol content (apigenin, cyanidin and malvidin) was performed in the presented study. Ultra-High Performance Liquid Chromatography / Mass Spectrometry (UHPLC/MS) was used for polyphenol quantification in red wine.

Experimental

Red wine, specifically Blue Portugal was selected for study. Wine (2 mL) was placed in quartz cuvettes which were placed to degradation chamber of Solarbox instrument (Cofomegra). Parameters of degradation were as follows: illumination of xenon lamp was set at 1000 W/m^2 and time of illumination was 1, 2, 6 and 24 hours. Reference wine sample was left without irradiation. Both samples were consequently diluted with 0.1% formic acid (1:1) and purified using Strata SDB-L columns (Phenomenex). Purified extracts were dried by a fine stream of nitrogen and residues were dissolved in a mixture of mobile phases (1 mL, A:B, 1:1, v/v). Prepared samples were analyzed by UHPLC (ACQUITY, Waters) / electrospray ionization MS (Select Series Cyclic IMS, Waters) in positive ionization mode. Separation of analytes was performed using Raptor ARC-18 2.7 µm column (Restek) thermostated at 30 °C. Mobile phase A consisted of water with 0.1% formic acid (v/v) and mobile phase B consisted of methanol with 0.1% formic acid (v/v). The flow rate of mobile phase was set to 0.200 mL/min and parameters of gradient elution were as follows: 1 % of mobile phase B in initial time and 100 % in time 14.00. Parameters of mass spectrometer were: spray voltage 2.5 kV, cone voltage 25 V, desolvation gas flow 600 L/hod, desolvation gas temperature 220°C.

Results

Quantification of selected polyphenols in wine was done using calibration curves of polyphenols standards. Changes in content of studied polyphenols due to UV irradiation are presented in Fig. 1. (anthocyanins) and Fig. 2. (apigenin). Significant decreasing of polyphenols was observed during time due to photolytic processes. This fast loss of analytes taking place in hours shows how sensitive compounds these polyphenols are and how easily the quality of wine can be deteriorated if treated and/or stored at unsuitable conditions. At the same time, degradation mechanism and products were studied in degraded wine.

Conclusion

Quantification of selected polyphenols in wine and its degraded forms was realized by UHPLC/MS technique. High energy UV radiation caused loss of studied polyphenols due to structure disintegration or their reaction with free radicals. Loss of health benefits and characteristic wine attributes as a result of degradation will be described.

Acknowledgements
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Figure 1. Content of cyanidin, malvidin and apigenin in reference and degraded wine and their changes during degradation.

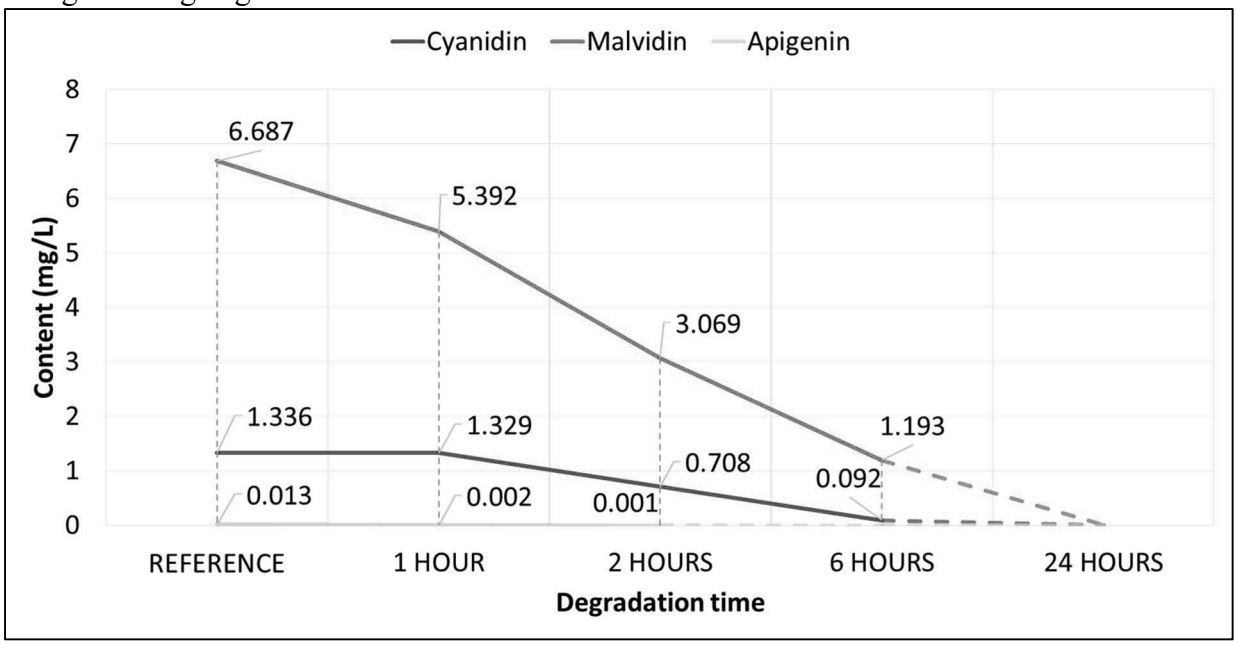
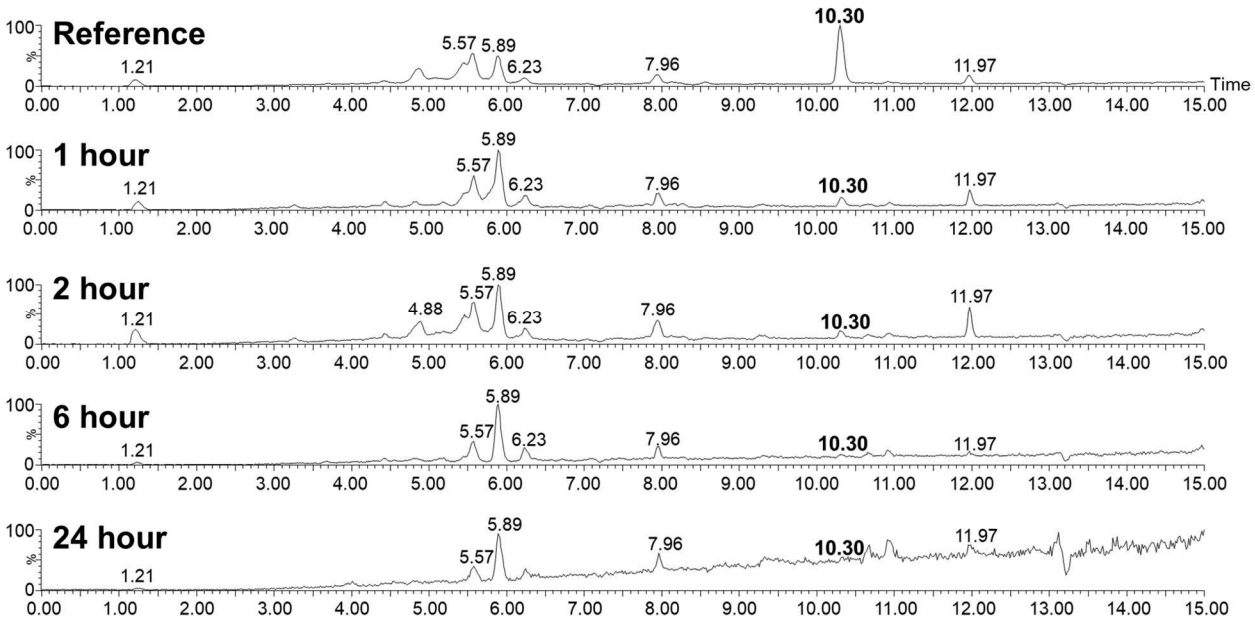


Figure 2. Chromatograms of reference and degraded samples and loss of apigenin during degradation in retention time 10.30 min.



Using microchip electrophoresis for the determination of melamine in infant formula

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Introduction

Melamine (1,3,5-triazine-2,4,6-triamine) is an organic heterocyclic compound that is used to make plastics, fertilizers and other products and is not approved as a food additive. Melamine is also a metabolite of pesticide, cyromazine, which is used as a foliar spray to control leaf miners in vegetables, mushrooms, and ornamentals. Therefore, it can be found in food as a low-level contaminant due to the degradation of cyromazine. Nitrogen-rich melamine is also illegally added to food or food-related products because protein content is usually estimated as nitrogen content. Added melamine increases the nitrogen content of the products, making them appear to contain more protein and reducing production costs. In September 2008, infant formula that was illegally adulterated with melamine resulted in health problems for thousands of infants in China. To protect public health and food safety, the US Food and Drug Administration, the European Commission and authorities in other countries and regions (Australia, Russia, New Zealand) have established a standard limit for content of melamine in various milk products, namely 1.0 mg/kg for infant formula and 2.5 mg/kg for other milk products [1]. Screening for the presence of melamine in food and protein-based animal feed has become an important quality control measure. The acute need for the determination of melamine and its negative impact on human health can be deduced from many developed analytical procedures. Melamine has been determined in several matrices such as food (dairy products, fish, rice, soy, eggs, poultry meat and flour), animal feed, wastewater, and soil. The determination of melamine has been carried out using various analytical methods, such as capillary electrophoresis [2], high-performance liquid chromatography [3] and gas chromatography-mass spectrometry [4].

Experimental

Microchip electrophoresis (MCE) is a suitable miniaturized analytical technique for the determination of melamine in complex food samples. MCE separations were performed on an electrophoretic microchip with coupled separation channels (IonChipTM 3.0, Merck, Germany) made of poly(methyl methacrylate). Separations were carried out in a cationic mode and monitored by a conductivity detector integrated at the end of the second separation channel. Adsorption of the analyte on the inner walls of the microchip channels was suppressed by the addition of triethylenetetramine to the background electrolyte, and methylhydroxyethyl cellulose, which was added to all electrolytes, suppressed the electroosmotic flow. The infant formula samples were pretreated by deproteinization, centrifugation, and, after appropriate dilution, injected into the microchip in the presence of discrete spacers.

Results

Due to the complexity of food samples, such as infant formula, it was necessary to eliminate potential interfering substances. For this reason, it was convenient to use a combination of

isotachophoresis (ITP), which has a high concentration power, and sensitive zone electrophoresis (ZE) for effective sample pretreatment on the microchip with coupled separation channels. The presence of discrete spacers in the analyzed samples made it possible to define a narrow ITP mobility interval in which melamine migrated without unwanted interferents. Cationic forms of butylamine and ε-aminocaproic acid were used as discrete spacers and interfering sample components were electrophoretically removed from the separation system using a column switching technology. The electropherograms from the ITP-ZE analysis of the infant formula sample are shown in Figure 1.

Conclusion

The developed ITP-ZE method on the microchip can be used in food quality control as a screening method for the determination of melamine in various food products.

Acknowledgements

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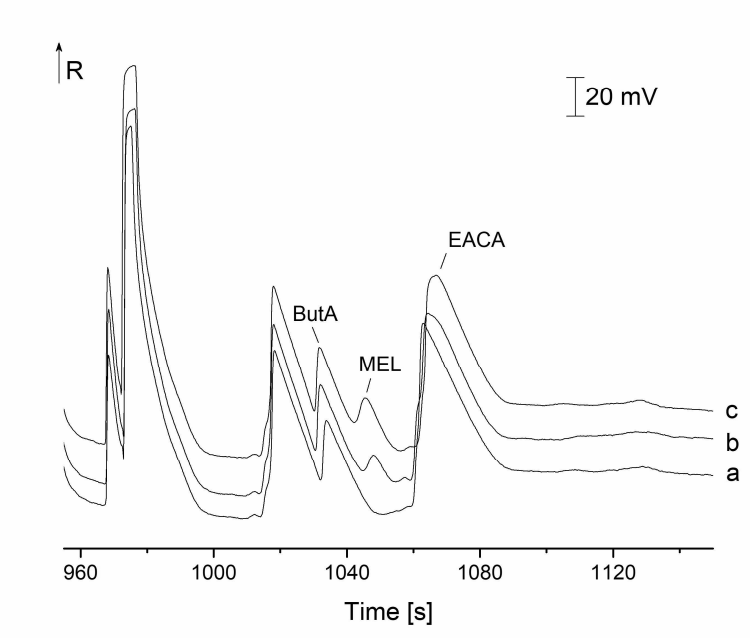
Figure 1. Electropherograms from the ITP-ZE analysis of the infant formula sample in the presence of discrete spacers (butylamine, ButA and ε-aminocaproic acid, EACA) and with the addition of (a) 0, (b) 50, and (c) 100 μg L^{-1} melamine (MEL).



Unlocking seed coat composition with Raman microscopy: Complementary tool to mass spectroscopy

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Introduction

The evolution of seed strongly enhanced the reproductive and dispersal potential of seed plants. It is mainly the seed coat which provides protection for the seed by physical and chemical barriers to prevent the entry of pathogens or the impact of adverse environment. Understanding of seed coat chemical composition is also important for seed quality, storage, and overall agricultural productivity.

The metabolic composition of peas has been extensively studied, including the use of LC-MS, ESI/MS [1], ASAP-MS [2], and MALDI-MS [3,4].

While traditional methods like mass spectrometry provide valuable insights into seed coat composition, they have limitations. These include sample preparation requirements and potential damage to the sample. Furthermore, data analysis is often challenging due to matrix effects and isobaric interferences, which can lead to ambiguities in compound identification. MALDI-MS in mass imaging mode has limitations in spatial resolution.

In this study, we employed the most powerful high-resolution imaging technique for label-free analysis of plant samples: confocal Raman microscopy. This technique was used to investigate the chemical distribution of the main components in pea hilum cross-sections.

Experimental

The excissed seed coats of mature dry pea seeds of domesticated (cv. Cameor) and wild pea (JI1794) genotypes were cross sectioned by Leica CM3050S cryostat. Raman images were acquired with confocal Raman microscope (alpha300RA, WITec, Germany), equipped with a 532nm laser. The laser power was set to 46 mW for domesticated and 8 mW for wild genotype. Integration time was 0.001s.

Results

The component distributions revealed for Cameor genotype were pectin [6], lipids [2] and cellulose, as shown in Figure 1 a, b and d, respectively. The component between the counter palisade tissue and the light line (Fig. 1, c) was identified as H-lignin monomer p-coumaric acid (1605 cm^{-1}) [5]. Furthermore, the tracheid bar (Fig. 1, c) was found to contain G-lignin (1600 cm^{-1}) [7]. In wild pea coat cross section pectin was not observed at all, probably due to low laser power (the maximum laser power caused burning of the sample due to high energy accumulation). In contrast to domesticated pea, the lignin component of the wild pea hilum was

found in both tracheid bar and counter palisade tissue (Fig.1, f). Furthermore, its signal has been observed at 1598 cm^{-1}, which is referred to in some papers as the S-lignin signal [8]. However, given that a peak was observed at 1582 cm^{-1} in the hilum region, it is likely that this is also a contribution of the eumelanin [9, 10].

Conclusion

The Raman mapping revealed a better understanding of the composition of pea seed coats and demonstrated the differences between the spatial distribution of various chemical components within the samples, especially with respect to lignin. These results provide a solid base for further in-depth multimodal research of the pea hilum cross-sections.

Acknowledgements

The financial support was provided by Czech Science Foundation (24-10730S) and Grant Agency of Palacký University Olomouc (IGA_PrF_2024_026).

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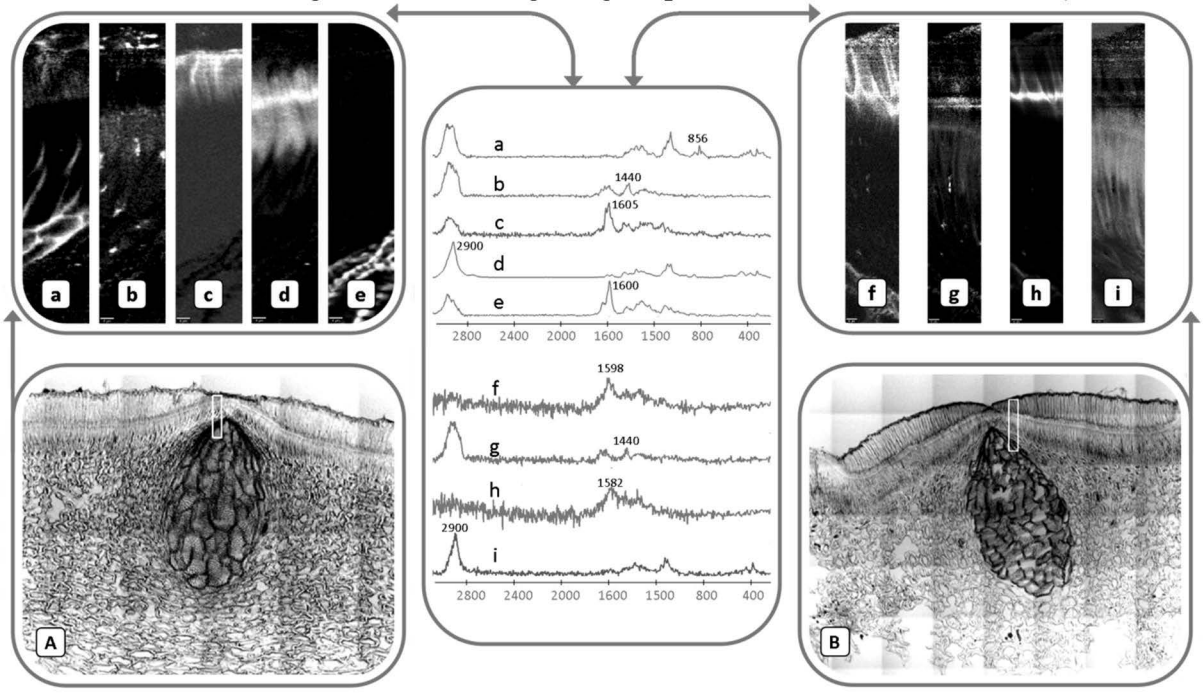
Figure 1. Raman images of the domesticated – Cameor (A) and wild – JI1794 (B) pea hilum cross section and its component average spectra. (For A: a – pectin, b – lipids, c – p-coumaric acid, d – cellulose, e – lignin; for B: f – lignin, g – lipids, h – melanin, i – cellulose).



Electromembrane extraction of methadone on-line coupled with capillary electrophoresis by flow-gating interface

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Introduction

Standard analysis of complex clinical, food and environmental samples, most commonly performed using HPLC, GC and CE techniques often in combination with MS, requires the implementation of an efficient extraction technique usually based on liquid or solid phase extraction prior to the final instrumental determination. A new type of efficient extraction technique for the separation of ionized analytes from complex matrices is electromembrane extraction (EME). Only a few on-line variants of EME/CE are known from the literature to date. In one of them, a fracture is created near the injection end of the separation fused silica capillary, over which the supported liquid membrane (SLM) is threaded and contacted with the donor solution [1]. Furthermore, on-line coupling of a miniature EME/SLM probe to CE via an air assisted flow gating interface (FGI) has been described [2-4].

Experimental

A schematic of the new design of the EME probe is shown in Fig. 1A [5]. The dialysis membrane is a polypropylene porous hollow fibre (HF, Accurel, ID 600 μm, wall thickness 200 μm, pore size 0.2 μm) with an active length of 6 mm, which is sealed at one end with UV cured adhesive. The SLM is formed by filling the pores of the HF membrane with extraction fluid nitrophenyloctyl ether (NPOE). The EME probe is on-line coupled with CE instrument by transversal flow gating interface (FGI) that is realized by a microfluidic PDMS microchip cast in the laboratory and the entire EME/CE analysis process is automated by the LabView system.

Results

The EME probe is tested on laboratory samples and methadone is extracted into 3.0 M acetic acid as acceptor, Fig. 1B. The concentration dependence is linear in the range of 0.1 - 1.0 $\mu g \cdot mL^{-1}$ at EME 300 s/150 V and in the range of 0.5 - 10.0 $\mu g \cdot mL^{-1}$ at EME 100 s/150 V. The enrichment factor is greater than 30 and the LOD is 0.21 $\mu g \cdot mL^{-1}$. The EME of methadone in clinical samples showed a linear concentration dependence in human urine and a nonlinear concentration dependence in serum. The distribution of methadone in each phase of the extraction system and the effect of extraction membrane thickness on the enrichment factor were studied. The EME probe can be applied repeatedly.

Conclusion

Flow-through probes with a coaxial arrangement of donor inlet and acceptor outlet are more stable in repeated extractions. Another advantage of the described arrangement with a PDMS

carrier chip is that the active part of the probe, the SLM, can be carefully withdrawn from the fused silica capillary when damaged and replaced with a new hollow fiber while keeping the overall probe arrangement unchanged.

Acknowledgements
The financial support from the Czech Science Foundation (22-22398S) is gratefully acknowledged.

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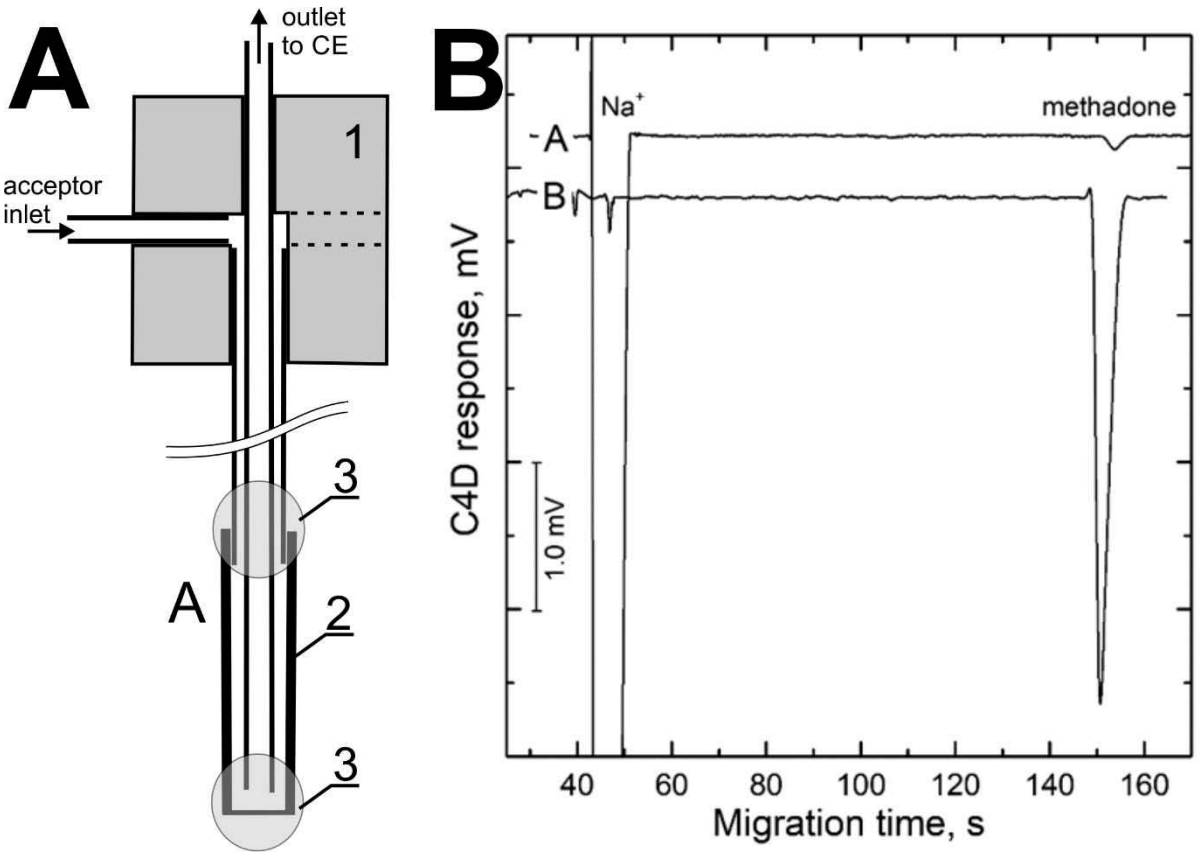
Figure 1. A – Schematic of the new coaxial probe for EME, 1 – PDMS chip 10 × 10 × 2 mm, 2 – polypropylene HF, 3 – UV cured glue. B – Electropherogram of 10 μg·mL^{-1} methadone solution in 0.1 M NaCl directly injected using a dummy cell without EME (A) and after EME 100 s/150 V under vibratory stirring; acceptor 3.0 M acetic acid (B).



Targeted, suspect and non-targeted liquid chromatography-high resolution mass spectrometry for identification of degradation products of selected pollutants in water samples

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Introduction

The presence of organic pollutants such as pharmaceuticals, drugs, pesticides, personal care products and many others, in various parts of the environment is an actual global problem. A major challenge in analyzing environmental water samples stems from their complexity, as they contain numerous anthropogenic substances with diverse physicochemical properties. Moreover, within these complex samples, many chemicals remain unidentified or emerge through various biotic and abiotic degradation and/or transformation processes of their parent compounds and may cause adverse effects to aquatic organisms at very low concentration e.g. especially endocrine disruption [1]. The presence of persistent pollutants in the environment is associated with the risk of adverse effects on living organisms and therefore new treatment technologies are intensively studied. The electrochemical advanced oxidation processes (EAOPs) belong to the water treatment technologies based on principles of electrochemistry and advanced oxidation to degrade organic pollutants present in water. Boron doped diamond electrodes (BDDEs) belong to the EAOPs, technologies based on the generation of highly reactive oxidizing species, typically hydroxyl radicals (•OH), that are able effectively degrade different types of organic pollutants [2, 3].

In this context, a combination of liquid chromatography and high-resolution mass spectrometry (LC-HRMS), allows the identification and quantification of many known and unknown compounds due to its high resolution, accuracy, and selectivity. The identity of all contaminants can be elucidated in three main workflows by target analysis, suspect screening, and non-target screening [4].

The main objective of our study was to employ LC-HRMS to identification of degradation and transformation products of selected pollutants formed during the EAOPs using BDDEs.

Experimental

The chemicals and solvent for preparation of mobile phases and analytical standards of selected pollutants were purchased from Sigma-Aldrich (Germany). The isotopically labeled standards were purchased from Chiron (Norway), Sigma Aldrich (Germany) and Toronto Research Chemicals (Canada). The electrochemical oxidation of specific pollutants was conducted employing various types of boron-doped diamond electrodes. Prior to analysis, water samples were filtered using syringe filters (regenerated cellulose with a pore size of 0.45 μm).
The water samples from the degradation experiments were analyzed by LC-MS/MS using the triple quadrupole mass analyzer TSQ Quantiva (Thermo Fisher Scientific, USA) with heated electrospray on positive and negative ionization mode. Identification of degradation products was performed by LC-HRMS instruments, LC-MS-IT-TOF™ (Shimadzu) and QExactive (Thermo Fisher Scientific). Compound Discoverer 3.3 (Thermo Fisher Scientific) with workflows containing retention time alignment, peak detection, accurate m/z (<5 ppm) elemental composition calculation, halogens isotope pattern detection and statistical analysis were used for data processing.

Results

Currently, the occurrence of various pollutants in the environment is of a big concern. Wastewaters, as a source of these pollutants contents a large scale of chemical substances with potential harmful effect on ecosystems. For this reason, techniques for sufficient micropollutant elimination and their safety evaluation mainly in term of formation of degradation and transformation products are needed. The pollutants chosen for electrochemical degradation experiments on BDDE exhibit diverse physicochemical properties, and their average removal efficiency in conventional WWTPs is typically below 50%.
Degradation and transformation by-products are commonly observed during EAOPs treatment, and they may possess heightened persistence and/or toxicity relative to their parent compounds. The identification of these by-products from selected pollutants utilized HPLC-HRMS, employing two approaches – suspect and non-target screening. First, a suspect list of degradation and transformation products of selected pollutants was compiled based on published research. In non-target analysis, peak searching was performed by comparison of mass spectra obtained before and during/after electrochemical oxidation to find a newly formed peaks belonging to potential products. Both methods enabled the detection of numerous degradation products across all tested pollutants, in both model and real wastewater samples.

Conclusion

Non-target screening of trace organic compounds complements routine monitoring of water bodies. This approach offers valuable insights into the fate of pollutants during advanced treatment processes, facilitating the development of more robust pollution control strategies.

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Characterization of Asian lacquers by atmospheric solids analysis probe high resolution tandem mass spectrometry coupled with cyclic ion mobility separation (ASAP-cIMS-HRTMS)

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Introduction

Asian lacquers are natural polymers produced from sap of trees *T. Vernicifluum*, *T. Succedaneum* and *G. Usitata*. Since antiquity, these lacquers are used for various goods. In restoration tasks of these objects, it is important to know, which type of lacquer was used. Chemically, all three lacquers are alkylcatechol- and alkylphenol-based polymers. Traditionally pyrolysis coupled with gas chromatography/mass spectrometry (py-GC/MS) is mostly used [1] however time-of-flight-secondary ions mass spectrometry (TOF-SIMS), high performance liquid chromatography (HPLC) [2] and Fourier transform infrared spectroscopy (FTIR)[3] are used. We developed a new method for differentiation of these lacquers based on atmospheric solids analysis probe cyclic ion mobility high resolution tandem mass spectrometry.

Experimental

Twenty-seven lacquer samples from 17th to 20th century were analyzed. Analyses were 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

All but three samples were identified as Asian lacquers. On these samples, principal component analysis (PCA) with selected signals used for PCA matrix was applied. In appropriate Score Plot, there were visible three separate clusters corresponding to each of these three lacquer types. Among important markers alkylcatechols were observed and identified. Ion of methyl-catechol was clearly visible among fragments of these markers in MS/MS spectra. Other ions belonging to fragmentation of alkyl chain were also observed. Besides these compounds, ASAP-MS method was able to confirm the presence diterpenic and triterpenic resins and arsene sulphides-based pigments. During analysis of lacquer layers taken from two Japanese

decorative boxes cyclic ion mobility was used for separation of parent ions to increase selectivity of MS/MS experiment for verification of particular alkylcatechols identity.

Conclusion

ASAP-cIMS-HRTMS in combination with PCA was proven to be a useful technique for classification of Asian lacquers. The method is fast, requires virtually no sample treatment and allows to detect broader range of compounds and it is thus attractive for routine analysis of ancient lacquer samples in context of modern care of cultural heritage.

Acknowledgements

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Wagon or no wagon: The new insight in Early Iron Age burial custom

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Introduction

Between 2012 and 2019, four Hallstatt C and Hallstatt D tumuli (7th-6th century BC) were excavated at Simmelsdorf-St. Helena, which is located at the western rim of the Franconian Alps, in Middle Franconia. Huge number of findings were recovered. In one tumulus, mound 2, a male grave was discovered along with numerous grave goods, including more than 13 ceramic vessels, eight bronze pins of various shapes, an iron sword, a large amber ring, numerous bronze objects, including bronze tacks, and horse-gear-related objects. An assumption about a wagon burial was made. The presence of wagon in the burial is related to the ability to transport goods, a sign of higher agricultural maturity and the capacity to create long-distance trade networks.
Whole wooden wagons are often not preserved in graves, which makes an accurate identification difficult. The discovery of wagon-related artefacts, such as linchpins or bronze decoration from the wagon box, as well as horse-gear can provide valuable analytical information identifying the presence of wagons in burial. The preserved or mineralised organic materials can be found on the surface mainly due to the biocidal properties of metals and metal corrosion products [1]. The extracted material can be subsequently studied with many widely used and established analytical methods such as GC-MS [2]. The aim of this study was to gain insight into the circumstances surrounding the placement of the bronze objects, including whether they were attached to materials like wood or leather, in order to verify or falsify the existence of a wagon in the burial mound.

Experimental

The samples and reference samples were extracted using acetone/chloroform solution (50:50, v/v), centrifuged, and evaporated with fine stream of nitrogen gas. Consequently, the sample was silanized using pyridine/N,O-Bis(trimethylsilyl)trifluoroacetamide (1:1, v/v). The measurement of all samples was performed by an Agilent 7010 Triple Quadrupole GC/MS system with Mass Hunter software (Agilent Technologies, Palo Alto, USA). The separation was performed on two (5%-Phenyl)-methylpolysiloxane HP-5ms Ultra Inert capillary columns connected in a series (15m × 0.25mm × 0.25µm, each) with a constant flow of 1.0 and 1.2 $ml \cdot min^{-1}$, respectively. Nitrogen (Messer Group GmbH, Germany) was used as a collision gas with a flow rate of 1.5 $ml \cdot min^{-1}$, and helium (He 5.0. Siad, Italy) as a quench gas with a flow rate of 2.25 $mL \cdot min^{-1}$. The initial oven temperature was 70 °C for 5 min, and the oven was heated at a rate of 15 $°C \cdot min^{-1}$ to the value of 320 °C, which was held for 10 min. The injection volume of the extracts was 1 µL with splitless injection. The identification of the compounds was done using the NIST 14 library and comparison with the authentic standard of cholesterol (Sigma-Aldrich, St. Louis, USA).

Results

The higher abundance of archaeologically important biomarkers was found. First marker detected by GC-MS technique in the studied samples is cholesterol, a marker of animal origin. The presence of cholesterol in the samples might be positively false due to the contamination of human factor. Therefore, positive results should always be evaluated with regards to suitable reference material. The highest cholesterol content was detected in four samples, which consists of organic residues from the inside of a rein-knob or bronze plate. Two higher abundances were also from reference samples. Second important marker is oleic acid, C18 unsaturated fatty acid. Its presence in a sample represents either animal or plant origin of the oil. High content of oleic acid was detected in the organic residues from eight rein-knobs and sediment collected around one of the pendants of the potential linchpin. Linchpins were used to secure the axle-caps of the wagon to the wheel, and these are an important clue to determine the burial style. The presence of 1-monooleoylglycerol, together with oleic acid, led to hypothesis that the samples contained vegetable oil. Other compounds have been identified that may serve as potential archaeological markers of wood presence and/or resins. Abietic acid and dehydroabietic acid are diterpenoid compounds that are mainly contained in pine resin and may be used as biomarkers of conifers. It is apparent that the highest concentration of both biomarkers was found in the sediment from pendant of the potential linchpin. In the remaining samples, the presence of abietic acid and dehydroabietic acid was insignificant. Another marker of resin is pimaric acid and its derivates levopimaric acid and isopimaric acid. The highest content of pimaric acid was detected in the samples of the surrounding sediment. The highest content of levopimaric acid was detected in organic samples attached to individual bronze plates. The samples with the highest contents of abietic, dehydroabietic, levopimaric, and pimaric acid, indicating a high pine resin content, were from the sediment surrounding individual objects (linchpins). This finding may not point to a negative result, but to the excess of already decomposed conifer wood.

Conclusion

The chemical analysis by GC-MS provided us with novel information about excavation from Simmelsdorf-St. Helena. Markers such as cholesterol, oleic acid, 1-monooleoylglycerol, linoleic acid were found in sediments within knobs and pins which can led us to believe that they were attached to leather (treated by plant oils) or other similar material. The second group of compounds founded indicates presence of resins which are main residues of decomposed wood. All these information, including the presence of linchpins, suggested that there was some kind of wooden object – the wagon. It is worth noting that only two linchpins are documented, instead of the usual four commonly used for four-wheeled wagon. Notably, most linchpins are made from iron, although a few are made of bronze. It is also possible that some linchpins were manufactured from organic materials that are no longer detectable. Several interpretations are possible: a *pars-pro-toto* contribution, a four-wheeled wagon with possibly two bronze and two organic linchpins, a wagon with detached wheels, a two-wheeled wagon, no wagon at all, but rather some kind of furniture or linchpins as a decoration of the chest-strap of a yoke. This could explain the organic residues – possibly leather – that seems to be wrapped around the bronze artefact.

Acknowledgements

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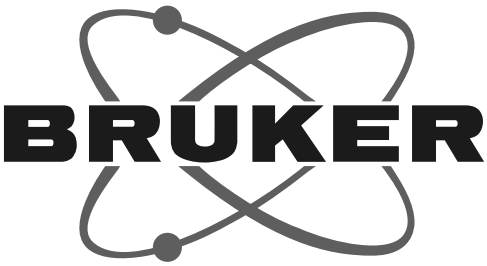

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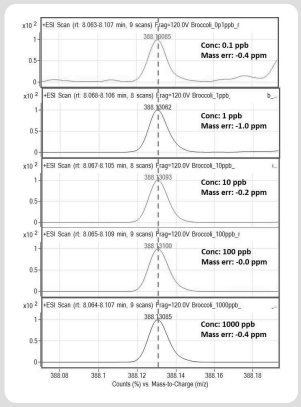

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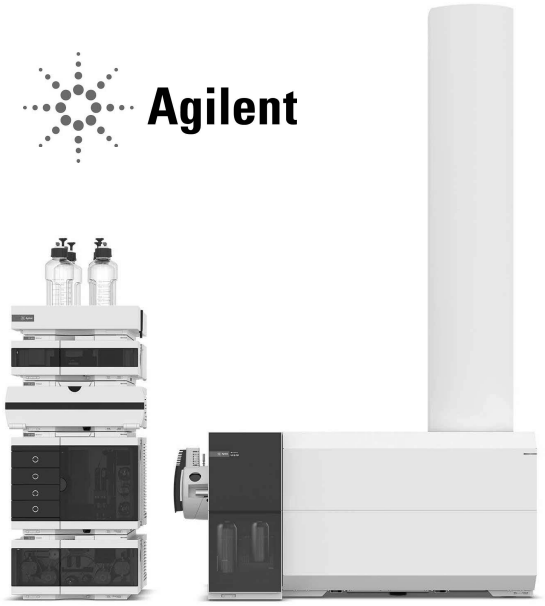

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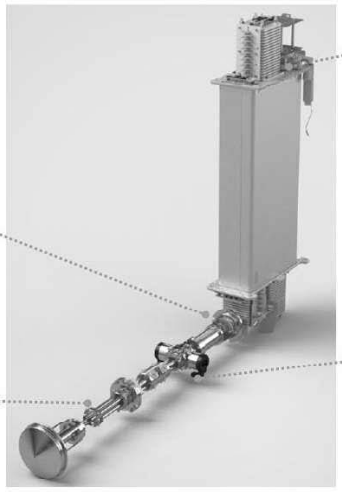

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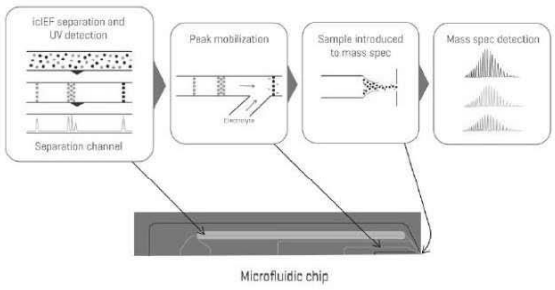

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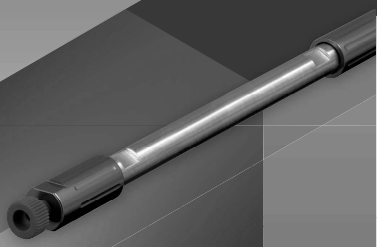

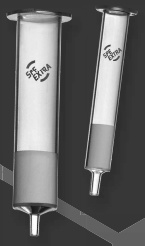

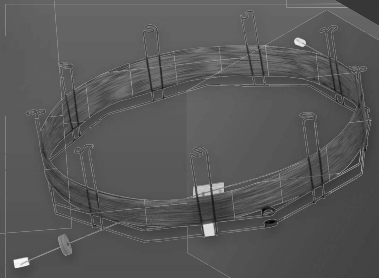

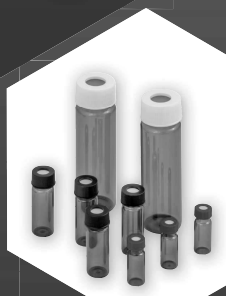

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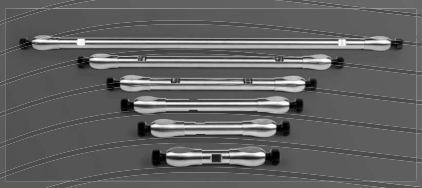

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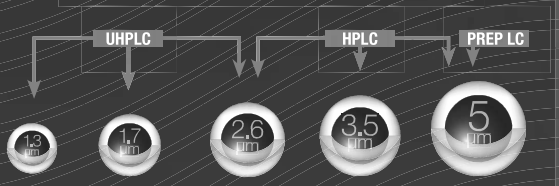
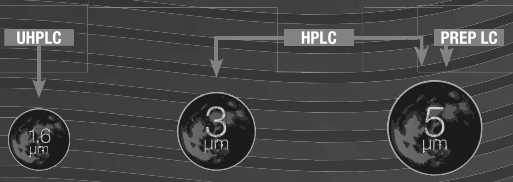
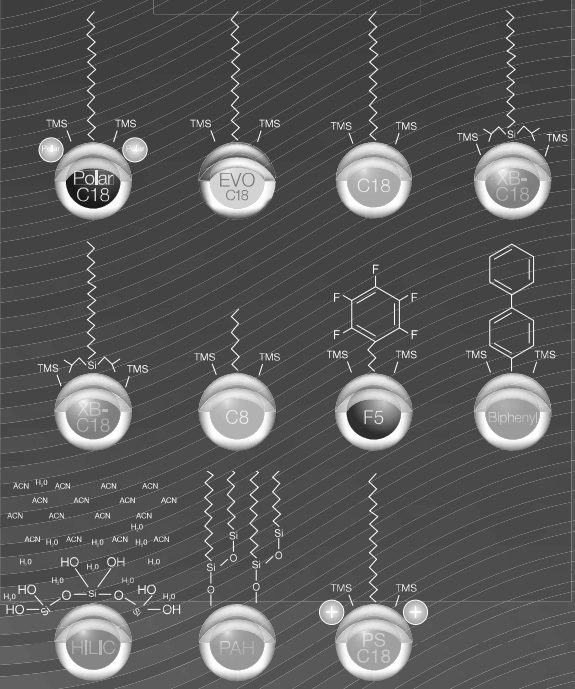
Výběr částic Kinetex Core-Shell



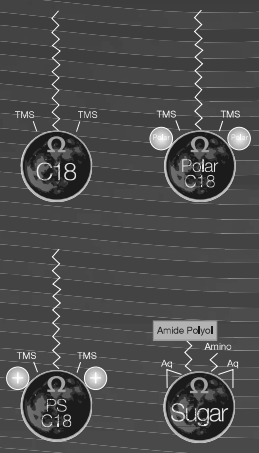
Výběr částic Luna Omega



Výběr fází Kinetex



Výběr fází Luna Omega



Naskenujte tento QR kód a získejte přehled **HPLC/UHPLC selektivit, které nabízíme**

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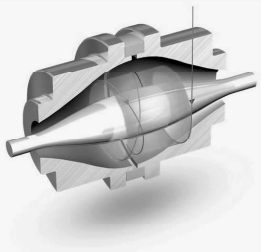

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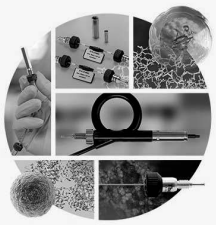

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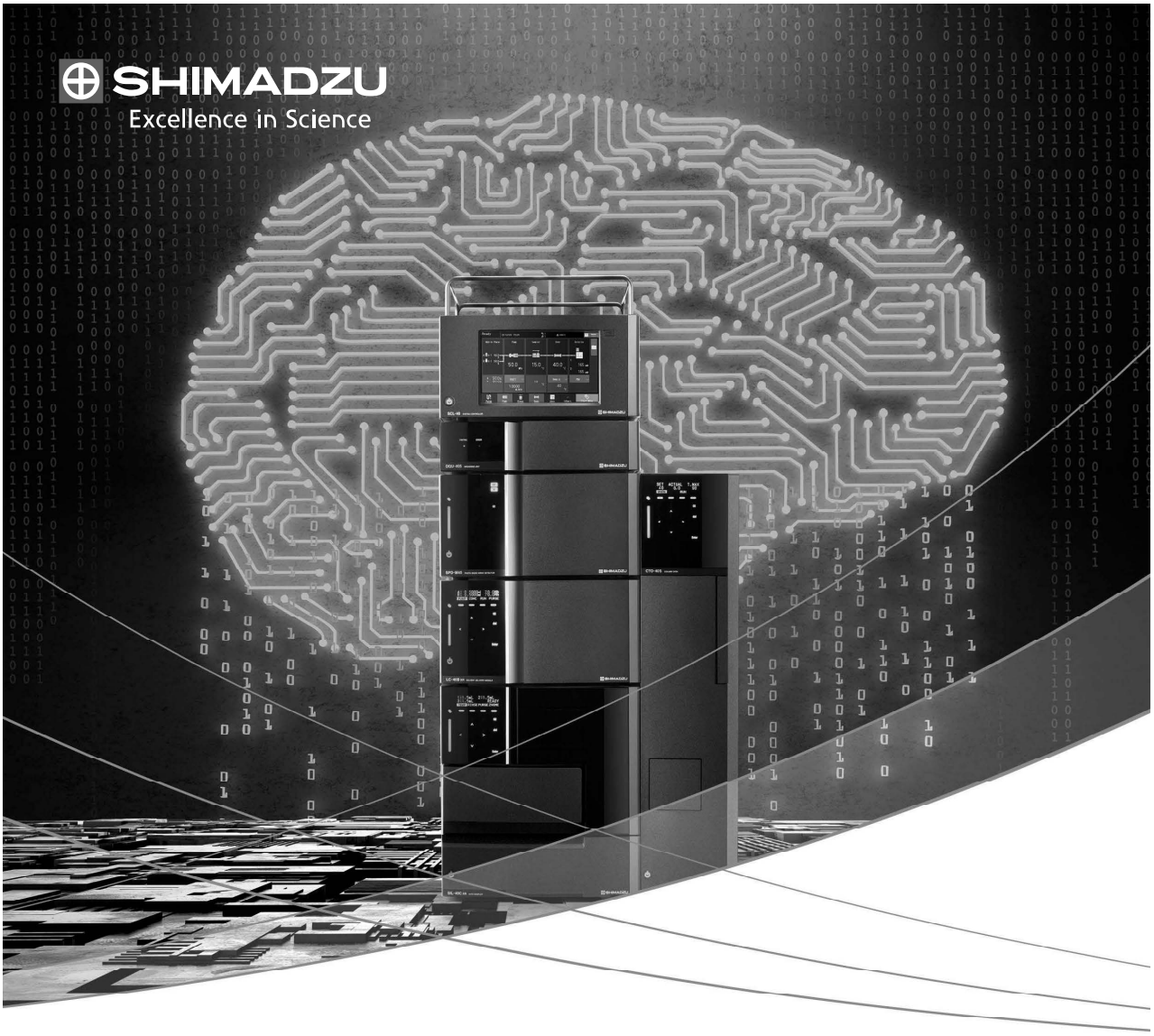

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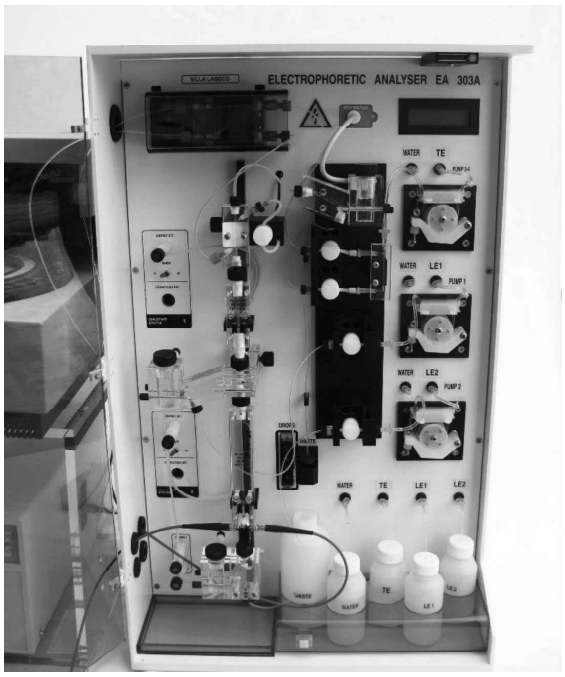

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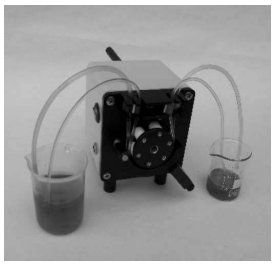

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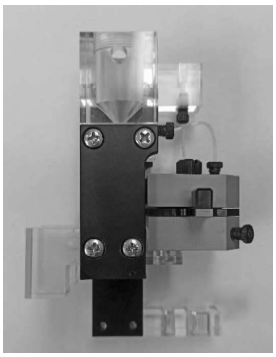

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