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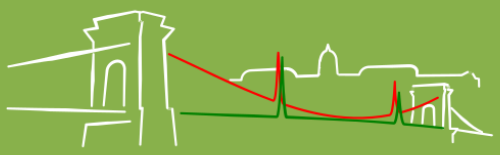
PLENARY LECTURES



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Journeys in GC×GC - Developing Ultra-High-Resolution Separations

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This presentation will review the scope of very high resolution techniques that our research has implemented over an extended period. We commenced with a simple idea for cryogenic modulation - which we continue to be intrigued by its general utility to provide effective focussing and rapid re mobilisation in GC. And since then have been on a mission to introduce unique and we hope useful separation solutions for volatile chemical analysis, based on multidimensional gas chromatography (MDGC) and comprehensive two-dimensional GC (GC×GC), plus innovative hybrid approaches which include elements of MDGC and GC×GC.

Amongst the chemical applications we have studied have been: essential oils, perfumes and allergens; pesticides; drugs; petrochemicals; fatty acids; amino acids; wine and beverages and others.

The solutions we have investigated are based on our unique longitudinal cryotrap movement, and microfluidic switching devices. Often these involve multiple columns beyond the two that are used in the basic GC×GC technique. Scattered within these methods include studies that we refer to as capabilities beyond simple high-resolution volatiles separations, and which reveal interesting chemical phenomena not readily apparent in a 1D GC separation. Here, we will plot our journey through 2, 3 and 4 column systems, using the cryotrap system with Dean's switches, and demonstrate new volatile chemical analysis.

Keywords: GC×GC, gas chromatography, high resolution, cryogenic modulation

The Human Factor in Scientific Thinking: The Illusions That We Live By

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The talk is based on a book published in 2015, and takes a candid look at science from an unusual perspective, attempting to offer some insights into how psychological factors secretly infiltrate its fabric.1 The common notion that science is the world of objective rationality based on methodological rigor, stringent logic, irrefutable experimental proofs, and unbiased peer reviewing and testing, is an idealistic myth which all too often dissents from what "scientific truth" is in reality, and from how scientists truly „function" in practice. Actually, our scientific thinking is influenced by deep-rooted human ("anthropic") factors of which we are not normally aware of. Some of these lead to what we call mental traps, i.e., the hidden sources of mistaken or misleading inferences, a phenomenon we refer to as the illusion of understanding, and mass misconceptions about apparently well-established "scientific truths". By their very nature, mental traps affect even the smartest, most knowledgeable, and most attentive scientists. However, by understanding the essence of the traps one can develop the enlightening faculty of detecting and avoiding them both in one's own and in others' thoughts. It is this mental aptitude/attitude of being keenly conscious about our human nature during scientific thinking which is captured in the phrase "anthropic awareness" in the title of the aforementioned book. The talk will address the concept of "anthropic awareness" as a kind of philosophy, will outline the reasons behind the mental traps, and will discuss some of the traps, including a few real-life examples. I will attempt to show that "anthropic awareness" has a general relevance throughout all of natural sciences, and is useful not only in our everyday professional lives as researchers, but also in our nonprofessional everyday lives.

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Separations, Dimensions, Actions and Reactions

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Most liquid-chromatography (LC) separations are performed with a one-dimensional setup, often with mass-spectrometric detection. However, for very complex samples we need more separation power and comprehensive two-dimensional LC (LC×LC) separations are increasingly common. A straightforward collection of fractions from the first-dimension effluent and re-injecting these on the second-dimension column does not always work, so we have learned to take action ("active modulation") in between the two dimensions. These developments in LC×LC also made many other manipulations of the sample or analytes in on-line integrated systems possible, such as dissolution of nanoparticles or digestion of proteins ("reaction modulation").

In principle, spatial separations are advantageous in multidimensional separations in comparison with the traditional multi-column separations. However, this requires a paradigm shift in instrumentation and separation devices and progress has been slow. However, working on new types of separation devices generates spin-offs for other projects, such as 3D-printed parts for specific LC operations, such as mixers, modulators and, perhaps, columns.

Progress in advanced multidimensional LC methods and possible benefits of 3D-printing will be discussed in this lecture.

UHPSFC/MS in Lipidomic Quantitation: Towards the Clinical Screening of Pancreatic Cancer

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Lipids are the main constituents of cellular membranes, energy deposits, but they also play an important role in signaling in relation to various diseases, such as cancer. The coupling of liquid phase separation techniques and mass spectrometry (MS) is a prevalent technology in the lipidomic analysis [1]. We have optimized and validated the high-throughput ultrahigh-performance supercritical fluid chromatography – mass spectrometry (UHPSFC/MS) method for lipidomic quantitation of biological samples [2]. Several hundred lipid species are typically quantified in biological samples, such as plasma, serum, cell lines, and tissues. The main issues in lipidomic quantification are the reliability of the data over a long period of time, the comparability of the results among different groups, absolute molar quantitation based on the use of exogenous internal standards, and harmonized data reporting [3,4]. At least one internal standard per each lipid class is used for reliable quantitation together with regular injection of quality control samples. Data are processed by LipidQuant software [5]. The comprehensive MS determination of a wide range of blood lipids reveals statistically significant differences between various types of cancer patients and healthy controls visualized by multivariate data analysis [6]. The most extensive results are obtained for pancreatic cancer [7], which showed the dysregulation of very long-chain sphingomyelins, ceramides, and some (lyso)phosphatidylcholines. The sensitivity and specificity to diagnose pancreatic cancer were more than 90%, which outperforms CA 19-9, especially at an early stage, and is comparable to established diagnostic imaging methods. Similar patterns of dysregulation were observed for kidney, breast, and prostate cancers [6]. The current focus is the performance of clinical validation to confirm the real utility for patient management and then the implementation of UHPSFC/MS method for the early detection of pancreatic cancer in high-risk groups. The work was supported by the grant project No. 21-20238S from the Czech Science Foundation.

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An Assessment of Stationary Phase Selectivity in SFC

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Supercritical Fluid Chromatography (SFC) has seen a recent resurgence in interest following investment in the development of instrument technology by numerous instrument manufacturers. Increased focus on sustainability in chromatographic science is likely to further drive the uptake of SFC in many sectors. As with any form of chromatography, optimising separation selectivity is a key variable in providing adequate resolution and accurate identification and quantification of target analytes. Stationary phase chemistry can be readily exploited to substantially alter the separation selectivity obtained. This article examines the selectivity differences offered by three prototype SFC phases.

Substantially different separations of a standard 8 component test mix were obtained using the three stationary phases under identical SFC gradient conditions. These differences in selectivity were then assessed in a quantitative manner by applying a modified Neue selectivity approach. Selectivity values were generated by screening 48 analytes with wide-ranging physicochemical properties (as demonstrated by PCA analysis) under generic gradient conditions using a CO2/MeOH solvent system, with ammonium formate used as an additive. The values obtain demonstrate a high degree of orthogonality between these phases ($S \geq 51$), ideal for method development purposes. Additionally, the impact of switching the additive to ammonium hydroxide was assessed and found to not have a profound impact on selectivity.

Keywords: Column Characterisation, SFC

KEYNOTE AND ORAL LECTURES

Kinetic Plots and What We Can Learn From Them

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Considering the current versatility of physicochemical conditions (GC, (U)HPLC, SFC, High-temperature LC,...) and support formats (open-tubular columns, fully porous or core-shell particles, silica and polymer monoliths, micro-machined columns,...), each with their own propagators, the chromatographic community would benefit a lot if the speed and efficiency data obtained on these systems would be compared in a system- and geometry-independent performance plot.

The kinetic plot method, mathematically not more demanding than establishing a Van Deemter curve, offers this possibility. The method uses two simple expressions, known from the very early days of chromatography on. In the present contribution, the audience will be taken through the few steps (only involving a few multiplications and divisions) needed to establish a kinetic plot. Subsequently, it will be shown how this method can easily be extended from isocratic LC to any type of gradient operation and to GC and SFC. Subsequently, a series of examples, taken both from own experiments and recent literature, will be given to demonstrate how kinetic plots can be used to compare the different chromatographic systems in each category (GC, SFC, LC).

Another advantage of the kinetic plot representation, i.e., that it can be used to unambiguously assess the packing quality of given column without having to measure or define a characteristic length or mean particle size will be illustrated as well by comparing the intrinsic packing performance of a number of state-of-the art LC columns packed with fully-porous and core-shell particles.

In addition, kinetic plots can also be used to compute and visualize the difference in peak capacity and required time between 1D-LC and on-line and off-line 2D-LC.

Pressure-Enhanced Liquid Chromatography: Tuning Selectivity with Pressure Changes

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In liquid chromatography, it is known that operating pressure can impact solute retention. However, pressure has not yet been comprehensively considered as a method parameter.

With this work, we have explored the use of pressure as a method development parameter to alter the selectivity of peptide, protein and other macromolecules' separations. A new apparatus for the facile manipulation of column pressure was assembled through a two-pump system and post column flow restriction. Using this setup, we were able to quickly program various constant pressure changes and even pressure gradients (positive, negative, linear, convex or concave). We found that unique selectivity can be obtained by intentionally changing operating pressure. Such changes in selectivity cannot be achieved by changing other common method variables, like mobile phase composition and temperature. Introducing pressure as a method variable will increase the degrees of freedom for method development strategies.

The addition of pressure to bring column operating pressure beyond 500 bar was enough to change the elution order of proteins in reversed phase LC. Moreover, with our setup, it is possible to combine mobile phase compositional- and pressure gradients in the same analytical run (dual gradient). This approach was applied for mRNAs in anion-exchange mode and for oligonucleotides in ion-pairing reversed phase mode. We have referred to this method as pressure-enhanced liquid chromatography (PE-LC) and believe it can offer unseen selectivity, for peptide and protein reversed phase separations and for any other large molecules (oligonucleotides, nucleic acids) in both denaturing and non-denaturing modes.

Keywords: pressure, new selectivity, method development, proteins, gradient

Measurement and Modelling of Longitudinal Diffusion in Supercritical Fluid Chromatography

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The improvement of supercritical fluid chromatography (SFC) instrumentation enhanced its reliability and utility over the past decade. The further development of high speed and high resolution separations is however obstructed by the lack of accurate models for axial dispersion in SFC. In a first step to enhance prediction and understanding of separation performance in SFC, methods need to be developed to measure molecular and longitudinal diffusion in SFC and to model their behavior. These parameters are essential to predict the separation efficiency in chromatography as it affects all contribution (A, B and C-term).

Using a commercially available SFC instrument with the addition of only a single rotor-stator valve, a flexible method to measure D_{mol} was developed and validated. By using a chromatographic column to separate the solutes from the injection solvent plug, and a small volume loop to capture and reinject the solute in the appropriate mobile phase, typical practical limitation of this technique could be overcome. The effective diffusion coefficient (D_{eff}) was measured using stop-flow experiments, also called peak parking, where the mobile phase flow is halted when the solute is halfway through the column. In SFC, the set-up needs to be modified since during the parking the mobile phase would decompress and elute from the column. In addition, due to the stronger compressibility of the mobile phase, restarting the flow rate is accompanied by transient start-up effects that vary flowrate and retention in a non-reproducible way. Therefore a two-column variant of the set-up was developed where two identical columns are coupled in parallel using rotor-stator valves, to maintain pressure in the parking column and flow and stable operating pressure in the SFC system.

Keywords: SFC, molecular diffusion, longitudinal diffusion, efficiency, modelling

Design and Optimization of Capacity Gradient Stationary Phases

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Modern analytical separation tasks and challenges require the design and development of more and more efficient chromatographic columns. In case of silica gel based stationary phases, in addition to the use of solid core packings, it is possible to use phases with a diameter of less than 2 µm at operating pressures of 800-1200 bar. Besides, there is a constant need to further increase the separation power. Thus, other creative solutions are needed to increase column efficiency. Recently, ion-exchange stationary phases have been synthesized with ion-exchange capacity that varies along the length of the column [1]. In our work, we investigate, based on theoretical approaches, under which conditions the capacity-gradients of stationary phases provide higher resolution and selectivity than conventional isocratic phases. We investigate the separation efficiency of molecules with significantly different properties, and the combined effect of capacity- and mobile phase gradients to increase resolution.

The authors would like to honour the memory of Dr. Péter Hajós (1947-2021) with this presentation.

Keywords: capacity gradient, stationary phase, optimization, column design

Disruptive Planar Innovations: Multiplex Assays, 12D-Hyphenations, and 2LabsToGo Systems

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A disruptive prioritization strategy [1] is introduced combining two disciplines, *i.e.* chemistry and biology, to gain understanding on true mechanisms and important compounds in a complex mixture that should be known. Multiplex bioassays are integrated in a generic non-target 12D-hyphenation, coupling separation and detection dimensions as needed [2,3] and record high-resolution mass spectra fully automated and straightforwardedly from the bioautogram [2-4]. The versatile hyphenation dimensions provide useful information for zone identification. Multiplex bioassays allow the detection of opposing effects in mixtures. The differentiation of agonistic, antagonistic, false-positive and synergistic effects [2,3] clearly show that the ubiquitously applied *in vitro* assays may fail in providing true results for mixtures. Beneficial or harmful compounds with antioxidative, enzyme inhibiting [5], genotoxic [6], cytotoxic [7], neurotoxic [8], and hormonal [2,3] activity are detectable non-targetedly. Simulated metabolism reactions are demonstrated on the same surface, termed nanoGIT$^{+active}$ system [9], which point to activity conversions during digestion or hepatic de-/toxification. Such hyphenations were miniaturized to a portable open-source all-in-one 2LabsToGo system [10].

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Keywords: Effect differentiation, Hyphenation, Prioritization strategy, Digestion, 2LabsToGo

Challenges in HPTLC and HPTLC-MS Analyses of Phytochemicals in Plant Materials and Food

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High-performance thin-layer chromatography (HPTLC) is a well-established powerful chromatographic technique, however, its potential has not been fully explored. What makes it especially unique is the separation which results in an image and the fact that it enables a variety of in-situ bioactivity testing under the umbrella of the so-called effect directed analyses. Detection techniques (e.g. image analysis, densitometry, mass spectrometry) that can be used before or after post-chromatographic derivatization provide a lot of data about the analytes and other compounds present in the sample. These data can be combined with data of effect directed analyses. Combining all the collected data for efficient evaluation remains a big challenge for instrumental and software setups even when upgraded with chemometrics. In spite of this, HPTLC can provide complementary data to other chromatographic techniques in solving problems in research and development.

This lecture will present the potential of one and multidimensional HPTLC combined with different detections (UV, Vis, fluorescence, MS) before and after derivatization for targeted and non-targeted analyses. The influence of different combinations of the sorbent, pre-developing solvent and developing solvent on stability of different analytes (before and after development) and ion suppression in HPTLC-MS analyses will also be discussed. Examples will cover analyses of phytochemicals in plant materials and food - including food supplements.

Keywords: HPTLC, HPTLC-MS, phytochemicals, plant materials, food

Two-Dimensional Multiple Heart-cut Liquid Chromatography–Mass Spectrometry to Reveal the Sphingolipidome of *Caenorhabditis elegans*

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The nematode *Caenorhabditis elegans* (*C. elegans*) is frequently employed as a model organism in studies concerning aging and disease, which makes it particularly interesting for studies in the Lipidomics field. Especially sphingolipids were recently associated with neurodegenerative and cardiovascular diseases. In the two-dimensional (2D) multiple heart-cut liquid chromatography–mass spectrometry method (LC-MS) presented here we focus on the analysis and identification of the species of the three most abundant sphingolipid classes: ceramides, hexosylceramides and sphingomyelins in *C. elegans*. The commonly employed alkaline depletion sample preparation step in sphingolipid analysis was avoided by separating the sphingolipids in the first dimension from the more abundant glycerophospholipids via hydrophilic interaction liquid chromatography (HILIC). The fractions were cut out, stored in a sample loop and transferred onto the second dimension reversed phase liquid chromatography (RP-LC). In addition, fragmentation experiments (MS/MS) were conducted to further elucidate the chemical structure of the sphingolipids. The 2D-separation, in contrast to the 1D-LC, allowed for a better identification of lower abundant species, e.g. dihydro-sphingolipids (DhSLs) due to the separation from the glycerophospholipids and reduced matrix effects. A total of 45 sphingolipids were detected in the larvae stage L4 lipid extract of *C. elegans*.

Keywords: 2D-LC-MS/MS, C. elegans, sphingolipid, HILIC, RP-LC

Two-dimensional Liquid Chromatography in Different Ways: 2D-HPTLC and HPTLC-HPLC

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High-performance thin-layer chromatography (HPTLC) is a flexible technique that enables various pre-chromatographic derivatization, sample preparation, and post-chromatographic detection, all *in situ* in the same stationary phase. Compared to the HPLC, HPTLC has benefits, such as the ease of its performance, the unnecessity of complex sample preparation, analysis of more samples at the same time, exclusion of cross contamination, and a wide range of applicable detection methods, including the use of chemical reagents, enzymes and viable cells. The bio-profiles of the samples obtained by HPTLC-effect-directed analysis (EDA) allow a subsequent targeted characterization of the compounds run in a parallel HPTLC track, directly by spectroscopic and spectrometric techniques or after elution off-line, e.g., by GC-MS or NMR. The discrimination of the bioactive compounds from the closely or co-migrated other compounds can be achieved by improving the separation, e.g., with a spherical stationary phase, forced-flow layer chromatographic technique, or two-dimensional separation. 2D-HPTLC can be performed with different modes of chromatography by using two distinct mobile phases or he same mobile phase but intermediate derivatization. Both procedures allow an even better resolution of complex samples. The online heart-cutting HPTLC-HPLC-(UV)-MS using an online elution head-based interface is suitable to determine if more compounds are coeluted in the bioactive HPTLC zone.

An orthogonal HPLC system with a higher separation efficiency is helpful for the discovery of the compounds present in the zones of interest, but in the case of coeluting compounds, it does not point to the active one(s). The applicability of 2D-LC systems to identify the compounds responsible for the bioactivity will be presented with examples.

This work was supported by the National Research, Development and Innovation Office of Hungary (NKFIH K128921, K128838, and SNN139496) and the Hungarian-Slovenian bilateral grants (2019-2.1.11-TÉT-2020-00115 from NKFIH and BI-HU/21-22-007 from the Slovenian Research Agency).

Keywords: high-performance thin-layer chromatography hyphenations, two-dimensional liquid chromatography, 2D-HPTLC with intermediate derivatization, HPTLC-HPLC

A Microfluidic Device for Digital Manipulation of Gaseous Samples: From Breakthrough Volumes Measurement to Enhanced Preconcentration of Volatile Compounds

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Digital microfluidics is known for fine manipulation of sub-millimeter samples, with applications from biological sample preparation to diagnostic testing. Unfortunately, until now, it is only limited to the manipulation of liquid phases. In this presentation, a new system based on a multipurpose digital microfluidic platform (DMFP) designed to digitally manipulate gaseous samples will be presented. The DMFP relies mostly on interconnected packed micropreconcentrators (µPC) to trap and release the samples depending on their temperature. It will be shown that the DMFP is capable to perform all basic operations of digital microfluidics: trapping/releasing and moving samples, adding samples and separating samples, i.e. making a substraction, using n-pentane as main test compound. More complex programmable use of the DMFP will also be presented : the automatic measurement of breakthrough volume of alkanes on Tenax TA adsorbent, and very promising results on the multi-step manipulation of n-heptane to enhance preconcentration capabilities for concentrating very volatile compounds. The latter involves original successive sets of trapping-transfer-concentration operations using 3 µPC of the DMFP to advantage for the preconcentration of very volatile trace compounds.

Keywords: microfluidics, micropreconcentrators, volatile compounds

Gas Chromatography by Using a Negative Thermal Gradient and Short GC Columns

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Among the several ways of speeding up chromatographic separations, (negative) thermal gradient GC (TGGC) has evolved as a useful technique with great potential for fast and highly efficient separations. The main idea is to use an experimental set-up where the sample is injected at the hot end of a short (2-5 m) capillary GC column and travels towards the detector at the cold end of the column. This leads to a thermal focusing effect as the peaks move along the column, enabling narrow peak widths and consequently high column efficiency. We report here our individual realization of TGGC which is based on a cylindrical ABS housing that has a spiral grove along its hull and is filled with a polymer foam material. The GC column is inserted in a directly resistively heated stainless steel capillary that is positioned just above the spiral grove of the cylinder. While the capillary is uniformly heated by direct current passage, it is non-uniformly cooled by an air stream that is passed through the foam filling of the cylinder, leading to a stronger cooling at the bottom end and at a reduced cooling at the top end of the cylinder. A negative thermal gradient is thus realized along the column as a consequence of the different heating and cooling rates.

We will describe here the different design considerations, leading to a TGGC instrument, and how they affect chromatographic performance. The feasibility of this set-up is demonstrated in the separation of real-world samples. Limitations of this TGGC set-up are discussed and ways to overcome these proposed.

Acknowledgment: The authors gratefully acknowledge the funding of this work by the Austrian Research Promotion Agency (FFG), Proj. no. 879613 ("OPERION").

Keywords: fast GC, thermal gradient GC, separation efficiency, chromatographic theory

Optimization of Organic Molecule Separation and Detection Using Gas Chromatographic Column Based on MEMS Technologies Developed for Planetary and Exobiology Studies

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The work presented here aimed to explore functionalization and characterization of square cross section channels of microchip columns for on board instruments for organic molecule measurements in solar system.

First, the influence of the analytical set-up used to integrate our microchips have been demonstrated and optimized to ensure the most real microchip performances and limit extra-column effects. Thus, several junction capillaries, liner internal diameter and detector parameters were compared and the best combination was chosen.

Then, the influence of stationary phase static coating method parameters was studied by varying the coating velocity and the coating thickness. Efficiency measurement and optical microscopy were used to compare microchips. Results showed that slowing down the coating velocity improved the coating. Moreover, the efficiency and the retention capacity of our microchips highly depend on the film thickness (10,000 ptx.m^{-a} with k = 0.12 for the thinnest film up to almost 5,000 ptx.m^{-a} with k = 0.93 for the thickest film) and varied following the same trend as with conventional GC columns, which is a way to validate our coating method on microchips.

Finally, we compared square cross section and circular cross section and both using conventional GC fused silica capillaries. Result demonstrated the anticipated complications of coating a stationary phase film on a square cross section.

Keywords: gas chromatography, miniaturization, microchips, MEMS, exobiology

Detailed Performance Analysis of Zwitterionic Hydrophilic Interaction Liquid Chromatography Polymer Columns

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In recent years, hydrophilic interaction liquid chromatography (HILIC) has become a popular separation mode for the analysis of polar compounds. Different polar functionalities have been successfully used as HILIC stationary phases, including amide, diol, amino, cyano and zwitterionic stationary phases. In case of zwitterionic (ZIC) HILIC stationary phases, two oppositely charged groups are present in a 1:1 molar ratio. Several manufacturers have developed zwitterionic HILIC columns, such as ZIC-pHILIC columns with permanent polymeric sulfoalkylbetaine zwitterionic functional groups covalently attached to porous polymer beads.

The current study aims to perform a detailed investigation of the performance characteristics of two types of zwitterionic HILIC polymer columns: particle packed and monolithic columns. Due to the different formats of these columns, kinetic plots are used to evaluate their performance in an unbiased way. For this purpose, experimentally determined plate height data are combined with the permeability values of the columns. Subsequently, the performance of the columns is investigated more in-depth by evaluating the individual contributions to mass transfer. For this, effective diffusion coefficients are determined via peak parking experiments, and confirmed by extrapolating the experimental plate height data to low velocities. This results in extremely low values of the B-term, even for compounds with relatively high retention factors, indicating a low amount of longitudinal diffusion in these columns.

To obtain more insight in the relationship between these low values of longitudinal diffusion and the porous zone morphology of and diffusion processes occurring in the columns, it is subsequently investigated whether the experimentally obtained effective diffusion coefficients can be fitted to an equation deduced from the effective medium theory, to deduce values of the corresponding porous zone (intra-particle) diffusion coefficients. It is, however, observed, that the effective medium theory falls short in describing effective diffusion in the studied zwitterionic HILIC polymer columns, and this is attributed to the extremely low intra-particle diffusion occurring in these columns. This low intra-particle diffusion is attributed to very low surface diffusion rates or a strong adsorption, wherein the analytes experience virtually no diffusion when in contact with the stationary phase.

Development and Characterization of HILIC Stationary Phases Modified with Polyacrylamide Prepared by different Polymerization Methods

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Hydrophilic interaction chromatography (HILIC) has been widely used for the separation of highly polar and hydrophilic compounds such as peptides, nucleic acids and saccharides. In this study, preparation and characterization of amide type HILIC stationary phases were carried out, by modifying polyacrylamide (PAAm) onto the surface of porous silica particles, to improve retention and selectivity for structural difference. Three different polymerization methods including free-radical polymerization (FRP), atom transfer radical polymerization (ATRP), and reversible-addition fragmentation-chain-transfer polymerization (RAFT) were employed to functionalize silica particles. Both ATRP and RAFT methods were carried out by surface-initiated polymerization on the silica particles modified with an activated bromoalkyl group in the presence of a copper(I) catalyst for ATRP [1], and water-soluble azo initiator in the presence of a trithiocarbonate derivative for RAFT. All PAAm HILIC columns were characterized by a test scheme [2]. The RAFT and FRP stationary phases contained PAAm with molecular weight (M_W) of 100,000 or more. The molecular weight of PAAm in the solvent reached to M_W = 700,000 in FRP, while it was suppressed to 20,000 in RAFT that helped better binding of PAAm to silica. The ATRP PAAm phases provides 2 to 3 times better retentivity than commercial HILIC phases, with 100 to 200 times higher distribution of shorter polymer chains (M_W = 6,000 to 43,000) compared to the FRP phases. Structural selectivity and the formation of water enriched phases by these columns are also discussed.

[1] A. Taniguchi, T. Ikegami, *J. Chromatogr. A*, 1650 (2021) 462207.
[2] T. Ikegami, A. Taniguchi, T. Okada, K. Horie, S. Arase, Y. Ikegami, *J. Chromatogr. A*, 1638 (2021) 461850.

Keywords: HILIC, Polyacrylamide, ATRP, RAFT, Characterization

The Potential of Hydrophilic Interaction Liquid Chromatography in the Separation of Glycopeptides

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Protein glycosylation is the most frequent and significant post–translation modification, which influences, among others, cell recognition, receptor activation, protein stabilization and folding. The analysis of glycopeptides is challenging due the requirement of analyzing both glycosylation sites and glycan structures, simultaneously. Hydrophilic interaction liquid chromatography (HILIC) is a powerful separation technique used in glycoproteomics, which provides better separation of different glycoforms than reversed phase chromatography. In this work, we studied the retention behavior of intact *N*–glycopeptides derived from different glycoproteins (human hemopexin and immunoglobulin G, RNase B) on different HILIC columns (HALO® penta–HILIC, Glycan BEH Amide, and ZIC–HILIC) based on their glycan composition. In general, with addition of neutral monosaccharide unit the retention time of the studied glycopeptides increased. Moreover, in case of sialylated glycoforms the retention enhanced more significantly. Additionally, we compared the above–mentioned HILIC columns in the separation of fucosylated and sialylated hemopexin glycopeptide isomers. The HALO® penta–HILIC column showed the best separation of fucosylated and sialylated isomers, followed by the Glycan BEH Amide column, whereas the ZIC–HILIC column provided a very poor chromatographic resolution.

Keywords: glycoproteomics, glycopeptide separation, hydrophilic interaction liquid chromatography

Quantitative Assessment of Retention Mechanisms in Hydrophilic Interaction Chromatography (HILIC)

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Decades of research has demonstrated that various mechanisms are involved in governing the retention of polar compounds in hydrophilic interaction chromatography (HILIC), including hydrophilic partitioning, surface adsorption, and electrostatic interactions between charged analytes and stationary phases. However, the current understanding is only at the qualitative level and the quantitative contribution of each mechanism to the overall retention is not known. This is largely due to the lack of appropriate methodologies to assess each mechanism quantitatively. To this end, we have developed a methodology based on the thermodynamic principle of partitioning to quantitatively investigate the retention contributed by partitioning and adsorption mechanisms. Based on phase ratio variation by changing salt concentration in the mobile phase, we are able to determine the distribution coefficient of a non-ionized analyte (cytosine) between the immobilized water layer and the mobile phase containing different levels of acetonitrile. Then the retention factors of cytosine attributed to partitioning and adsorption are calculated and the percent contribution of each mechanism provides a quantitative understanding of the retention mechanisms in HILIC. Preliminary results demonstrate that hydrophilic partitioning is the dominant retention mechanism for cytosine on ZIC-HILIC, XBridge Amide and LUNA-HILIC columns. The current work is only proof-of-concept which validates the proposed methodology. More work is under progress to investigate a wide range of polar compounds on more polar stationary phases.

Keywords: HILIC, retention, mechanisms, partitioning, adsorption

New Frontier in Ultra Fast-GC from Theory to Pratice

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The present lecture is focussed on the optimization of very fast GC analysis. Narrow-bore columns allow fast separation as proven by the Golay equation. Maintaining the phase ratio β very high the contribute of resistance to mass transfer in the liquid phase to band broadening is very small, and can be neglected. In this case the minimum plate height at the optimum linear velocity will approach the value of the column diameter.

These parameters for the selection of experimental conditions were optimized for the fast analysis of different matrices.

This approach permits the separation of the components in less than two minutes, maintaining the same resolution as a conventional GC analysis of about 50 minutes, and with quantitative results that well agree each other. In addition, fast GC/MS coupling permits to acquire MS spectra free from interference, easier to compare with those of standard components, since capillary columns used for fast GC analysis give a very low bleeding due to the very thin film of stationary phase.

Keywords: Fast GC, narrow bore columns, Golay equation,

Investigation of Analytical Artifacts in the Application of Thermodesorption – Comprehensive Two-Dimensional Gas Chromatography - Mass Spectrometry for the Study of Particulate Matter and Atmospheric Processes

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In this study, thermal desorption (TD) two-dimensional gas chromatography coupled to time-of-flight mass spectrometry (GC×GC-TOFMS) is applied to investigate the chemical composition of the organic fraction of atmospheric particulate matter (PM). The challenges of combining a TD process with GC×GC-TOFMS will be herein addressed with focus directed to the investigation of analytical artifacts. The objective of this study is to demonstrate how the TD process might alter the sample matrix, thus generating uncertain qualitative and quantitative results.

Laboratory-generated secondary organic aerosol (SOA) derived from β-pinene oxidation and PM from an urban site (Munich, Germany) were herein investigated. A multistep TD/pyrolysis approach was applied for monitoring thermal decomposition products and oxidations products from TD by varying the maximum desorption/pyrolysis temperature and the temperature gradient ramp. Generally, it has been shown how the thermally labile fraction of the sample matrix will thermally decompose following a trend which is a function of the desorption temperature, the temperature gradient ramp of the heating process, as well as the aerosol chemical composition.

Keywords: comprehensive two-dimensional GC, aerosols analysis, thermal desorption, analytical artifacts.

Optimization of GC Columns with Radially-Elongated Pillars with Different Coatings as Second Dimension of Comprehensive two-Dimensional gas Chromatography (GC×µGC)

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Miniaturization of GC column is of primary importance in space exploration and field analysis. In this study, GC columns with radially-elongated pillars (REP) (L= 70 cm, 75 µm deep and 6.195 mm wide) were coated, then tested in comprehensive two-dimensional gas chromatography. Stationary phases (apolar PDMS, medium polar RTIL based on monocationic phosphonium derivative and polar PEG-1000) were successfully coated using the static method. The solvent evaporation step was optimized using programed vacuum pressure improving the efficiency by approximately 15%. The best efficiency reached up to N = 62,000 theoretical plates. A separation of a mixture of 11 volatile compounds using a temperature program on a PDMS-coated chip was achieved in less than 36 s with conventional GC system. The coated chips were tested using a microfluidic reverse fill/flush flow modulator in a GC×µGC system. The REP columns showed a high compatibility with the operating conditions in terms of flow rate. A particularly promising feature of the REP columns is their capability to combine high efficiency with high flow rate which is important to obtain an efficient re-injection using microfluidic modulators.

Keywords: Radially-elongated pillars stationary phase coating, bidimensional gas chromatography, multi-capillary column

Short-Column Gas-Chromatography for the Analysis of Thermally Labile and High Boiling Compounds of Forensic Interest

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Gas chromatography (GC) is a widely used technique for separation, identification, and characterization of substances. However, GC is limited to the analyses of volatile and thermally stable substances. Special techniques have been developed to allow the analysis of thermolabile and high boiling compounds. Some make use of special injectors such as cold on-column, others rely on interface or ionization hardware such as field ionization and supersonic molecular beams. There is also the possibility of derivatizing high boiling and thermolabile compounds to a more volatile and stable ones, but derivatization techniques are not universal, and some previous knowledge of what is in the sample is fundamental for the success of such approach. In this work, we developed a short-column GC method to analyze thermolabile and high boiling compounds of forensic interest in a standard GC–mass spectrometer (7890A / 5975C models, Agilent). A 7.5-fold reduction in the column length (from a standard 30 meters to 4 meters) drastically reduced the harsh interactions of thermolabile compounds with the column, while decreasing method's resolution by only 2.7 times. The short-column method also reduced the system pressure, allowing for higher gas flow rate (4 mL/min) and lower analyte elution temperature. A split injection (20:1) reduced the residence time at the injection port. These characteristics together allowed the detection of the 25R-NBOH (R= Br, Cl, I, Et) family of thermolabile drugs, and high boiling cocaine cutting agents (HBCCA) together with cocaine and other 16 commonly observed CCA. HBCCA were detected for the first time in seized cocaine samples with a prevalence of 84.2% in crack cocaine and 21.5% in cocaine salt samples.

Keywords: Short-column gas chromatography, thermally labile, high boiling point, forensic chemistry

Boosting the Downstream Processing of Biopharmaceuticals by Means of Multicolumn Continuous Chromatography

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Purification procedures (downstream processing) still represents the bottleneck, in terms of both cost, time and sustainability, of the entire production process of biopharmaceuticals. Purification is usually carried out through single-column preparative liquid chromatography with two or more chromatographic steps. The first one (capture step) is needed to remove all non-product-related impurities (e.g., host cells or DNA). Afterwards, one or more polishing steps remove all the product-related impurities (e.g., truncated species or diastereoisomers). However, single-column processes suffer of some intrinsic limitations. Indeed, the trade-off between capacity utilization and productivity can be very relevant in the capture step, while polishing processes are characterized by yield-purity trade-off.

These limitations can be partially overcome with multicolumn countercurrent continuous chromatography, which allows for the internal recycle of the product into the system. Thus, the purification process can be completely automated, with minimum need of human intervention, meantime reproducibility is improved, and solvent consumption is reduced. This communication will show, through a series of case studies, the great potential of innovative multicolumn platforms for the capture and the polishing steps in the manufacturing of biopharmaceuticals.

Keywords: biopharmaceuticals, purification, multicolumn countercurrent chromatography, preparative chromatography, process chromatography

Recycling Liquid Chromatography for the Analysis of Polymer Composition

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Synthetic polymers typically show dispersity in molecular weight and potentially in chemical composition. For the analysis of the chemical composition distribution gradient liquid chromatography may be used. The chemical composition distribution obtained using this method is often convoluted with an underlying molecular weight distribution. In this work we illustrate that the influence of this molecular weight distribution can be reduced using very steep gradients and that such gradients are best realized utilizing recycling gradient-elution liquid chromatography. This method allows for a more accurate determination of a polymer's chemical composition distribution and allows one to determine (approximate) critical conditions if these exist. Several practical aspects regarding the successful use of this method are first presented. The approach is then demonstrated for several polystyrene standards, and for the separation of statistical copolymers consisting of styrene/methyl methacrylate and methyl methacrylate/butyl methacrylate. Finally, remaining opportunities are highlighted.

Keywords: Gradient Recycling, Chromatography, Gradient elution, Polymer analysis.

The Quest for Evermore Speed and Efficiency in Chiral Chromatography: Considerations on the Effect of Particle Geometry, Particle Pore Size, and the Loading of Chiral Selector

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This work aims at presenting some fundamental considerations regarding the most innovative trends towards the preparation of more and more efficient chiral stationary phases (CSPs) for fast chiral separations. By considering different typologies of CSPs, including polysaccharide-based, macrocyclic antibiotics, brush-type, and different kinds of chiral analytes and chromatographic modes (RP and NP, HILIC, SFC, etc.), data will be presented to reason on the effect of particle geometry, particle pore size, and the loading of chiral selector on the kinetic performance of chiral separations.

Machine-learning for Chiral Method-development in High-throughput Laboratory – Status Update

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Selection of chiral stationary-phase (CSP) for enantiomer separation in a high-throughput laboratory, even after decades of research, remains more of a guess-work rather than a strategy based on science. The main reason is the complexity of the retention mechanism inside a CSP. Chiral selectivity depends on multitudes of inter-molecular interactions between the CSP and the racemates, making it difficult, if not impossible, to develop a general set of rules for CSP selection. Most prevalent practice is to screen a number of CSPs assuming one of them will generate the required resolution.

Increasing examples of success of machine-learning (ML) algorithms in "learning" the behavior of a complex phenomenon, rather than trying to develop a rule-based approach, have created optimism of having a program that can select the right CSP based on compound structure. Publications on this topic, however, demonstrates the difficulties in developing such a program. Even its practical utility in high-throughput environment is questioned. Availability of ultra-performance instruments, allowing significant screen-time reduction, coupled with the practice of over-night screening, reduces the incentives of investing resources to develop such a model.

In this presentation we describe a strategy that has a simple, achievable objective but still can have a significant impact in drug-discovery. The objective is to determine from the compound structure whether it is going to be a routine or problematic separation. An early warning on a problematic separation can be a significant time-saver for both the analysts and the medicinal chemists. In this presentation we will (a) describe different solution options with ML, (b) present the proposed solution with supporting results.

Keywords: purification, machine-learning, solvent-gradient, focused-gradient

Enantiomeric Separations Based on High-Performance Liquid Chromatography Utilizing Different Types of Chiral Stationary Phases

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To separate enantiomers of chiral compounds, especially those of biological and/or pharmaceutical importance high-performance liquid chromatography (HPLC) is one of the best solutions. Depending on the nature of analyte chiral stationary phases based on modified polysaccharides, macrocyclic antibiotics, or Cinchona alkaloids may offer a choice for efficient enantiorecognition. In this study, enantioseparations were examined in reversed-phase, normal-phase, polar organic, and polar ionic modes depending on the chiral stationary phase and the properties of the analytes. Based on the structural peculiarities of the analytes focus has been placed on the evaluation of structure-retention relationships. For the thermodynamic characterization, temperature-dependence studies were also carried out.

Keywords: enantioselective separation; HPLC;

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Chiral Liquid Crystals – Neverending Separation Challenge

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Liquid crystalline materials containing a chiral center are priced for their unique and useful properties as compared to their achiral analogues. Mesomorphic properties of chiral liquid crystals strongly depend on the enantiomeric purity of the material. Therefore, there is a big demand for fast and reliable separation methods for the analysis of their optical purity.

Fast and efficient ultra-performance chromatographic methods (UHPLC, SFC) were developed for enantioseparation of newly synthesized liquid crystals of different structures. The compounds differentiate primarily in the type of chiral center (lactic acid, octanol, etc.) but also in a number of benzene rings, length of alkyl side chains, and presence of halogen as a lateral substituent. Both techniques proved to be suitable and baseline enantioseparation of all compounds was achieved with tris(3,5-dimethylphenylcarbamate) derivative of amylose or cellulose as chiral selector. The effect of the analyte structure on the enantioseparation was assessed. Significant effect of type of mobile phase modifier was observed in both systems including switching the enantiomer elution order based on both the type of the modifier and its portion in the mobile phase.

Keywords: chirality, liquid crystals, enantioseparation, UHPLC, SFC

Modular Closed-Loop Open-Source Platform for Automated Interpretive Method Development in (2d) Liquid Chromatography Using Retention Modelling and Machine Learning

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One and two-dimensional liquid chromatography (LC) technology is making remarkable advancements, however the number of potential users remains lagging behind for 2D separations. A prime obstacle for large-scale usage is the considerable investment needed for method development and data processing. Automation in any step of the method development reduces the required investment.

Our groups have previously proposed a workflow for the optimization of gradient parameters in comprehensive 2D-LC (LC×LC), inspired by the work of Dolan and co-workers, and Schoenmakers. Schoenmakers was inspired by the interpretive design of the work by Laub and Purnell for GC. The term interpretive refers to the applicability of the workflow to samples of unknown composition, which is in stark contrast to almost all of the above approaches in which the user is required to specify chemical structural information or retention times of all compounds of interest, and – to facilitate the latter – ensure that the sample complexity is limited. This prospect is not feasible for the highly complex mixtures usually targeted by UHPLC and LC×LC methods. Indeed, also our previous 2D workflow was arguably wellreceived, but also received the criticism that manual assignment of all peaks was not viable. At the same time, many of the above approaches have highly unique and useful traits for method development.

To address this challenge, we present a comprehensive, modular, closed-loop and interpretive algorithm for automated LC method development for complex samples of unknown composition. Novel metrics have been developed to facilitate automation. Our computer platform directly and iteratively programs the LC with new method parameters obtained from previous raw experimental data until convergence of a specified objective function is reached. To our knowledge, this is the first time such an interpretive closed-loop system is reported. Our aim is not to present a final – best – solution, but rather to give a state-of-the-art roadmap supported by experimental data and the newly developed metrics for this project. Our algorithm is designed to be modular so as to be inclusive towards all efforts globally in method development for LC.

This presentation will detail this algorithm, its design, and performance tested using a retention modelling strategy as well as a machine learning strategy.

Self-Organizing Maps for the Classification and Exploration of Thin-Layer Chromatograms

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The Self-Organizing Map (SOM) is a data-processing algorithm that allows visualizing high-dimensional data on a two-dimensional map. Similar samples are placed close together, and the difference between adjacent grid squares is plotted as well. On such a map, a data set can be explored intuitively for associated and unusual samples. In addition, unknown samples can be assigned automatically to a class, and the signals in the input that are common or different between regions can be explored.

We established the use of SOMs for the analysis of high-performance thin-layer chromatograms (HPTLCs; DOI: 10.1016/j.talanta.2021.122460). HPTLC is most useful for the analysis of biological samples with little sample preparation due to its matrix tolerance. It allows generating fairly-sized data sets with few resources, offering a choice of detection modes, which can readily be combined for SOM calculation. The samples of two datasets (495 essential oil samples, 40 wheat anthocyanin samples) were classified correctly using videodensitometric data (visual, reflectance) only. For each incorrect assignments, a cause was identified either in the chromatographic data or in the composition of the sample. Furthermore, we found SOMs useful for automated one-class and multi-class classification; selecting unusual samples for closer examination; identifying the peaks that contributed to a certain classification; uncovering interfering signals in misclassified samples, which in turn contributed to method development; and for validating the robustness of the chromatographic method by dissimilarity values.

Keywords: Anthocyanins, Essential oils, Principal component analysis, SOMQC Index, Wheat

Analytical Quality-by-Design (AQbD) Model-based Comparison of HPLC-Separation Systems using Multivariate Eluent Design Spaces – Establishing Stationary Phase-independent Separation of Ezetimibe and Related Impurities Across Several Column Chemistries

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The selectivity provided by the given column chemistry (C18, C8, embedded, etc.) is always considered to play a substantial role in high-pressure liquid chromatography separation. Selecting appropriate – ideally the best – stationary phase is therefore one of the first objectives during the early-stage method development. At later stages of development work however, the main objective shifts more towards selectivity optimization and validation with the outcome of tight method specification – clear definition of the stationary phase, including column brand name, dimensions, and physicochemical properties. Nevertheless, finding backup/replacement columns with identical or similar selectivities for the developed method is still of great interest, for a worst case, if the primary chosen stationary phase becomes unavailable (supply chain issues or discontinued production).

There are different method development strategies applied (trial-and-error, one-factor-at-the-time and systematic) in the analytical field today, to find the most attractive stationary phase and method parameter combination for a specific separation problem. Since the early days of chromatography however, there has always been a clear reference to employing systematic and highly predictive modeling tools for a consistent method design. Furthermore, the newest (currently draft) ICH guidelines Q14 and Q2(R2) along with the accepted Q12 set new standards to analytical procedure development by urging HPLC-specialists to follow «enhanced» modeling approaches to build a science-driven, risk-, and knowledge-oriented development routine which in turn, would also allow the transition from the earlier fixed setpoint concept more towards a flexible Method Operable Design Region (MODR).

This said, in our work, we used a chromatography-based modeling software (DryLab), with the main focus on building 3-dimensional separation models of ezetimibe and its related substances on 9 different RP-columns. Based on only twelve model input experiments per column, we investigated the impact of all chromatographically relevant method parameters – such as gradient, column temperature, ternary composition of the mobile phase and other instrumental factors – on the efficiency of the separation process. The acquired multivariate eluent design spaces (DS) provided in-depth characterization of

each separation systems with certain tolerances of relevant method parameters, as fostered by the recent analytical Quality-by-Design (AQbD) methodology. We used the design spaces of the individual separation units to identify both dissimilar and interchangeable areas in their method operable design regions (MODRs). Advanced multi-attribute modeling options of the software also allowed us not only to pinpoint optimum setpoints of each separation systems but also to customize method goals by considering concurrent fulfillments of multiple method-specific criteria – resolution, runtime, peak-shape. Finally, from the overlapping MODR areas, a common – virtually stationary-phase independent setpoint – was chosen and as a proof-of-concept, experimentally verified on all 9 modeled separation systems.

Keywords: Column-interchangeability Study, Analytical Quality by Design, Design Space Modeling, (U)HPLC Column Characterization & Comparison, Reversed-phase analysis

In silico Separation of Overlapped Chromatographic 2 Peaks with the Expectation-Maximization Algorithm

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Chromatographic separation has always been dependent on physical instrumentation. However, as computer science prevailed, the urge to enhance separation science with just implemented mathematics has also been ever-present. We are using an expectation-maximization algorithm to discover and separate overlapping peaks. This method is capable of narrowing the chromatographic peaks with iteration calculations. While doing so, it keeps the areas intact and retention times consistent, securing precise evaluation. Over in silico experiments, we are exploring the effects of its parameters. Meanwhile, we are showing the boundaries and capabilities of this method. It can separate almost merged peaks. Also, it not only can separate two but multiple highly overlapping components. And it can work in low signal per noise. Even lower than the limit of detection. And finally, we are constructing calibration curves on unseparated components using measured chromatograms. By comparing it to commercial evaluation software, we display this algorithm's ability to enhance chromatographic separations. This method is capable of successfully determining the concentration of two overlapping peaks. It has the same efficiency as separated components. The two are so overlapping that the commercial software can only detect one.

Keywords: chromatography, expectation-maximization, chemometry, resolution enhancement

Fundamentals of Adsorption Applied to Analytical and Preparative Liquid Chromatography for Cannabinoids Separations

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The popularity and the recent legalization of cannabis products have contributed to the increase of the demand for accurate analytical methods, able to provide a detailed characterization of cannabis samples and extracts, and fast, efficient, and cost-effective methods for the isolation and purification of cannabinoids.

Despite cannabis has become a very hot topic in the last years, just a few fundamental studies about retention and adsorption of cannabinoids in liquid chromatography have been performed. As a consequence, the development of separation processes is still based on trial and error method, constituting an important limitation, especially from the industrial view point, where time and operative costs need to be accordingly optimized. In this context, the investigation and the understanding of the main factors affecting the separation (i.e. kinetic and thermodynamic factors) may be useful for the selection of the proper stationary phase and other experimental conditions (mobile phase type, flow rate, etc.) for both the separation and isolation of cannabinoids.

In this work, a detailed study of the influence of the stationary phase chemistry (apolar, polar, chiral, etc.) and elution mode (normal and reversed phase) on retention and selectivity of a series of cannabinoids will be presented. The acquired information will also be used to model the purification process under preparative scale, with reduced waste of sample, solvents and time, and avoiding trial and error strategy.

Keywords: cannabis, liquid chromatography, kinetics, thermodynamics, preparative LC

Application of Prediction Intervals to the Interpretation of the Robustness Study of a UHPLC Method for the Separation of Cannabinoids

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Among the analytical methods used to analyze cannabinoids (CNBs) and other compounds in different matrices, in the last decade, ultra-high-performance liquid chromatography (UHPLC) coupled with UV or MS detectors has been used to increase the chromatographic performance of the analysis of cannabinoids present in different matrices.

In this context, the aim of this work is to discuss the robustness of a UHPLC-UV method developed for the analysis of the major cannabinoids present in plant materials by applying prediction intervals calculations to the Design of Experiments (DoE) results.

As already reported [1], a specific tool based on the prediction interval of the matrix experimental results can be used to verify that the joint effect of the studied factors does not influence significantly the final responses and to estimate the day to day variation interval of the analytical procedure results.

In addition to this calculation, two others original tools are proposed in this work, aiming at increasing the information level obtained from the DoE results. The first one, is based on the results experimental error, and gives information about the measurements dispersion impact on the calculated effects of each studied factor. The second one uses the estimated prediction interval at the factors significance limits to fine tune the Method Operable Design Region (MODR).

[1] J.M. Roussel, M. Righezza, J. Pharm. Biomed. Anal. 193 (2021) 113706

Keywords: Cannabinoids, UHPLC, Robustness, Design of Experiments, Prediction Intervals

Cannabinoids in Sport: Innovative Analytical Methods Can Help

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The World Anti-Doping Agency (WADA) issues the list of prohibited and monitored compounds in sport annually. The prohibited list is not a static and constant one, but it is changing dynamically according to the state-of-art of related medical and chemical studies. However, cannabidiol (CBD) was removed recently from the list of banned substances, other natural and synthetic cannabinoids are still remained prohibited. Furthermore, since 2021 Δ^9-tetrahydrocannabinol (THC) has been considered as a "Substance of abuse" by WADA. Many top athletes try to find "legal" performance enhancing products, especially containing CBD, but instead of being safer, their use can lead to a positive doping test. The analysis of dietary supplements (DS) and sports food containing CBD can be very important. Consequently, our scope was to analyse these products with suitable and innovative chromatographic techniques (liquid (LC) and gas (GC)) coupled to mass spectrometry (MS). Our analytical methods were optimized to obtain low, but also practical limit of detection of the 10 most present cannabinoids. Two well-complementary and substitutive limit test methods were developed using LC-MS/MS and GC-MS/MS techniques prior to a common sample preparation (except for derivatization for GC). At the validation, detection limits of 100 ng/g and 100 ng/ml were determined. Among 11 different analysed products with cannabis extract or CBD (e.g. chewing gum, chocolate, DS, oil)) 4 of them contained almost one prohibited cannabinoid. At the same time in 3 cases the measured content of the desired active substances, CBD, was not detected. Due to the analysis of the products prior to use by elite athletes can support the choice of safe DS and sport foods and reduce the possibility of unintentional violation of doping rules.

Keywords: dietary supplement, cannabinoids, prohibited substances, chromatography, mass spectrometry

Use of Synergistic Systems in EKC, HPLC and LC-MS for Improved Separations – Evaluation of Extraction Procedures for the Quantification of Cannabinoids, Polyphenols and Aloins in Plants and Products

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This presentation will basically cover recent advances and the most important applications of capillary electrophoresis and liquid chromatography performed in the Instrumental Analysis Laboratory. In our work, the relatively new chiral selectors, called cyclofructans (CFs), and the new class of chiral ionic liquids derived from amino acid esters (AAILs) are employed as chiral selectors in electrokinetic chromatography (EKC) and high-performance liquid chromatography (HPLC), and their potential chiral discrimination capabilities are evaluated. In addition, it is examined whether the combination of either CFs or cyclodextrins with AAILs can improve separations of chiral compounds, whose enantiomers, according to literature, are difficult to be separated. Finally, the investigation and the evaluation of different extraction procedures, such as solid phase extraction, soxhlet and ultrasound assisted extraction, is discussed. All methods of extraction and purification were compared in order to determine the one that is the most effective, in regard to analyte recovery, time, difficulty, and precision, for the extraction of cannabinoids, polyphenols and compounds with laxative action.

Keywords: chiral selectors, ionic liquids, liquid chromatography, electrokinetic chromatography, extraction

Multidimensional Liquid Chromatography as a Powerful Tool to Enhance Selectivity in Pharmaceutical Analysis

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Pharmaceutical analysis is challenged nowadays by new therapeutic modalities and formulations which bring about higher degree of structural complexity, uncommon challenges due to larger molecular dispersity, extended molecular mass ranges, microheterogeneities, and polydisperse lipid excipients. All those peculiarities require careful structural characterization and stringent batch-to-batch process control. At the same time impurity profiles become more complex, a fact that is amplified by new formulations e.g. lipid emulsions which may contribute to the complexity of impurity profiles.

Standard analytical methodologies have shortcomings for analytical profiling of such pharmaceutical products and may need an array of assays for a full characterization. One-dimensional analytical methods may suffer from a variety of limitations, like insufficient peak capacity, inadequate selectivity to deal with two independent structural dimensions of sample constituents, and incompatibility of various chromatographic modes with ESI-MS detection. 2D-LC with multi-detector approach can be an effective solution for many of those problems. Its potential for pharmaceutical analysis will be discussed by selected applications from impurity profiling, oligonucleotide analysis, peptide therapeutics characterization, biopharmaceutical process control and enantioselective analysis.

Keywords: UHPLC, Multidimensional LC, Pharmaceutical Analysis, Selectivity

Retention Modelling on Stationary-Phase-Assisted-Modulation Columns for Application in Two-Dimensional Liquid Chromatography

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Liquid chromatography (LC) is a key technique in the arsenal of an analytical chemist. The peak capacity of LC can be further increased by going from one-dimensional to comprehensive two-dimensional liquid chromatography (LC×LC). In conventional LC×LC, incompatibility issues could arise, and the sensitivity is decreased because of the dilution. These problems can be overcome by using stationary-phase-assisted modulation (SPAM), which utilizes trapping columns to only retain the analytes on the column and discard the ^{a}D eluent. With SPAM, the analytes are preconcentrated before entering the ^{b}D column. It reduces the injection volume of the ^{b}D separation and the associated band broadening. The major requirement for SPAM is that the analytes must be effectively retained on (and later released from) the trapping column. If the analytes are not retained, this will result in sample loss. A possible solution could be the addition of a dilution flow to increase the retention of the analytes on the trap column. These are often chosen randomly, which does not eliminate the possibility of losing the early eluting compounds.

In retention modelling, analyte-specific parameters are obtained by performing scanning experiments. These can be used to predict retention at different concentrations of organic modifier or for different gradients. In this presentation, the use of retention modelling will be demonstrated for developing and optimizing SPAM in LC×LC. First, the retention on trap columns is compared with that on analytical columns. Then, the best set of scanning-experiments on the analytical column is discussed for predictions on the trap columns. Finally, the retention is predicted as a function of the dilution flow. We will present a new tool that allows scientist to predict the applicability of SPAM in their setup without having to test trap columns.

Keywords: two-dimensional liquid chromatography, stationary-phase-assisted modulation, active

The Incentive of Dual Injection Capabilities for Method Development and Quantitation in Heart-cut 2D-LC

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Heart-cut-based 2D-LC workflows are continuously gaining popularity in liquid chromatography, as they add either orthogonal or specific separation criteria, or they enable additional detection capabilities, when the mobile phase of the first dimension is not compatible with a certain detector type. Cutting peaks from a phosphate bearing mobile phase LC-UV dimension into an MS-friendly second dimension method for peak identity confirmation by mass is one example.

While 2D-LC instrumentation is becoming increasingly user-friendly, challenges with method development and optimization, as well as calibration and quantification are still impeding success. Dual arm injection devices (as in open autosamplers) are mostly used for sample preparation automation and/or throughput increase, but they can also open a plethora of capabilities in 2D-LC. A major benefit for 2D-LC is the direct injection into the 2nd dimension, which is far more cumbersome and restricted, when a single arm sampler is switched or manually replumbed between the two dimensions.

In this presentation we will demonstrate the incentive of a compact dual injection arm sampler in a 2D-LC setup for the simultaneous development and optimization of separation and detection methods in both dimensions, but also to verify transfer ratios and analyte mass balances. We also highlight the incentive to inject calibrants into an LC-MS-MS 2nd dimension with a time offset (ECHO injection) relative to fraction switch, as an alternative to isotope dilution for matrix effect compensation, when no isotope labelled calibrants are available. The dual sampler concept is finally compared to an advanced 2D-LC setup with switched single arm injector, which employs a multi detection configuration with radioactivity detector and orbitrap tribrid MS and is applied in a drug metabolism and pharmacokinetics laboratory.

Keywords: dual split sampler, multi-heartcut 2D-LC, multi-detector setup, ECHO peak technique, 2D-LC to dual 1D-LC switch

Improved Analytical and Preparative Separation Methods of Therapeutic Oligonucleotides using Digital Tools

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Oligonucleotide (ON) therapeutics constitute a growing class of drugs meeting society's need for more effective interventions in hitherto incurable diseases. There are several FDAapproved ON -based therapeutics and many more are awaiting approval/undergoing clinical trials, so this area has potential for strong growth in the near future. However, the complicated synthesis and degradation pathways of ONs, with sophisticated new chemical modifications, generate hundreds of impurities such as shortmers/longmers, in contrast to yesterday's quality-controlled and bestselling small-size drug molecules, which typically contain only around three to five well-defined impurities. Therefore, this new class of putative drugs entails challenging separation tasks: for example, a small mass change such as 1 Da must be distinguished in a 10,000 Da parent molecule for purposes of both quantification and purification and at extremely high resolution. As well, ON therapeutics must be chemically modified before entering the body, in turn generating extreme challenges. One such challenge is the phosphorothioate modification generating diastereomers: for a 20-nucleotide-long fully phosphorothioated ON, this exceeds half a million diastereomers. For obvious practical reasons, the tendency of current separation systems to separate diastereomers is usually mitigated.

The analyzing, characterization and purifications of these medium-sized drugs are therefore most challenging and in this lecture we will present strategies based on digitalization concept to overcome these challenges. We will also present our most recent research findings for the analysis and purification of therapeutic oligonucleotides. We will concentrate on today's best generic separation method (i.e., ion-pair reversed-phase liquid chromatography), on selecting the proper operational conditions based on a fundamental understanding of the separation processes and of the impact of important operational settings and experimental parameters (e.g., the selection of proper stationary phases) based on surface chemistry, particle size, and pore size, and on selecting the best mobile-phase composition, gradient composition, and type.

The Impact of Low Adsorption Surfaces for the Chromatographic Analysis of Oligonucleotides

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As interest in oligonucleotide therapeutics is increasing, there is a need to develop sensitive analytical methods to properly analyze the oligonucleotide itself and its related impurities. However, sensitivity of oligonucleotide separations has always been a major concern. It has been widely reported that oligonucleotides, because of their electronrich backbone, suffer from undesired and often adsorptive interactions with chromatographic surfaces. Known as non-specific adsorption, this phenomenon negatively impacts chromatographic performance by reducing recovery and altering peak shapes (tailing, asymmetry). Metal components of the chromatographic hardware are particularly prone to adsorption. Hence, there is a need of permanent solutions to minimize the interactions of analytes with the chromatographic surfaces. To this end, novel surface technologies applied to sample vials, chromatographic columns and liquid chromatography systems are emerging in the last few years. Referred to as being bioinert, they are composed of hybrid organic-inorganic material, polyether ether ketone or titanium, and they show great promise in mitigation of adsorption. In this context, we comprehensively evaluated the impact of these low adsorption surfaces for the analysis of 15- to 100-mer RNA and DNA oligonucleotides. The whole sample flow path was considered, including vials, chromatographic columns, and instrumentation. This study highlights the importance of using bioinert surfaces for the analysis of oligonucleotides and it helps understand the contribution of each surface on the overall oligonucleotide adsorption.

Keywords: oligonucleotides, liquid chromatography, bioinert surfaces, low adsorption surfaces, metal-free surfaces

Investigation of Supercritical Fluid Chromatography (SFC) Analytical Conditions for Oligonucleotides

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In the synthetic process of oligonucleotides, shorter oligonucleotides or phosphodiester (PO) variants of phosphorotioate (PS) oligonucleotides, are also produced as related impurities. Ion-pair reversed-phased liquid chromatography (RPLC) is the gold standard methodology for the impurities profiling; however, the accurate profiling might be compromised due to co-elution of structurally related impurities. Hence, the better separation techniques are desired.

In this study, we focus on SFC because of its superior structural recognition ability, which is expected to provide a different separation from that of RPLC. The applicability of SFC to oligonucleotide analysis was investigated using short-chain oligothymidilates, T2, T4 and T10, as model compounds. After several attempts, T2, T4 and T10 were separated using a column modified with cyanopropyl groups and general SFC mobile phase containing ammonium formate. We also performed SFC analysis of T4 containing PS linkages and found that it can be separated from the corresponding PO variants.

The preliminary results indicated the possibility of SFC to be applied to the impurity profiling of oligonucleotide therapeutics.

Keywords: Oligonucleotide, impurity, supercritical fluid chromatography, SFC

Oligonucleotide Sequencing by LC-MS/MS: A novel Approach for Characterization and Quality control of mRNA-based Vaccines and Biotherapeutics

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The pandemic pressure of SARS-CoV-2 vastly accelerated the development and acceptance of mRNA-based vaccines. Propelled by this, oligonucleotide-based biologics now generally step out of the shade of their protein counterparts and demand corresponding analytical strategies for their characterization to keep up to pace. The unique, highly charged, linear chain structure of these entities creates novel analytical challenges for sample preparation, chromatography, and mass spectrometry. Protein sequence verification by LCMS the standard for therapeutic proteins, however, a successful method has never been developed for large oligonucleotides so far. Here we present an approach for the sequence analysis of large mRNA using UHPLC and HRMS fragmentation. Partial digestion using RNase T1 is used to create a mRNA map, akin to a protein's peptide map. The digest is tuned to create oligonucleotide fragments of a size that allows their analysis by LC-MS/MS. We show that the chromatographic conditions used predetermine the successful fragmentation required for sequence analysis and propose a model for the ion pair chromatography mechanism behind. Optimization of the HCD fragmentation under these conditions allows sequence analysis of fragments as large as 50 nt. Commercially available software is used to annotate and map the fragment sequence to the known mRNA sequence. Data filters are applied to prevent false identifications in the analysis. Using these combined approaches >80% sequence coverage of a range of mRNA therapeutics was achieved, including the SARS Co-V2 spike protein. The digestion reproducibly produces the same cleavages and missed cleavages to allow a specific chromatographic fragmentation pattern which could be used in a QC environment. The ability to rapidly identify, characterize large mRNA therapeutics with high sequence coverage provides important information for identity testing, sequence validation and impurity analysis.

Keywords: mRNA, mRNA vaccine, sequencing, LC-MS/MS

Establishing the Next-generation Multi-dimensional Liquid Chromatography

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In spatial comprehensive three-dimensional chromatography (3D-LC) components are separated within a three-dimensional separation space that can lead to unprecedented resolving power, in terms of peak capacity and peak-production rate. The maximum peak capacity is the product of the peak capacities achieved in the individual dimensions when orthogonal retention mechanisms are incorporated. The parallel development of the second- and third-dimension separation stages overcomes the fundamental limitation of conventional multi-dimensional approaches, in which sampled fractions are analyzed sequentially. General considerations for chip design are discussed and possibilities and prospects to establish spatial comprehensive 3D-LC analysis are presented.

Keywords: multi-dimensional liquid chromatography, microfluidic chips

Novel Approaches Minimizing Dilution in Comprehensive Two-Dimensional Liquid Chromatography

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Over the past decades, the two-dimensional comprehensive LC has matured into routinely used technique with several commercially available instruments on the market. Theoretically a higher number of compounds can be separated and quantified in a single analysis compared to that of unidimensional LC. The main drawback of the technique is however the dilution of the analytes during analysis, mainly also due to the contribution of the interface for the transfer of analyzed fractions.

In this work, the factors affecting the dilution of the fractions transferred between the dimensions for combination of reversed-phase systems and hydrophilic interaction LC mode are addressed and discussed for the analyses of naturally occurring flavonoid compounds, and for non-ionic surfactants. The benefits of microscale separations are compared with the application of conventional columns connected using focusing interfaces. Further, various types of focusing interfaces have been tested and compared, and its relation to the optimization of gradient elution profiles is discussed.

The financial support by the Czech Science Foundation, project No. 22-09556S is gratefully acknowledged.

Keywords: two-dimensional comprehensive liquid chromatography; gradient elution; focusing interfaces

Combining Temperature Gradient Elution with Refractive Index Detection through Temperature-Responsive Liquid Chromatography

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Refractive Index Detection (RID) is the liquid chromatography detector most approaching the characteristics of universal detection (allowing ideally for standard independent analysis). Because the refractive index is a bulk property of a substance it gives much more homogeneous response between different analytes as compared to other detectors such as UV or mass spectrometry [1]. Unfortunately, the applicability of RID is limited to isocratic HPLC (High-Performance Liquid Chromatography) because of its extreme sensitivity to compositional solvent changes.

However, temperature-responsive stationary phases can be suitable to circumvent this issue. Temperature-responsive polymers are, typically, water soluble smart polymers, that change their conformation when exposed to an external stimulus, in this case temperature. While various types exist therein the polymers depicting a Lower Critical Solution Temperature have only been used in TRLC so far. At high temperature such polymer dehydrates because monomer-monomer forces are dominant, while at lower temperature the polymer chains interact with water molecule and a unique phase is formed. This mechanism, depicting a gradual transition when the polymer is coupled to silica, allows for the control of analyte retention as a function of temperature as they pass through the column when using only water as mobile phase. Consequently, solvent gradient can be avoided in TRLC, and replaced by temperature gradient, this opens new possibilities for the coupling of RID with this separation mode [2].

In this work, is introduced the hyphenating of TRLC with RID for the analysis of non-UV visible molecules. The method is demonstrated through a downward temperature gradient that shows comparable results to what can be obtain with solvent gradient. Overlapping calibration curves are obtained for chemically similar compounds, meaning that the detector response is identical within a marginal error.

The approach is demonstrated for the analysis of molecules which are challenging to detect by UV (free fatty acids and long chain alcohols). Emphasis is thereby set on the demonstrations of the proof of concept that quantitative temperature gradient TRLC-RID is possible. For this purpose, home-made columns packed with PNIPAAm (Poly(N-isopropylacrylamide)) and end-capped with acetic anhydride were developed and operated with various gradient slopes (-0.25 to -1 °C/min) between 45 and 5 °C. Excellent overlap is thereby obtained between the calibration curves for different free fatty acids. As only about negligible baseline drift was observed, this illustrates the potential of TRLC-RID for the quantitative analysis of analytes depicting too large retention differences via conventional HPLC analyses.

Keywords: temperature gradient, Temperature-Responsive Liquid Chromatography, Refractive Index Detector, hyphenated techniques, PNIPAAm

External Cavity Quantum Cascade Laser (EC-QCL) Coupled to SEC for Online Detection of Functional Groups at ppm Level

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External Cavity Quantum Cascade Laser (EC-QCL) is utilized as a very sensitive IR detector for hyphenation with SEC. Compared to conventional IR laser sources, EC-QCL has a superior spectral power density (i.e., 10^4 more photons per wavenumber), leading to a much higher sensitivity, which allows us to detect low-concentrated functional groups in polymer samples and mixtures. With our custom-designed EC-QCL-based spectrometer, our research group has demonstrated its capability by detecting one carbonyl stretching vibration of PMMA in ~500 000 g/mol when pulsing at 1730 cm^{-1}, with a 5 % duty cycle pulsing frequency. During the studies for achieving the best possible sensitivity by operating the laser in continuous wave (CW) mode (where the duty cycle is 100 %; that is a continuous photon flux onto SEC eluent), it is shown that increasing the duty cycle from 5% to 10% enabled us to detect a functional group in ~700 000 g/mol under identical conditions. A specifically designed flow cell is also discussed for the best possible coupling of SEC with this unique EC-QCL spectrometer. While attaining the ppm level of detection with different duty cycle values in pulse mode, the challenges and the possible solutions (i.e., detector overloading, data handling, and processing) of working with CW mode are also discussed. Coupling of SEC with EC-QCL for IR detection promises a unique and unprecedented sensitive detection scheme for polymer samples, especially when CW mode measurement is activated in the near future.

Keywords: IR spectroscopy, Quantum Cascade Laser, SEC-FTIR coupling, Online detection

Vacuum Jacketed Column Technology for Improved Ultra-High Pressure Liquid Chromatography Mass Spectrometry Performance

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Significant losses in peak resolution are encountered in ultra-high performance liquid chromatography (UHPLC) coupled to mass spectrometry (MS) detection due to excessive post-column dispersion. This issue is of great concern for short (2-10 cm long) narrow-bore (2.1 mm i.d.) columns packed with sub-2 mm particles. In this presentation, a solution consisting of deploying the column as close as possible to the ionization source and running it in absence of a LC oven is proposed, described, and tested. Briefly, a vacuum jacket (VJ) is placed around the column in order to maintain uniform the column temperature along its length. Additionally, a Joule heater is placed at the column outlet to cope with 1) the residual heat leaks observed at both column extremities and 2) the nefarious impact of radial temperature gradients on band deformation expected at high speeds/pressure. The advantages of the VJ column placed in still-air environment directly connected to the MS ionization probe over the same column but installed in the oven of conventional UHPLC-MS systems is confirmed by the simulation of the radial temperature profiles and by the optimum sample band refocusing at the column outlet. In practice, it is shown how it can be successfully applied for the high-throughput gradient (2-98% ACN/Water + 0.1% formic acid, T=296 K) separations of several drug components (< 2 min, 2.1 mm x 100 mm CORTECS-C_{18}), 20 metabolites (< 2.5 min, 2.1 mm x 50 mm CORTECS-C_{18}), and 12 substrates/metabolites (< 1.0 min, 2.1 mm x 30 mm XBridge-C_{18}).

Keywords: Vacuum Jacket Column, UHPLC-MS hyphenation, Peak capacity.

Band Broadening in Multicapillary Columns with Diffusional Bridging – Alleviating the Polydispersity Problem

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It is well-established that capillary columns offer the best compromise between separation performance and pressure drop. Although they are indeed the standard in gas chromatography, they do not have the same status in liquid chromatography. This is partially because limiting their diameters to a few micrometres, as necessary to achieve plate heights comparable to those of contemporary packed bed columns, severely limits sample capacity and thus detection sensitivity. Multicapillary columns comprising up to thousand(s) of such microcapillaries, as proposed by the end of the 20th century, would however not suffer from this limitation.

Alas, multicapillary columns suffer from the so-called polydispersity problem. Variations in capillary diameter cause variations in flow velocity among the capillaries, such that analytes in different capillaries diverge from one another during the separation. This source of band broadening only gets worse when increasing the length of the column, thus limiting the plate count. As a potential solution to this problem, technology has been developed to manufacture multicapillary columns where the capillaries are surrounded by a mesoporous matrix. This facilitates diffusive exchange between the capillaries, reducing the polydispersity problem to a mass transfer problem and alleviating the ensuing band broadening.

In our recent work, we have studied the potential of this diffusional bridging by means of mathematical models and computational simulations. In a first approach, we analysed a system of two capillaries, one broad and one narrow. Since the band broadening in such a system was amenable to an analytical expression, it allowed to gain detailed insight into the diffusional bridging's dependence on the system parameters. In a second approach, we expanded our analysis to a generalised system of N capillaries with random diameters. This system revealed that the dynamics of diffusional bridging are in fact more complex and that the two-capillary approach underestimates the band broadening in a multicapillary column. Nevertheless, both approaches confirm the potential of multicapillary columns with diffusional bridging, encouraging further study.

Keywords: band broadening, column technology, diffusional bridging, multicapillary column, plate height equations

The Importance of Column Compartment Thermostatting and Preheating for Temperature Sensitive Separations in Liquid Chromatography

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Separation temperature plays an important role in high performance liquid chromatography (HPLC) since it can influence retention time, selectivity and peak shape. While temperature is one of the key factors to control during developing and transferring LC methods, the understanding of column thermostatting and mobile phase preheating and their impact on chromatography are often incomplete.

In today's analytical instrument market, most LC systems include either a dedicated column compartment or provide options of various types of column compartments. In general, there are two common types of column compartments, block heater and circulation air types. The different thermostatting mechanism can result in differences in heat transfer efficiency, temperature equilibrium, temperature gradient in the column, etc. For adequate mobile phase preheating, most LC vendors provide preheaters that will heat the mobile phase to the set method temperature prior to solvent introduction into the column. The preheater includes passive or active preheaters. Passive preheaters are capillaries with a certain length that are in direct solid contact to a temperature-controlled surface in the column compartment. Active preheaters use its own internal heating element to actively control the eluent temperature. All these variables can impact the transferability of temperature sensitive separations.

To illustrate this, we conducted a temperature sensitive separation across UHPLC systems from various vendors with different column thermostatting and preheater designs. While many instrument parameters can impact method transfer, this study focuses on the impact of column compartment characteristics and mobile phase preheating. General consideration of column compartments and preheating during method development and method transfer is provided based on the study.

Keywords: column thermostatting, method transfer, temperature sensitive, preheating

Computational Fluid Dynamics Study of the Fine Details of the Viscous Heating Band Broadening and Potential Solutions to Alleviate it in Liquid Chromatography at Pressures up to 2500bar in 2.1 Millimeter Columns

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In the present numerical study, we investigate the fine details of the viscous heating band broadening under extreme high-pressure conditions (2500 bar). We first show that viscous heating leads to two clearly distinguishable band broadening effects, one originating from the radial differences in the species migration velocity and the other from the axial variation in velocity, diffusion coefficient and retention. It was found that the radial contribution is independent of the intrinsic band broadening of the bed. On the other hand, the axial contribution is strongly dependent on it and it is found to be 4 to 5 times lower than the radial contribution.

A number of potential column technology solutions to minimize the additional plate height originating from the viscous heating effect were investigated. For the considered column and conditions, it is found that, in order to keep the global plate height as measured at the column outlet ($H_{vh,glob,out}$) below 1 µm at an operating pressure of 2500 bar, the bed conductivity would need to be raised to 2.4 W/m·K, i.e., 4 times higher than a typical packed bed of fully-porous or core-shell silica particles. An equivalent effect on the band broadening could be obtained if it would be possible to replace the steel column wall with a low conductivity material. In this case, a wall conductivity of 0.25 W/m·K, i.e., 64 times smaller than the conductivity of steel, would be needed to keep $H_{vh,glob,out}$ below 1 µm.

Keywords: UHPLC, Viscous heating, band broadening, plate height, CFD simulations

Tackling Pharmaceutical and Biomedical Questions using Thiolene-based Microfluidic Devices

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Microfluidic devices have long since been established to provide solutions that are not equally well offered by more conventional formats, if at all. Our group has focused on thiol-ene polymers both as building materials for such devices, but also because this material family is easily functionalized using click chemistry, and also still offers a number of little exploited (and little understood) physico-chemical characteristics. I will present recent results from several collaborative projects. This includes progress in using thiolene devices for hydrogen-deuterium exchange mass spectrometry for protein structure elucidation, where microfluidic devices can help probe very short labeling time scales. High-surface structures ("monoliths") made from thiolene have been exploited as efficient immobilized enzyme reactors, both for HDX-MS but also for more specialized uses with expensive or rare enzymes. I will show their use for isolating circular DNA from clinical samples for diagnostic purposes. Finally, thiolene materials are promising within the organ-on-a-chip field, e.g., when realizing soft extraction from cell cultures for mass spectrometric analysis, or when providing oxygen gradients under flow conditions by their intrinsic property to scavenge oxygen from solutions.

Keywords: microfluidic devices, thiolene polymers, HDX-MS, IMERs, cancer diagnostics

A Continuous Microfluidic Sieve for the Size-based Fractionation of Particle Suspensions and Colloids

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We report of a novel microfluidic device concept for the continuous fractionation of a size-dispersed suspension of micrometric/nanometric particles. The device geometry consists of parallel channels communicating through a narrow slit of assigned width, which act as a cutoff length for the size-based separation of the particles. The suspension is fed to one of the channels, and particles of size below the cutoff length spread throughout the device cross-section as they flow downstream, whereas larger particles remain confined to the injection channel. Two regimes are investigated, namely (i): a Brownian-sieving regime, where particle transport across the channels relies entirely on transversal diffusion, and (ii): a convective-sieving regime, where pressure between the channels is made different, and small particles are (primarily) driven by convection from one channel to the other(s). This latter regime is ideally suited for scaling up the device to micrometer-sized suspensions, in that the diminished particle diffusivity has negligible impact on the separation performance. Potentialities and limitations of the method proposed are compared and contrasted to those of other emerging microfluidics-assisted techniques, such as Field-FlowFractionation (FFF) and Deterministic Lateral Displacement (DLD).

Keywords: Hydrodynamic Chromatography, Continuous Fractionation, Brownian sieving, Preparative separation, Microfluidics

Peak Broadening Caused by Using Different Micro Liquid Chromatography Detectors

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Continuing developments in column technology have led to smaller particle diameter and more efficient phases. In parallel, the use of columns with reduced inner diameters is becoming more common. Reduction of the column dimension reduces the effective column volume, thereby making the systems more susceptible to the effects of band broadening due to extra-column volume. Band broadening after the separation column has a considerable negative effect on efficiency. In this study, different mass flow and concentration dependent detectors were examined for their influence on band broadening using a micro-LC-system. A mass spectrometric detector, an evaporative light scattering detector, two ultraviolet detectors, and a newly developed fluorescent detector were compared. The influence on efficiency is compared using plate height vs. linear velocity data and peak variance. It is shown that an increase in inner diameter after the post-column transfer capillary can lead to significant loss in plate height. When comparing the UV detectors, it could be shown that the dispersion was reduced by 38% by the reduction of the post-column volume. These effects could also be confirmed when using the peak standard deviation. The largest variance was found for the ELSD, which was 368% higher compared to the variance of the detector with the least effect on band broadening.

Keywords: Miniaturization; extra-post-column band broadening; micro bore column; green analytical chemistry

High Aspect Ratio Pillar Array Columns for Deep Proteome Profiling at Moderate LC Pump Pressures

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In search of increased separation power, LC column technology has been continuously evolving towards using smaller packing materials to present a continuous feed of peptides to the mass spectrometer. In this contribution, we report the evaluation of a novel type of pillar array column where the combination of reduced inter pillar distance and increased etching aspect ratio result in improved separation performance at moderate operating pressures. Previously, we have reported on a novel generation of pillar array columns where pillar dimensions had been scaled down by a factor of 2 to increase resolving power. Even though separation performance was improved by a factor of up to 1.75, column permeability was decreased by a factor of 12. This seriously limited the range of flow rates at which columns could be operated, but also the maximum length at which they could be designed. By modifying the aspect ratio (AR, pillar height/inter pillar distance) of the separation bed, permeability could again be increased by a factor of 4, opening up opportunities to design LC columns with increased separation length and wider LC flow rate acceptances. Using a 2nd generation pillar array column with a length of 110 cm, peak capacities up to 1600 could be obtained. Significantly more precursors could be characterized when performing single data-dependent LC-MS/MS analyses of a tryptic digest of a human cell line with FAIMS. With LC-MS run times of 90, 120 and 150 min, we respectively identified 6521, 7165 and 7539 protein groups with 1000 ng of sample loaded on column.

Keywords: pillar array column, microfluidics, nanoLC, proteomics, LC-MS

Fit for Purpose Metabolomic Workflows – Key Aspects of Validation

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Metabolomics is a prime example of interdisciplinary science. At its core, the analytical task of measuring the full scope of small molecules within a few analytical runs remains challenging. Successful mass spectrometry approaches tackle multi-platform measurements defining and addressing sub-omes (such as metabolites and lipids) and analytical tasks (such as targeted and non-targeted analysis), individually. While targeted absolute quantification involves a well- defined validation practice, non-target analysis being far more complex, accepts multiple lines of evidence, harmonized by guidelines. Our group proposed different metabolomic/lipidomic strategies relying on high resolution mass spectrometry methods based on isotopically enriched biomass, multiplexed extractions and dual chromatographic separations. In the presented lecture, different aspects of validation will be emphasized as enabled by isotopologue measurement and evaluation.

Quantification of *N*-glycosylation Signatures of Monoclonal Antibodies Using Liquid Chromatography – Mass Spectrometry

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Therapeutic monoclonal antibodies (mAbs) are usually recombinantly expressed by cells that secrete not only a single mAb product, but a mAb pool with different *N*-glycosylation signatures. The chemical composition of *N*-glycans is critical for the proper folding, functionality, efficacy, and safety of therapeutic mAbs. A small portion of mAbs is afucosylated, meaning that they do not carry core-fucosylated *N*-glycans. Afucosylated mAbs have increased FcγRIIIa-affinity, which in turn leads to an enhanced effect of antibody-dependent cellular cytotoxicity (ADCC)[1,2]. For this reason, less abundant glycosylation variants need to be accurately quantified.

Here, we aim to quantify mAb *N*-glycosylation profiles at several protein structural levels using liquid chromatography in conjunction with mass spectrometry. These include glycans released by PNGase F, tryptic glycopeptides, subunit level after IdeS-digestion and after disulfide reduction. In these approaches, the combination of glycosylation pairs is lost and artificial protein modifications can be introduced. Thus, the quantification is supported by intact mAb analysis under denaturing and native conditions. The combination of these approaches also allows for accurate quantification of minor abundant glycosylation variants.

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Keywords: therapeutic monoclonal antibody, quantification, N-glycosylation, protein structural level, LC-MS

Anion-Exchange Chromatography at the Service of Gene Therapy: Boosting the separation of Full/Empty Adeno-Associated Virus Capsids

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Gene therapy is opening unprecedented opportunities for novel therapeutic approaches. Based on the concept of directly replacing malfunctioning genes to allow biological functions to be restored, it requires the use of biological vectors to ensure the proper delivery of therapeutic genes. In this context, recombinant adeno-associated virus (AAV) are the most widely used vectors. Their biomanufacturing process requires the insertion of the therapeutic gene into the AAV (full capsids). However, a percentage of AAV that do not contain the desired gene (empty capsids) might also be produced, potentially impacting the efficiency of the therapy. Therefore, the determination of the full/empty ratio of AAV capsids needs to be monitored to ensure consistent product quality and efficacy. Anion-exchange chromatography (AEX) can serve this need. In this contribution, a thorough AEX method development, including mobile phase and gradient scouting, will be presented to highlight the potential of this approach in supporting gene therapy. Specifically, the analytical workflow will be illustrated with commercial AAV consisting of different serotypes.

Keywords: Anion-exchange chromatography (AEX), recombinant adeno-associated virus (AAV), gene therapy

Expediting the Characterization of COVID-19 Therapeutic mAbs using Innovative Ultra-Short Column Formats and Automated Chromatography-Based Method Modeling

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Recent studies showed that IgG-type monoclonal antibodies (mAbs), in particular tocilizumab, casirivimab and imdevimab, could be administered to aid the recovery of hospitalized COVID-19 patients. However, like all cell culture expressed immunoglobulins, these mAbs are complex molecules with a high degree of heterogenicity. Ultrahigh-pressure liquid chromatography (UHPLC) separation techniques, such as reverse phase (RP), hydrophilic interaction chromatography (HILIC) and ion exchange chromatography (IEX) are well suited for the comprehensive mAb analysis that is used to ensure safe and efficacious treatments.

Starting method development work on new drug candidates can be a difficult, time-intensive process, sometimes leading to delays in drug development and release. In this work, we introduce new, state-of-the-art, ultra-short RP-, HILIC- and IEX column formats (1 – 2 cm long) along with an automated, model-facilitated method development approach for intact and subunit level analysis of the COVID-19 authorized mAb cocktail including casirivimab and imdevimab. Apart from discussing the multifold benefits of this streamlined approach, we will highlight several practical advantages, including (1) the automation of experiments, (2) systematic comparison of different elution modes (e.g. pH gradient vs salt gradient IEX) and (3) virtual method transfer between different column formats (e.g. 50 x 2.1 mm to 10 x 2.1 mm) and chromatographic systems.

With our new approach, the development and verification of three or four complementary analytical methods requires only 1-2 days of experimental work. In the end, one chromatographic analysis can be performed within 1-2 minute run times such that it has become feasible to comprehensively characterize this COVID-19 mAb cocktail by 3 different profiling techniques within a 1 hour turnaround time.

Keywords: COVID-19, Ultra-short columns, Automation, Modelling, mAbs

Characterization of Retention Interactions in Reversed Phase and Hydrophilic Interaction Liquid Chromatography

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Hydrophilic Interaction Liquid Chromatography (HILIC) is a modern mode of liquid chromatography complementary of Reversed Phase Liquid Chromatography (RPLC). HILIC uses polar bonded phase (or even bare silica) columns in combination with water-organic solvent eluents. Water from the eluent is preferentially adsorbed on the polar phase creating immobilized and/or semi-immobilized water-rich layers which act as stationary phase. Solute retention is thus, reversed from that of RPLC retention.

In this presentation, retention interactions in several RPLC and HILIC columns (C18, IAM, zwitterionic, diol, cyano, amino, underivatized silica) with acetonitrile/water and methanol/water mobile phases have been characterized by the Abraham Linear Free Energy Relationship model (LFER) in terms of cavity creation, hydrogen bond acidity and basicity, dipolarity/polarizability, and excess molar refraction. Results show clear differences between RPLC and HILIC columns and some differences in HILIC columns between acetonitrile and methanol eluents.

Moreover, and since the RPLC method requires a lot of measures, simpler methods such as the Tanaka method that uses pairs of indicators to characterize columns in terms of hydrophobicity, shape/steric selectivity, and hydrogen bond capacity are investigated and compared to LFER method. New pairs of LFER-based indicators are also proposed for measuring cavity formation, hydrogen bond acidity, hydrogen bond basicity, dipolarity/polarizability, and excess molar refractivity LFER-interactions.

Keywords: HILIC, Abraham LFERs, Retention models, Tanaka model

The Impact of Organic Modifiers on Hydrophilic Interaction Liquid Chromatography (HILIC) Retention Mechanisms

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Hydrophilic interaction liquid chromatography (HILIC) continues to be an important technique for the analysis of polar analytes. Although acetonitrile is commonly the organic solvent of choice in HILIC, many publications and applications incorporate alternative organic solvents as either the primary component or as a smaller fraction of the overall mobile phase composition. Surprisingly, little is found in the literature regarding how these alternative solvents impact the retention and selectivity from a fundamental perspective.

In this work, the addition of methanol and other water-miscible organic solvents into standard HILIC systems is systematically studied using a select set of acidic, basic and neutral polar probes. Changes in retention and selectivity of these probes are then with respect to the underlying HILIC retention mechanisms that are impacted. Through a better understanding of retention mechanisms, this work promises to aid method developers in intelligently utilizing alternative solvents as additives as a tool to manipulate analyte retention and selectivity. The results are also likely to provide a stronger fundamental picture of HILIC retention mechanisms in general.

Keywords: HILIC, retention mechanisms, solvent systems

Stationary Phases for Hydrophilic Interaction Liquid Chromatography and Use of Multivariate Analysis to Classify Materials

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Hydrophilic Interaction Liquid Chromatography (HILIC) is a powerful separation technique for the retention and resolution of a diverse range of polar and hydrophilic molecules. Analytes retain and elute based on multiple factors – partitioning into and from labile water layer(s) enriched off the hydrophilic stationary phase surface, ionic interactions with charged analytes, hydrogen bonding, dipole-dipole interactions, and even adsorption in some cases. The intricacy of HILIC retention mechanisms, combined with a vast array of stationary phases to choose from, makes column selection potentially time consuming, unclear, and misleading. This work presents data from experiments attained with over fifteen commercially available columns under defined HILIC conditions (a standardized approach that gives information about the hydrophobic selectivity, hydrophilic selectivity, shape selectivity, cation exchange and anion exchange selectivity, and surface acidity/basicity). The use of principal component analysis allowed for clustering of the columns based on their chemical modification. The combination of selectivity data and multivariate analysis tools has helped elucidate retention properties of different stationary phases in HILIC. The study, furthermore, revealed important information about overall separation efficiency and peak shape as a function of column choice. The methodology can be used as an initial column screening approach for HILIC method development as well as an effective tool for the development of new HILIC stationary phase materials.

Keywords: Hydrophilic Interaction Liquid Chromatography, HILIC, Analyte retention, Selectivity, Multivariate Analysis, Principal component analysis

Advances in the Design of Anion Exchange Stationary Phases for Ion Chromatography with Various Bonding Chemistries

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Polymer-based anion exchangers with electrostatically bonded functional layers are known to be the best performing stationary phases for suppressed ion chromatography. Their high efficiency results from the presence of negatively charged barrier of sulfonic groups on the polymer surface, which prevents the diffusion of analytes into the particle. Combination of electrostatic attachment of functional layers with hyperbranching technology, which includes cyclic treatment of resin with diepoxides and amines, adds versatility to the system and provides several options for manipulating selectivity of the prepared anion exchangers. The most obvious parameters affecting selectivity of such phases are the structures of diepoxides and amines used for hyperbranching, however, crosslink of the functional layer increasing with every reaction step adds the number of reaction cycles to the list of key factors defining the elution order and separation of target analytes. Covalent bonding of the functional layer in general doesn't allow one to compete with electrostatically bonded phases in terms of efficiency, but high performance can still be achieved by adjusting surface coating density and hydrophilicity and limiting polymer beads modification to the surface, which was the purpose of the present work. Differences in surface hydrophilicity of electrostatically and covalently bonded anion exchangers result in significant differences in their selectivity. This work describes new opportunities in IC stationary phase design using covalent bonding of the anion exchange functional layers. Direct step-by-step comparison of electrostatically and covalently bonded hyperbranched phases reveals the differences in the behavior of analytes in both systems and demonstrates for the first time a unique relationship between the number of reaction cycles and the selectivity of various hyperbranched anion exchangers.

Keywords: ion chromatography, stationary phase, hyperbranched anion exchanger, bonding chemistry, selectivity.

A Detailed Investigation of Mass Transfer Phenomena in Core-shell Particles with Different Particle and Pore Sizes

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Since their re-introduction in 2006, columns packed with core-shell particles have revolutionized liquid chromatography separations [1]. These particles, also known as fused-core or superficially porous particles, are commercially available in column lengths as short as 3 cm, particle sizes smaller than 2 µm and pore sizes ranging from 90Å to 1000Å [1–3]. These columns have shown superior performance in terms of reduced plate height, lower backpressure, and increased analysis speed [1,3,4].

Core-shell particles are employed to analyze compounds of any size, from small molecules to DNA fragments, monoclonal antibodies, large proteins, and polystyrene standards. In general, for small molecules, pore sizes of 90-100Å are employed. Columns with larger pore sizes are required to analyze large (bio-) molecules, as the pores should be sufficiently large to allow molecules to diffuse inside the pores freely.

This study aims to provide a detailed investigation of mass transfer mechanisms in RPLC columns with core-shell particles for representative small and large (bio-) molecules. This is obtained by a detailed assessment of the individual contributions to their mass transfer for a set of 6 columns, with pore sizes between 90 and 1000 Å, particle sizes of 2.7 µm and 3.4 µm, and different stationary phase ligands (C4 and C18). For this purpose, plate heights are measured on the columns of interest over a range of different flow rates. The individual contributions to mass transfer are determined next and should, summed together, result in the overall determined plate heights. Peak parking experiments are performed to determine the effective diffusion coefficient (D_{eff}). Knowledge of D_{eff} allows to accurately extract the intra-particle diffusion coefficient (D_{part}) [5]. The observed differences in intra-particle diffusion are then related to differences in pore size, porosity and stationary phase functionality.

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Keywords: core-shell, mass transfer, RPLC, small molecules, large molecules

Understanding New Porous Graphitic Carbon Chromatography Columns: Retention Mechanisms and Applications

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Porous graphitic carbon (PGC) has been used for multiple applications in liquid chromatographic separations since the 1970's. Although its retention mechanisms are yet to be elucidated, it is clear that PGC has unique retentive properties towards polar compounds. Recent advances in synthetic procedures have led to the development of a new PGC particle that displays somewhat different retentive properties than what has been investigated so far in the past. This lecture will focus on fundamental investigations of polar retention effects using this new PGC particle. After a brief review of fundamental studies that have been done in the past with PGC particles, data showcasing the fundamental differences between the two PGC particles now commercially available will be discussed to elucidate how analytes interact with the newly designed particles. In addition, application data involving difficulty to retain compounds will be shared highlighting how the theoretical studies can aid application and method development. Finally, PGC is a unique stationary phase amongst more conventional HPLC stationary phases and further advancements in PGC particle design may result in even better resolving power for a wider range of compounds.

Design of Next-generation Polymer-monolithic Stationary Phases Targeting Ultra-high Resolving Power of Proteomic Sequencing

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Polymer monolithic stationary phases have emerged as an good alternative for packed column formats. To increase the resolving power of packed bed columns, the particle size can be optimized. However, downscaling the particle size leads to increase in pressure proportional to the fourth power. The porous structure of polymer monoliths can be to some extent optimized such that macropore size and globule size are tuned independently. By simultaneously tuning phase ratio, microglobule sizes and macropore sizes, here we report a highly repeatable poly(styrene-co-divinylbenzene) capillary monolithic column fabrication approach to further push forward the kinetic performance of the state-of-art polymer monolithic column. The optimized columns have been characterized both morphologically and chromatographically, producing a separation impedance of 924 (reduced by 10-fold of the commercial monolith column as benchmark). A peak capacity of 400 has been achieved for separation of BSA tryptic digest within 60 min. The column-to-column repeatability has been demonstrated by comparing 12 columns made from different batches in different days, resulting RSDs of 6.7%, 1.5% and 3.2% for peak width, retention time and back pressure normalized by length, respectively.

Keywords: nanoLC, capillary column, high resolution, monoliths, column technology, proteomics

Potentialities and Limitations of Vortex Chromatography

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Open Tubular Liquid Chromatography (OTLC) is attracting increasing attention as a viable technique for specific analytical applications where the size of the sample is intrinsically limited (e.g. proteomics, forensics). When compared to the performance of standard packed HPLC columns, two major drawbacks afflict OTLC, namely the low ratio of absorbing area to the column volume, and the swift increase of the dispersion bandwidth at increasing eluent velocities, which is ultimately caused by the Taylor-Aris dispersion effect. Recent theoretical and experimental work showed how for a non-adsorbing solute it is possible to tame sizeably the Taylor-Aris regime by triggering cross-sectional vortices in the empty capillary acting alongside the pressure driven axial flow. Here, we extend the analysis of dispersion to the case where the solute undergoes simultaneous equilibrium adsorption with a stationary phase deposited onto the channel walls. We show how the positive effect of transversal vortices in containing axial dispersion is progressively lost as the adsorption constant increases. On a practical level, our results suggest that the potential of vortex LC is confined to systems where the column adsorption constant is of oder unity or below.

Keywords: Open Channel, Liquid Chromatography, Transversal Flow, Taylor-Aris, Efficiency Enhancement

Chromatography Study of Fundamental Properties of Medical Radioisotope Astatine-211

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Astatine-211 is considered one of the most promising radionuclides for Targeted Alpha Therapy. In order to develop reliable procedures to label biomolecules and utilize efficient delivery vehicle principles, one should understand the main chemical characteristics of astatine. The short half-life of ^{211}At (~7.2 h) and absence of any stable isotopes of this element are limiting factors towards studying the behavior of astatine. Our team has developed a procedure for rapid and efficient isolation of astatine from irradiated bismuth material in nitric acid media based on 3-octanone and 1-octanol extraction chromatography resins. This process has been automated and it takes 20 min from the beginning of the target dissolution to the At-211 fraction elution. Our next step is to consider commercially available chromatography resins and their applicability in astatine purification in the same media. Results obtained along with the corresponding sorption mechanisms will be discussed.

Keywords: astatine-211, chromatography, automation, mechanism, radiopharmaceuticals

Separation of Heavy Metals from Aqueous Solution using Scallop Shells as Adsorbent Material. Investigation of the Interactions of Metals with Shell Matrix Components

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Mollusc shells are formed by a biologically controlled mineralisation that leads to the formation of superimposed $CaCO_3$ layers [1]. The reuse of mollusc shells, generated as a waste by seafood processing, as adsorbents could potentially be a cost-effective approach for the removal of heavy metals in water remediation technologies. Indeed, the composition of the shell matrix is suitable for the uptake of metal ions dissolved in water. Heavy metals are common pollutants found in natural waters, especially nearby mining sites and metalworking industries. For example, cadmium represent a contaminant of major interest because of its toxicity even at low concentrations: it substitutes calcium and zinc in biological processes leading to the alteration of cellular metabolism [2]. In this study we investigated the adsorption and diffusion of metals through the shell layers, and the interactions with the shell components. In the case of cadmium, the main uptake mechanism resulted to be ion exchange between Ca of the shell and Cd present in solution, with the formation of $CdCO_3$. LA-ICP-MS 2D-images showed that Cd is adsorbed mainly on the outer layers with little diffusion towards the shell interior. Moreover, the presence of organic substances in the shell matrix, in particular pigments, proved to increase the metal uptake from the aqueous solution.

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Keywords: Adsorption, separation, water remediation, heavy metals, bioadsorbents

Flax Fibers as New Filter for Metals Removal from Runoff Waters

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Industrial activities and road traffic provoke the release of atmospheric micropollutants into the environment, especially during a rainy event. Because of an important soil sealing, spontaneous infiltration of water is inhibited, leading to their accumulation in runoff waters. Among these pollutants, metals constitute a real menace to the environment and health (Es-sahbany et al., 2022) and this type of pollution affects the quality of the water released into the environment. The treatment of water by rapid and inexpensive methods, including biomass adsorption, is nowadays an important issue. Today, Europe, and especially France, is the world leader in the production of fiber flax, and during the production process, some become unusable. A previous study (Kajeiou et al., 2020) proved that flax fibers can remove Cu^{2+}, Pb^{2+} and Zn^{2+} ions in high percentages (between 80 and 94% for monometallic solutions, and between 60 and 94% for multi-metallics) and that they could be used, in combination with sand, for the development of a natural filtering system. The idea is to extend to Cd^{2+}, Cr^{2+}, Hg^{2+}, Ni^{2+}, and Pb^{2+}, by conducting competitive and non-competitive batch experiments on flax fibers to study metal ions biosorption performance. The biosorption efficiency is dependent of contact time, metal initial concentration, and flax fibers concentration, and measured by ICP-MS. Adsorption behavior of each metal and of a seven metals mixture are studied at levels close to those of real runoff (concentrations in the µg/L). The biosorption data are fitted with different models in order to determine the kinetics and sorption process.

Keywords: runoff, metals, ICP-MS, kinetic models, isotherm models

Adsorption Treatments for Water Remediation by Zeolite Y: Removal of Per-poly Fluoroalkyl Substances (PFAS)

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Perfluoroalkyl chemicals (PFAS) compounds present high thermal stability and lipid/water repelling properties that legitimated the wide use of PFAS, in an array of different formulations, as surfactants intermediates, waterproofing treatment for textiles, and household products. The extensive use of PFAS has caused their ubiquitous presence in natural waters. Among the different technologies and methods applied for water remediation, adsorption is widely use for the low cost, the wide range of application, selectivity and regeneration of adsorbent materials, such as activated carbon, mesoporous silica, nanocarbon tubes and zeolites. In this work the adsorption efficiency of zeolites Y towards perfluorooctanoic acid, and perfluorooctane sulfonate was investigated. The hydrophobic/hydrophilic behaviour of zeolites was evaluated by varying the silica/alumina ratio. Furthermore, zeolites Y have been used to obtain Ag-exchanged zeolites. The adsorption efficiency of the several materials considered was evaluated by adsorption kinetic and isotherm studies. Adsorption isotherms show that the saturation capacity of Y390 as-synthesized and Ag-exchanged sample, was respectively 43 mg/g and 62 mg/g for PFOA, and 17mg/g and 32mg/g for PFOS. These findings indicated that these materials can be successfully used for the decontamination of water from perfluoroalkyl chemicals. Additionally, the introduction of Ag onto zeolites could confer combined antifouling and adsorption properties, thus exhibiting improved performances compared to zeolite as-synthesized, working synergistically in the removal of PFAS from water.

[1] Kotthoff et al., Environmental Science and Pollution Research volume 22, pages 14546–14559 (2015)

Keywords: PFAS, zeolites, adsorption, water remediation

Why use GC-EI-MS When You Can Do Better?

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Although the coupling of GC/MS with atmospheric pressure ionisation (API) was reported as early as the 1970s, interest in coupling GC with atmospheric pressure ion sources has increased in the last decade. The requirement for a "soft" ion source to obtain highly diagnostic molecular ions is desirable, in contrast to "hard" ionisation techniques such as electron ionisation (EI) in conventional GC/MS, which fragments the molecule to a high degree. Here, the ion sources we have developed for atmospheric pressure chemical ionisation (APCI), atmospheric pressure photoionisation (APPI), atmospheric pressure laser ionisation (APLI) and low-temperature plasma (LTP) for coupling with GC-MS are presented, compared with each other and the advantages and disadvantages of these analytical platforms are carefully discussed in theory and with applications.

Keywords: GC-APCI, GC-APPI, GC-APLI, GC-LTP

Intact Protein Analysis by Capillary Zone Electrophoresis – Mass Spectrometry

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Capillary zone electrophoresis (CZE) is well-known for its high resolving power, simple instrumentation, minimal sample consumption and short analysis time and is relatively easy to on-line hyphenate with ESI-MS, thereby providing molecular mass and fragmentation pattern information about the separated proteins.

One of the most significant issues in CZE analysis is the adsorption of proteins onto the capillary wall through electrostatic or other intermolecular interactions due to the forces acting between charged or neutral surfaces and the biomolecules of interest. In order to overcome this problem, several strategies have been applied, including the appropriate choice of pH or capillary coatings. With an uncoated capillary, the simplest way to reduce interactions is if very low or high pH values are chosen for the BGE. In our work, human insulin and its several analogues were separated and determined using CZE-MS. Three different capillaries (bare fused silica (BFS), dynamic successive multiple ionic-polymer layer (SMIL) and static linear polyacrylamide (LPA) coated) were compared based on their separation performances in their optimal operating conditions [1]. The applicability of CZE for the separation of the deamidated forms of insulin has been also studied. 50 mM ammonium acetate (pH = 9) with 20 % v/v isopropylalcohol was found optimal for efficient separation of insulin from its even 10 deamidated forms. The developed method was efficiently applied for monitoring the degradation rate of insulin and the formation of different deamidation isoforms [2].

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Keywords: capillary electrophoresis, mass spectrometry, intact protein, top-down

FastRet: Fast and Simple Retention Time Prediction with SMILES

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Feature annotation in untargeted metabolomics still poses a challenge in Liquid Chromatography-Mass Spectrometry (LC-MS)-based analysis. By using Retention time (RT) prediction, potential annotations can be excluded or confirmed. We approached that challenge bioinformatically with two algorithms – a Lasso regression and a xgboost tree. We trained the models on an in-house RT library of around 380 metabolites. As predictors for our models, we used chemical properties acquired with the Chemistry Development Kit (CDK[1]) using Simplified Molecular-Input Line-Entry System (SMILES). The process is facilitated by a shiny Graphical User Interface (GUI) for retention time prediction of candidate metabolites. We also developed an algorithm for reducing the training set to only 25 metabolites, on which new regression models were trained to adjust for modifications in chromatography (e.g. flow rate, column temperature, gradient). Validation of the xgboost models showed a mean absolute error (MAE) of 0.5 and 0.86 minutes in prediction for reversed phase and hydrophilic interaction LC, respectively. An improvement in estimating the chemical properties will yield a more accurate RT prediction.

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Keywords: metabolomics, HPLC-MS, retention time prediction, untargeted analysis

Trace Analysis of Haloacetic Acids in Environmental Water Samples using High Pressure Ion Chromatography-Mass Spectrometry

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Ion chromatography (IC) is a technique for the analysis of charged inorganic and organic species. Recently, there has been increased interest in coupling IC to mass spectrometry (MS) in order to gather structural information at high sensitivity, especially in the area of trace analysis. A previous limitation with IC was the speed of analysis, as it is generally not run at high pressures due to instrument limitations. However, with the recent emergence of high pressure IC (HPIC), there is great potential to improve separation times, therefore increasing sample throughput, without compromising on resolution and efficiency. Herein, we present the development and performance of a fast and sensitive HPIC-MS method for the detection of haloacetic acids in environmental water samples. The single quadrupole MS offers excellent sensitivity with limits of detection achieved in the low ppb range. This approach was then applied to water samples collected from various localities in Ireland.

Keywords: ion chromatography, mass spectrometry, haloacetic acids, water analysis

Development of Immobilized Enzymatic Microreactors for Bottom-Up Proteomic Studies

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Bottom-up proteomic workflows include a multi-step sample pretreatment procedure of which the most time-consuming stage is proteolysis, potentially hindering high-throughput proteome analysis. Typically, low enzyme concentrations are applied in the standard in-solution digestion protocol to limit autolysis, necessitating long incubation times (2-16 h). However, if enzymes are confined to solid supports, their concentration can be largely increased, bringing about a significant reduction in reaction time.

Microfluidic platforms offer a favourable environment for housing such IMERs mostly due to their high specific surface area. These can be further classified into microchip- and capillary-based implementations. An advantage of microchip-based devices is the freedom to design channel structures to fit a specific application. For proteolytic IMER devices, therefore, channel patterns with either increased surface area-to-volume ratio [1] or integrated passive mixers [2] can be easily created. In addition to these off-line chip-IMER arrangements, an in-line μ-IMER was also developed, which allowed the full automation of the digestion-separation-detection workflow in a capillary electrophoresis-mass spectrometry (CE-MS) system [3]. The efficiency of the three different μ-IMERs was demonstrated using real samples (saliva, tear, venom).

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Keywords: microfluidics, proteomics, immobilization, enzyme reactor

A Flow-Through Atmospheric Pressure-Atomic Layer Deposition Reactor for Thin-Film Deposition inside Capillary Columns

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We report the development of a new atmospheric pressure-atomic layer deposition (AP-ALD) system to coat the inner walls of capillary columns for gas chromatography (GC) (see Linford et al. *Anal. Chem.* **2022**, *94,* 7483–7491). Unlike traditional ALD, this reactor operates at near-atmospheric pressure and addresses the challenges of depositing thin films inside long capillaries, which include long pump down times, deposition in high-aspect-ratio materials, and temperature control. We show ALD of alumina in 5 and 12 m capillaries (0.53 mm ID) via sequential half reactions of trimethylaluminum and water. Our system yields pinhole-free, uniform thin films. To confirm and quantify deposition, it includes small witness chambers for witness silicon shards before and after the capillary. An engineering flow/transport analysis of the device was performed. Our ALD alumina thin films are characterized by multiple surface and material analysis methods. Alumina film growth achieved is 1.4−1.5 Å/ cycle, which is consistent with previously reported results. Film thickness measurements by spectroscopic ellipsometry, SE, on witness shards of silicon and by transmission electron microscopy, TEM, at both ends of the capillary are in good agreement. A capillary column coated with alumina is used to separate different gases by GC. This successful deposition of ALD alumina in long capillaries has opened the door for exploring other surface functionality with the use of additional ALD precursors – new applications and developments of this technology will be discussed in this presentation.

Keywords: ALD, AP-ALD, GC, TEM, SE

Fast Online LC Reaction Monitoring Using Sub-minute UHPLC Separations in Combination with "Feed" Injection

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Chemical reaction monitoring requires appropriate analytical tools to determine speed of reaction and types & quantities of end products. Online analysis is an elegant way to monitor reactions in real time. In chemical processes online GC and online FT-IR/Raman spectroscopy are widely used. In contrast, the application of online LC is still limited. High degree of complexity, lack of robustness and slow speed are cited as the main bottlenecks for the technology. With the introduction of ultra-high performance LC (UHPLC) more than a decade ago, improved column technology and other instrument features, analysis speed and robustness of LC instrumentation has increased significantly.

This present study employs UHPLC to monitor a set of different chemical reactions. For several examples, a short sub-2 µm silica particle column (30 mm x 2.1 mm) is operated at high linear flow velocities. As such separations of less than one minute are obtained. Novel "Feed injection", in which strong injection solvent is diluted with weak mobile phase, provides high chromatographic fidelity for sample matrices which are strong solvents at reversed-phase LC conditions, such as acetonitrile, ethanol and propanol. Full online LC cycle times (separation & sampling) are as low as 1.5 min enabling high-speed reaction monitoring of chemical reactions. Run charting displays quantitative results in real-time.

The methodology has been applied to three areas: temperature and pH dependent hydrolysis of a vinyl succinic ester, where reactions as fast as ten min could be followed by LC. Second, monitoring the derivatization speed of aldehydes in polyethylene glycols with 2,4-dinitrophenylhydrazine (DNPH). It is shown that DNPH reaction speed for formaldehyde is dependent on the PEG type / molecular weight. Finally, the reaction of isocyanates with short alkyl chain alcohols revealed that the starting isocyanate compound and intermediates can be observed in addition to the reaction product. Ultra-fast chromatography at the one minute and sub-minute level provides details in the reaction process and makes online LC a viable technology for process monitoring. Chemical reactions in various sample matrices can be studied in reaction times as low as ten min.

The Role of Universal Detection in Modern Liquid Chromatography

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From the increasing popularity of aminoglycoside based antibiotics, to the use of sugars and excipients in modern biotherapeutics up to the spike in lipid nanoparticle carried mRNA vaccines due to the CoVid-19 pandemic, all these developments have reignited the decade old discussion of the use of more specific detectors versus the use of more universal detectors in liquid chromatography.

The history of chromatography has seen the advent and often also decline of various techniques which were claimed to enable universal detection, such as: Refractive Index Detection, Flame Ionization Detection, Chemiluminescent Nitrogen Detection, Evaporative Light Scattering Detection, Condensation Nucleation Light Scattering Detection, Charged Aerosol Detection and potentially more to come, each are associated with their own advantages and limitations.

Using meaningful examples, we want to address the recent developments in the field of universal and uniform response detectors highlighting benefits and challenges of various detector types with the goal of summarizing the state-of-the-art as well as providing an outlook on strategies for improving the performance of some current detector designs. Special emphasis is put on the relative influence of factors like mobile phase organic modifier and buffer / additive type and concentration; isocratic vs. gradient elution, analyte properties, etc.

Keywords: universal detectors, charged aerosol detection, instrumentation

Gradient Supercritical Fluid Chromatography Coupled to Mass Spectrometry with a Gradient Flow of a Make-up Solvent

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In this study, we have focused on the chiral analysis of synthetic cathinones using chiral zwitterion ion exchangers Chiralpak ZWIX (+) and Chiralpak ZWIX (–), which possess a positively and negatively charged unit in the molecular structure of the selectors. The presence of the positive charge in the selector's structure, functioning as a counter-ion for charged basic analytes, significantly reduces the required amount of a buffer, which is plausible for hyphenation of such separation system with mass spectrometry. In SFC-MS, the use of a make-up solvent is usually required to avoid precipitation and low ionization efficiency of analyte when using a low concentration of a polar protic organic co-solvent (modifier). Hereby, we introduce a unique approach, which is based on the introduction of the make-up solvent in gradient mode to the post-column effluent. Using this approach, it is possible to keep constant the overall amount of the organic solvent (modifier and make-up) introduced into the mass spectrometer. Moreover, systematic optimization of MS conditions has been performed to achieve high signal intensities.

Acknowledgement: The authors acknowledge the support from CSF grant No. 21-31139J.

Keywords: supercritical fluid chromatography, hyphenation, mass detection, chiral separation, make-up solvent gradient.

On-line Supercritical Fluid Extraction - Supercritical Fluid Chromatography (SFE-SFC) for Nonpolar and Polar compounds from Milk Thistle Seeds

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Plant seeds, as those from milk thistle (*Silybum marianum*), are a valuable source of nonpolar and polar compounds with interesting biological effects. The main nonpolar compounds are triglycerides, which are also the main components of all vegetable oils. In addition, specific polar compounds – flavonolignans, have been found in large amounts in milk thistle seeds extract. Flavonolignans are a group of organic chemical compounds that are derivatives of flavonoids and have different biological activity.

In order to extract and analyse both nonpolar (triglycerides) and polar compounds (flavonolignans) from milk thistle seeds through a unique methodology, an on-line supercritical fluid extraction - supercritical fluid chromatography (SFE-SFC) method was developed. Different transfer approaches were compared to enhance chromatographic quality; i.e. direct on-column transfer and loop transfer. In this respect, nonpolar and polar compounds caused different issues, especially as polar compounds required a significant portion of co-solvent in the extraction step.

First, on-line SFE-SFC was used for triglycerides analysis and allowed the comparison of transfer modes. Then, on-line kinetics were performed to measure defatting time before polar molecules extraction. Finally, the eventual benefit of loop transfer was also investigated for the analysis of flavonolignans, polar molecules whose analysis can be difficult by on-line SFE-SFC. The objective is to discuss the versatility of on-line SFE-SFC and how challenging the coupling can be, especially when both non-polar and polar molecules must be analysed in a single sample.

Keywords: On-line SFE-SFC, Supercritical fluids, Hyphenated approach, Transfer investigation

Supercritical Fluids in Analysis and Extraction of *Amaryllidaceae* Alkaloids from *Narcissus* sp.

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Amaryllidaceae alkaloids have important biological effects, such as antiviral, antimalaric, anticancer, and anticholinesterasic. Their analysis is typically carried out using gas chromatography or liquid chromatography with mass spectrometry (MS) detection. However, the methods are often suffering from the poor separation of structurally related compounds and long analysis time. Therefore, ultra-high performance supercritical fluid chromatography (UHPSFC) with high separation efficiency and different selectivity can bring many advantages. In our study, we focused on the development of UHPSFC-MS/MS method for analysis of 33 weak to strong basic alkaloids, including various types and many isomeric structures. Detailed column screening and optimization of the mobile phase composition was carried out followed by fine tuning of back pressure, temperature, and flow rate. Resulting 10 min UHPSFC separation was achieved using 2-picolylamine stationary phase. MS/MS with electrospray ionization in positive ion mode allowed adequate selectivity and sensitivity with limits of quantification < 1 ng/mL in analytical UHPSFC. The separation method was subsequently transferred to preparative scale SFC with UV detection. *Narcissus* sp. bulbs and leaves were extracted using newly optimized supercritical fluid extraction. The extract composition was evaluated using UHPSFC-MS/MS.

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Keywords: UHPSFC-MS/MS, Preparative SFC, SFE, Amaryllidaceae, alkaloids, analysis

Understanding the Atypical Chromatographic Behavior of Semi-Crystalline Polyamides

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Polyamides (PA) are widely applied engineering materials. Characterization of PA products is essential for quality control and understanding of the material properties. Gradient-elution liquid chromatography (GELC) is often used for the analysis of chemically heterogeneous polymers. However, the application of GELC to semi-crystalline PA is hampered by irregular and often irreproducible retention and performance issues. GELC behavior of these PAs is marked by a broad chromatographic band that obeys chemistry-based retention followed by one or more atypical sharp peak(s). These peaks are thought to be caused by on-column crystallization phenomena. In order to better understand the observed performance, various experimental conditions potentially affecting PA solubility were thoroughly studied. The elution gradient comprised of 0.1% formic acid in water (A) mixed with hexafluoro-2-propanol (HFIP; B) was performed at different starting percentages of A. The column temperature was varied between 5 and 80 °C. For injection, the effect of PA sample concentration and mass loads were studied. Moreover, formic acid was added to the injection solvent. From these experiments, parameters that induced or alleviated potential crystallization were established. Related issues were best mitigated when employing a gradient from 50% A to 100% B at a relatively low column temperature of 5 °C. Potential explanations for this surprising result were further investigated and will be postulated.

Keywords: gradient elution liquid chromatography, polymer analysis, semi-crystalline polyamides

RP-HPLC-MS Analysis of Intact Proteins: How to use Deconvolution?

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One of the most commonly used technique for the study of intact proteins, including monoclonal antibodies (MABs), is the HPLC-MS. Study of pure, large amounts of protein is fairly easy, but for mixtures the situation is more complicated. However, the real challenge is to study small amounts of protein mixtures having large molecular mass. In this case, automatic evaluation (e.g. deconvolution of mass spectra) can give incorrect results and HPLC separation of the mixture is becoming increasingly important.

In this presentation, I would like to illustrate the process, problems and difficulties of evaluation from simple proteins to the study of complex antibody-drug conjugates. In the latter case, when the mixture is too complex, a reliable result can only be obtained using a detailed manual evaluation of the ion chromatograms and mass spectra.

Achieving Rapid LC-MS Analyses using Short 10 mm Columns

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Achieving fast LC analyses is essential in high sample throughput laboratories, such as clinical, drug discovery and environmental labs. Over the last two decades, UHPLC has enabled many labs to dramatically increase sample throughput, through use of shorter columns packed with sub-2 micron particles. However, at the same time, the performance of modern mass spectrometers has continued to evolve. Improved sensitivity and ultra-fast data acquisition capabilities, provide opportunities to further reduce analytical run times, using specially designed, high throughput columns.

This presentation will look at the use of 10 mm columns for the analysis of complex samples, either derived from biological or environmental sources. The presentation will initially outline the theoretical considerations of short columns, starting with van Deemter theory, before moving to a more detailed kinetic plot interpretation of their application. From here, practical considerations of the use of such short columns will be discussed, specifically looking at dwell volumes, tubing and data acquisition rates.

The analysis of complex samples requires a degree of sample preparation prior to application of high throughput LC-MSMS, due to matrix issues. The presentation will investigate the impact that the level of sample preparation has on the data integrity when applied to high throughput scenarios. Finally, the presentation will conclude with a series of applications detailing the benefits that the use of 10 mm length columns can have for high throughput analysis, without the loss of data integrity. The applications will include a series of therapeutic drugs, environmental pollutants and some samples from a hospital laboratory.

Keywords: High throughput, clinical, ion suppression, matrix removal

Smart Samplers for Mass Spectrometric Bioanalysis of Proteins

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In bioanalysis, there is an increasing trend to move from conventional plasma and serum samples towards less invasive microsampling techniques such as dried blood spots (DBS). The simplicity of DBS makes the technique suitable in patient-centric settings where the patient takes the sample at home.

For protein analysis DBS is often combined with immunometric assays, although methods based on liquid chromatography tandem mass spectrometry (LC-MS/MS) are also available. LC-MS/MS based methods provide high specificity and are regarded to be a more reliable alternative. Disadvantageous in bioanalysis of proteins by LC-MS/MS is the extensive sample preparation often needed, especially if the proteins are of low abundance. Typical sample preparation steps include protein proteolysis to digest the protein into peptides and affinity extraction for selective enrichment of a target protein.

We are addressing the need for extensive and often time-consuming sample preparation of proteins from DBS prior to LC-MS/MS by making smart paper-based sampling units. In these, we have incorporated typical sample preparation steps such as tryptic digestion and affinity extraction. By utilizing the time for sampling, drying and transport to carry out sample preparation, we can free time and labor in the analytical laboratory. In the current paper, we describe production and usability of both smart proteolysis samplers and smart affinity samplers.

Keywords: Protein analysis, affinity capture, LC-MS/MS, microsampling, bottom-up

Strategy for Structural Elucidation of Lipid A: Elucidation of Protonated and Disodiated Molecules using CID based MS/MS and MS^n Methods in Mass Spectrometry

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Lipid A, the most bioactive component of lipopolysaccharides (LPS, endotoxins), is anchored in the outer leaflet of the external part of Gram-negative bacteria. It varies between organisms and is essential in imparting specific pathogenic attributes to the bacteria. In this study, the application of mass spectrometric methodology based on the use of positive electrospray ionization, collision-induced dissociation (CID) and tandem mass spectrometry (MS/MS and MS^n) has been applied to the structural characterization of the protonated form and disodium adduct of monophosphorylated lipid A derivatives. A number of synthetic and native lipid A samples were included in the present work. We have found that there is considerable similarity between the fragmentation pattern of lipid A as a disodium adduct $[M - H + 2Na]^+$ and a deprotonated molecule $[M - H]^-$ (typically detected in the negative-ion mode), both revealing fatty acid distribution, and that the fragmentation pattern of lipid A as a protonated molecule $[M + H]^+$ is important for the phosphorylation-site determination. In short, the additional information gained from the CID fragmentation of these two types of precursor ions allow for the full structural characterization of a monophosphoryl lipid A species in a single ionization mode (i.e., only the positive-ion mode). Moreover, this study provides a better understanding of the overlap of MS signals often seen during the fragmentation of native lipid A species containing both, C-1 and C-4' phosphorylation isomers.

Keywords: CID fragmentation pathway, disodiated lipid A, positive-ion mode, structure elucidation, tandem mass spectrometry

Carrier Ampholyte-Based Isoelectric Focusing in Capillaries and Gels Is Not a Steady-State Process, It Is Transient Bidirectional Isotachophoresis

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Over time, through a series of insightful, but often neglected suggestions the current, widely held notion emerged that carrier ampholyte based isoelectric focusing (IEF) in polyacrylamide gels and capillaries is a two-stage process. In the first phase, Svensson's steady-state pH gradient develops rapidly, in the second stage, the gradient decays slowly as isotachophoretic processes (ITP) begin to move the extreme pI carrier ampholytes into the electrode vessels. In this lecture will briefly trace the development of this two-stage IEF model, point out critical suggestions that the IEF community should have embraced but missed, and explain our heretic opinion that in open systems with free ion transport into the electrode vessels (in gels and capillaries) there is no such thing as a (quasi)-steady-state two-stage IEF process, there is only a single electrophoretic mechanism that acts from the first to the last moment of the experiement: transient, bidirectional isotachophoresis (TBD-ITP).

TBD-ITP is a process that *(i)* has an outcome determined by the ionic mobilities, pK_a values and loaded amounts of all ionic and ionizable components; *(ii)* is constrained by both the total amount of charge transported through the separation space and the migration space available for the leading ions, and *(iii)* can never reach steady-state with respect to the spatial coordinate of the separation channel. Using simulations obtained by the new Simul 6 (freeware at https://echmet.natur.cz/) we will demonstrate the salient features of the TBD-ITP ampholyte separation method.

An Adventurous Journey Around the Thermodynamics of Liquid Chromatography

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Thermodynamics, especially the calculation of the standard molar enthalpy and entropy through the van 't Hoff plot is a popular field among chiral chromatographers. However, it is already known that this representation was adopted to chromatography without critical consideration. In our work, chiral chromatography is modelled with the column-coupling method to obtain the contribution of each adsorption sites. Besides the original problems of the van 't Hoff plot, it was found that the ΔS and ΔH values of each adsorption sites differ significantly from the values obtained when both adsorption sites are present. The circumstances of the chromatographic adsorption were also examined to see whether the calculated values are important, or unique values are obtained for each system. The pressure especially affects the determined amounts, thus the chromatographic system used (HPLC or UPLC) has to be distinguished, the determinations made in different ways must not be compared. The main question is: Is there any suitable equation that is less problematic to calculate thermodynamic quantities?

Computer-aided Multi-level Characterization of Functional Cellulose Ethers using Liquid Chromatography with Triple and Mass Spectrometric Detection

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Cellulose ethers (CEs) are a class of semi-synthetic polymers of high molecular complexity, which are extensively applied as e.g. pharmaceutical excipients and thickening agents in paint and drymix mortars. CE functional properties and biostability may be strongly affected by the substitution pattern obtained after synthesis. In order to understand performance differences among CE batches, in-depth molecular characterization is needed. Here we present new approaches for the comprehensive analysis of substituted CEs at the intact and fragmented level. Liquid chromatography (LC) was combined with 'triple' detection (TD) and mass spectrometry (MS), and novel chemometric tools were designed for more effective data handling and interpretation. Next to molecular-weight distribution, size-exclusion chromatography (SEC)-TD can provide information on the average CE conformation through the Mark-Houwink equation employing intrinsic viscosities. Using an in-house computational tool, we determined the change in CE conformation as function of molecular weight, revealing CE-specific differences. We also developed an LC-MS method for the comprehensive characterization of the substitution degree and composition of β-glucose monomers of CE samples obtained after acid hydrolysis. LC-MS provided monomer resolution and assignment based on ethyloxide, hydroxyl, and terminating methyl/ethyl content. Further distinction of constitutional isomer distributions was achieved using a novel probability-based deconvolution algorithm. To link the obtained information on intact conformation and monomer composition to biostability, SEC of enzymatically hydrolyzed CEs was performed as well.

Keywords: Biobased polymers, Characterization, SEC-TD, LC-MS, Chemometric tools

Introduction, Application, and Extension of ChromaRIM: A Liquid Chromatography Retention Index Model for Enhancing Structural Identification of Small $C_xH_yO_z$ Molecules

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In the past decade, high-performance liquid chromatography, coupled with highresolution mass spectrometry (HPLC-HRMS), is increasingly used in the analysis of non-targeted environmental solutes. Although HRMS prediction software can in general reliably predict the elemental composition of small molecules, the structural information remains limited, which further hinders the identification and structural conformation of unknowns.

Quantitative Structure- Retention Relationship (QSRR) modeling technique was used to build a predictive chromatographic retention index model (ChromaRIM) to support the structural elucidation of unknowns where only HRMS data and experimental retention indices are obtained. The developed predictive model is purely based on RPLC retentive data and molecular descriptors, which allow confirmation and/or invalidation of all possible structural formulas through the implementation of the optimized predictive retention index algorithm. A number of molecular descriptors were thereby compared and eventually selected in a broadly applicable model in the intended chemical space for retention index prediction. The model is specifically designed to operate under easily and transferrable generic linear gradients on one of the most frequently used stationary phases in liquid chromatography. *De novo* fully transferrable retention index-based workflow is effectively allowing narrowing down possibilities in the structural elucidation of carbon, hydrogen, or oxygen-containing organic solute (< 500 Da). As a proof-of-concept, the methodology was further tested with both known and unknown solutes of various micropollutants that are of wastewater treatment relevance.

ChromaRIM offers the prospect for the near future transfer of the complete workflow into integrated LC-MS software to assist in the structural identification of unknowns, via comparison of the experimental and predicted retention of all hypothesized structures. The transferability of the approach concomitantly seeds the development of the long overdue, yet necessary, broadly applicable retention index databases in RPLC.

Keywords: HPLC-HRMS, Quantitative Structure- Retention Relationship model, In silico prediction, Retention index, structural elucidation

Proteomic Analysis of Small Cell and Non-Small Cell Lung Cancer Tissue Samples

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Lung cancer is the leading cause of tumor-related mortality worldwide; therefore, significant effort is directed towards understanding molecular alterations occurring at the origin of the disease to improve current treatment options. We aimed to carry out a detailed proteomic analysis of formalin-fixed paraffin-embedded tissue sections from patients with small cell (n=9) or non-small cell lung cancer (adenocarcinoma, squamous cell carcinoma, and large cell carcinoma, n=10, 9, 10, respectively). Tissue surface digestion was performed on cancerous and tumor-adjacent normal regions and differentially expressed proteins were identified using label-free quantitative mass spectrometry and subsequent statistical analysis. We have identified several biological processes disrupted in all investigated cancer types such as the degradation of the extracellular matrix and suppression of the complement and coagulation cascade as well as the activation of the MTORC1 signaling pathway. Gene set enrichment analysis revealed dysregulated pathways specific to small cell and non-small cell lung cancer types like regulation of cell adhesion, actin filament-based processes and calcium ion binding. The dysregulated pathways revealed could contribute to a more precise classification of lung cancer. Furthermore, proteins with altered expression unique to a specific lung cancer type were identified and could be the targets of future studies.

Keywords: proteomics, lung cancer, FFPE tissue, gene set enrichment analysis

H/D Pooling of Benzoyl Derivatives: Internal Standard per Each Analyte for Lipidomic Quantitation by RP-UHPLC/MS

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Chemical derivatization of lipid functional groups can improve extraction efficiency, chromatographic separation, and sensitivity. Benzoyl chloride is a non-hazardous derivatization agent with high reactivity for several functional groups. The optimized benzoylation method increased the sensitivity 2 to 10-fold for almost all investigated lipid classes and even more than 100-fold for monoacylglycerols compared to the non-derivatization approach. Reversed-phase ultrahigh-performance liquid chromatography enables to resolve isomeric lipids, but for accurate quantitation a large number of internal standards must be used. H/D pooling means the mixing of non-labelled derivatives (H) and labelled derivatives (D) in the same ratio, which helps to easier identification and accurate quantitation. Our approach creates an internal standard per each derivatized lipid with the coelution of analytes and internal standards (ΔRT ± 0.1 min), ensuring the same matrix effect and ionization efficiency. Labeled derivatives are used as an internal standard mixture, producing the identical lipidomic profile and corresponding concentration for all analytes. The created doublets in the mass spectrum with known Δ*m/z* facilitate identification and bring the next parameter for a high confidence identification. The method was validated and used for targeted quantitative analysis and lipidomic profiling of healthy controls and cancer patients. This work was supported by Czech Science Foundation (GAČR) project No. 21-20238S.

Keywords: derivatization, lipidomics, H/D pooling, reversed-phased UHPLC, human plasma

HILIC and Graphite-based Solid-phase Extraction of Heparan Sulfate Disaccharides

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Heparan sulfate (HS) is a class of glycosaminoglycans built up of disaccharide repeating units comprised of glucuronic/iduronic acid and glucosamine residues; sulfation of the disaccharide building blocks may happen at four different positions. The most common workflow for HS analysis is enzymatic digestion followed by solid-phase extraction (SPE) purification and HPLC-MS measurement. Optimization of the SPE purification of HS disaccharides from biological matrices is an important, but usually neglected part of the analysis.

We aimed to develop SPE methods for maximizing the recovery of the HS disaccharides during sample preparation. First, we developed a HILIC purification method based on self-packed cotton pipet tips. The sample loading, elution conditions, and solvent volumes were thoroughly optimized. We found that the cotton-HILIC method provided excellent (ca. 80%) recovery for the highly hydrophilic doubly and triply sulfated disaccharides, but sample loss up to 60% was observed during the sample loading step for the less hydrophilic ones. Second, we tested three different commercial graphite SPE systems with three different loading and elution protocols, respectively. Excellent recovery was observed for the less hydrophilic non-sulfated and monosulfated components with most of the methods, while the highly-sulfated ones were not eluted from the column.

As a result, we designed a combined method where the flow-through of the cotton-HILIC SPE is further purified with the graphite-based system. The method was applied for biological matrices like HS chain isolated from different species, and we concluded that it was suitable for bias-free analysis of small amounts of biological samples.

Keywords: heparan sulfate, glycosaminoglycan, purification, solid-phase extraction, HILIC

Development of Sample Preparation Methods for the Proteomic Analysis of Phosphopeptides

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For efficient investigation of phosphorylation of small amounts of complex protein mixtures, it is essential to perform purification and enrichment steps before nano liquid chromatography coupled to tandem mass spectrometry (nanoLC-MS/MS) analysis. The most common types of these sample preparation methods include solid-phase extraction (SPE). Our goal was to improve the currently available SPE methods to minimize losses during sample preparation and to create an efficient phosphoproteomic workflow. Experiments were performed to determine the optimal loading- and elution buffer compositions, including the concentration of ion-pairing reagents, and to select the most suitable solid phase. Altogether, the performance of 16 different solid phases were compared for the purification of 1 µg rat smooth muscle (8 reversed-phase, RP; 5 graphite; 2 ion-exchange, IEX; and Oasis HLB). Besides commercial SPE cartridges/tips, home-made centrifugal SPE tips were also investigated. Regarding the number of identified phosphopeptides, HLB, and two RP tips gave promising results (40-70% increase). Regarding the recovery, a graphite tip was also found to give excellent results (34% increase, on average). Further investigation of tips with the best performance, and implementation of the developed methods into the phosphoproteomic workflow for tissue analysis are ongoing.

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Keywords: solid-phase extraction, purification, phosphopeptide, mass spectrometry

Exploring the Difficulties of Neat Carbon Dioxide as the Mobile Phase in Supercritical Fluid Chromatography

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In supercritical fluid chromatography (SFC), the variation of some of the mobile phase properties, including pressure, temperature and density is most notable, when the mobile phase contains only neat carbon dioxide. This can be attributed to the compressibility of CO_2, that introduces several difficulties to the work of chromatographers, including changes in volumetric flow-rate and chromatographic efficiency.

The goal of this presentation is to uncover some of the challenges that can be encountered when only carbon dioxide is used in the mobile phase. Although the practical usefulness of excluding any sort of modifier is debated, the information gained can be of assistance in method transfer and scale-up.

The only flow parameter considered to be constant across the SFC system is the mass flow-rate. It has been shown that the Coriolis flow meter (CFM) provides different types of information depending on its placement in the instrument. Our work focuses on the investigation of several factors affecting the potential variation of mass flow-rate in SFC, including different configurations around the column, different experimental conditions and different columns.

Keywords: supercritical fluid chromatography, carbon dioxide, mass flow-rate, Coriolis flow meter

POSTER ABSTRACTS

Chromatographic Surprises: Are the Injected Molecules Always in the Peak?

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Almost 60 years ago, Helfferich and Peterson published an article in Science suggesting that when an excess of sample molecules is injected into a chromatographic column equilibrated with a constant stream of identical molecules, the observed peak will not contain the injected individual molecules [1]. Instead, the observed peak will contain molecules from the stream while the injected individual molecules will exit the column in a slower moving, "invisible" peak. They considered it paradoxical that a single injection into a one-component system could cause the successive elution of two peaks [2]. Many years later, the paradox was experimentally proven for the first time, by us, using different experimental strategies: (i) a radiochemical approach [3] and (ii) a method based on the use of two enantiomers in a non-enantioselective separation system [3] and the use of stable isotopes in supercritical fluid chromatography (SFC) [4]; the experiments were confirmed with computer simulations. Here, we will tell this fascinating story, and also give examples of how the visualization of the "invisible peaks" can provide new reliable insights into solvent adsorption in modern supercritical fluid chromatographic systems [4].

Keywords: Invisible peaks, Paradox, Tracer peaks, Adsorption isotherms

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Uncertainty in the Determination of Thermodynamic Parameters in High-performance Liquid Chromatography

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In the separation of enantiomers, it is common to calculate thermodynamic parameters from the temperature (T) dependence of the retention factor (k) of a single compound using the van 't Hoff plot (ln$k$ vs. 1/T) [1]. However, the retention factor (k) cannot be equated to an interaction with only one type of binding site, even in reversed-phase chromatography, especially in chiral chromatography where enantioselective and non-enantioselective binding sites are present [2]. Since they cannot be separated experimentally, two different types of reversed phase chromatographic stationary phase were used to model the environment. To get to know each binding site, we first determined their thermodynamic parameters (standard molar enthalpy and entropy values) separately, then combined them using capillary to obtain a system with two known binding sites and performed the analysis. The effect of changes in liquid chromatography conditions, such as the flow rate used, the type of apparatus and the column length, on the thermodynamic parameters for both single and coupled columns was investigated. Our results showed that the type of device is an important factor in the results obtained. Similar conclusions can be made for the column length but extrapolating to zero mean pressure may reduce the discrepancies in the calculated thermodynamic data. It can be concluded that the linear combination of the thermodynamic data of each stationary phase does not give similar parameters to the coupled column. In addition, the Eyring equation, another possible method for calculating thermodynamic parameters, was investigated. The measurements were mainly focused on reverse phase separations, but the conclusions drawn are general and may also be true for other separation mechanisms.

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Keywords: chiral separation, van 't Hoff plot, thermodynamic parameters

Effect of Column Design on the Heterogeneity of Stationary Phases in Fast Liquid Chromatography

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Sample band broadening in the immediate vicinity of the column ends can be characterized by column reversal experiments [1]. In the case of packed columns, measurements have shown that the columns are heterogeneous, and some differences can be observed between the two ends of the column.

Column reversal has a peak compression effect, the peaks obtained with reversed flow are always narrower and more symmetrical than those without flow reversal, therefore column reversal is suitable for determining the local plate height values of the columns and for detecting the difference between the two column ends.

We can conclude that shorter columns are more homogeneous axially than longer columns, so that column length is an influential factor in the column packing procedure.

Column reversal was also attempted with macromolecules to eliminate the effect of pores. Similar conclusions could be drawn, however, due to the complexity of the measurements, we will continue to perform the column reversal with small molecules in the following.

In addition to this, electron microscopy measurements were also carried out, where the purpose of the measurements was to determine whether any damage was visible on the frit or on the particles as a result of the column packing procedure. The inlet and outlet frits of five types of columns were examined. None of the cases showed visible damage to either the frit or the particles. However, the silica particles were visibly entrapped in the pores of the frit due to their size and the heterogeneous structure of the pressed frit. The particles embedded in the frit may also be responsible for the band broadening effect near the frit.

Keywords: column reversal, monolith column, heterogeneity, UHPLC, frit, macromolecule, electron microscope

Comparison of Mixer Performance for Challenging Long Shallow Gradients

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With the increase in biotherapeutics, reversed-phase (RP) chromatography on high performance liquid chromatography UV (HPLC UV) systems is becoming more prevalent for the routine analysis of peptides. Along with these analyses, the separation requirements of peptide mapping applications can be challenging as these methods include long shallow gradients, along with the use of UV absorbing mobile phases containing trifluoracetic acid (TFA) and low wavelength detection (e.g. 214 nm). Such conditions, which are very commonly used for peptides, are challenging because TFA not only absorbs UV light at the same wavelength that is used to detect peptides (most commonly 214nm) but may be slightly retained by reversed-phase columns in water-rich mobile phases. This phenomenon can result in short-term variations in the mobile phase, leading to waves of TFA along the length of the columns as pulses of acetonitrile-rich mobile phase cause localized decreases in retention of TFA. This results in transient increases in elution of TFA from the column causing perturbations in the baseline noise, and irreproducible peak areas.

Given these challenging sets of conditions, HPLC system performance requirements, such as baseline noise, are becoming ever more important. To address the challenges with TFA containing mobile phases at low wavelength detection, newly designed mixers can improve the system performance of baseline noise from a standard 50 µL bead mixer to a diffusion bonded mixer using a long shallow gradient and TFA modifier. The evaluation of the mixers will include peptide standards eluted under a long shallow gradient, with evaluation of noise at specific regions of the chromatogram. Improved mixing performance of the diffusion bonded mixer was shown to have an 80% reduction in baseline noise when compared to the standard 50 µL bead mixer. In addition, the reduced baseline noise produced slight improvements in area %RSD and retention time reproducibility.

Keywords: Peptide mapping, HPLC, mixers, long shallow gradients

Solvent Maps in Liquid Chromatography

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In many liquid chromatography systems, the retention of the solute is conditioned by the partition between the two chromatographic phases: the mobile and the stationary phase. The partition of the solute is determined by the difference in the interactions of the solute with both phases, mainly: easiness of creation of a cavity in the solvents, hydrogen bonding and dipolarity/polarizability interactions. Selection and tuning of the appropriate chromatographic phases require an estimation of these interactions.

In this work, the Kamlet–Taft solvatochromic parameters (α, β and π^*) and the Hildebrand solubility parameter (δ) were examined for the most common solvents used in liquid chromatography (including solvent estimations for stationary phases), but also for some exotic (but suitable) and green ones. The α and β scales account for the hydrogen-bond donor acidity and hydrogen-bond acceptor basicity, respectively, of the solvent towards a solute-to-solvent hydrogen bond, and the π^* scale is an index of solvent dipolarity/polarizability, meaning the ability of the solvent to stabilize a charge or a dipole. δ is the work necessary to disrupt and reorganize solvent/solvent interactions to create a cavity in a solubility process, and thus a measure of the molecular cohesion of the solvent.

The selected solvents were studied by principal component analysis from the perspective of the chromatographic mode (reversed-phase, non-polar stationary phase; HILIC, polar) and the solute properties (polarity, hydrogen-bond acidity and basicity).

Keywords: Mobile phase, Selectivity, Solvent

Combination of Stochastic Theory and Mass Balance Models of High Performance Liquid Chromatography

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Chromatographic processes are usually described by differential mass balance equations that characterize the chromatographic processes in a desired detail. The chromatographic band profiles are obtained by the integration of these equations. Stochastic models, on the other hand, study the chromatographic processes at a molecular level via the random walk of individual molecules through the column. The chromatographic peak is obtained as the probability density function of the solute molecules at the outlet of the column.

In this work, the combination of stochastic and differential mass balance models are studied. Both the reaction-dispersive model and the stochastic theory are solved to describe the first and second moments of chromatographic peaks. The relationships between microscopic and macroscopic descriptors are identified and discussed. Based on these results, a general method is introduced for the determination of microscopic descriptors, such as the number of adsorption steps and the average time of adsorption. Our results show that microscopic models reveals important information about the chromatographic processes take place in the column during elution.

Keywords: capacity gradient, stationary phase, optimization, column design

Investigation of the Effect of Acidic Modification on Polyethylene Glycol Stationary Phase Columns

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In gas chromatography, the polyethylene glycol stationary phase is widely used for the analysis of polar compounds. Only the strong acid-base interactions are unfavorable, but with acidic or basic deactivation, this phase is capable for efficient separation of free acids or bases. Stationary phases with acidic modification are the simplest way to analyze free acids, instead of the derivatives of these compounds.

In our work, we tested and compared 3 acid modified and 2 unmodified columns with similar dimensions from different manufacturers, to investigate the effect of the modification and the different production technology. We elaborated a new test mixture with 11 compounds to examine all the possible interactions with the stationary phase. The chromatograms visualized the retention and the resolution of the columns and showed the strong interactions through the peak symmetry. The efficiency (HETP) of the columns were tested at isothermal conditions and different linear velocities (u), to edit the HETP - u charts and find out the optimal linear velocity for the best efficiency on each column. The strength of the interactions for each compound was compared with the determination of the sorption enthalpies and entropies.

The results showed many differences between the acidic modified columns. The unmodified WAX columns were slightly different and showed possibilities for measuring acidic compounds too. In this study, we show some important differences of these stationary phases. With this kind of comparison, we are able to choose the proper column for the analysis of free acids without any derivatization and other polar molecules.

Keywords: gas chromatography, column comparison, polyethylene glycol, efficiency, sorption enthalpy

Mix-mode Polymer-based Stationary Phases with Covalently Attached Polyelectrolytes

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The development of mix-mode stationary phases is a topical direction in the field of HPLC. At the moment, the vast majority of mix-mode resins are silica-based phases suitable for hydrophilic chromatography (HILIC) and RP HPLC modes, as well as for non-suppressed ion chromatography (IC). Mix-mode phases with ion-exchange properties almost always have weak ion-exchange functional groups on the surface of silica gel, that is stable only in the pH range from 2 to 8, as a result they cannot be used in more sensitive suppressed IC mode with strongly acidic or basic eluents. In this regard, it is promising to substitute silica gel with aromatic substrates which are stable over the entire pH range and compatible with organic solvents, as well as to introduce positively charged quaternary ammonium groups into the structure of the functional layer.

In this work, mix-mode phases based on aminated poly(styrene-divinylbenzene) with covalently attached polyelectrolytes were synthesized by polymerization of a secondary amine and diepoxide in situ. For additional shielding of the hydrophobic substrate, stationary phases with polyamine preliminarily fixed on its surface were obtained. The effect of the grafting density of polyelectrolyte chains, as well as the structure of the secondary amine used for their formation, on the selectivity and hydrophilicity of the resins was studied in suppressed IC mode. The use of the best phases in HILIC and RP HPLC modes has been demonstrated.

The combination of coating the surface of a hydrophobic core with a layer of polyamine with grafting of polyelectrolyte chains provided its best shielding and maximum hydrophilization degree. Significant hydrophilization of ion-exchange sites was also achieved using more hydrophilic secondary amine. In HILIC mode the stationary phase with grafted polyamine and polyelectrolyte chains on its surface had the highest hydrophilicity and provided the separation of 6 sugars, 6 vitamins, 9 nitrogenous bases and nucleosides, as well as 7 amino acids. In addition, due to the hydrophobic substrate, the resulting phases were suitable for using in RP HPLC mode and provided the separation of 7 alkylbenzenes.

This work was supported by the Russian Foundation for Basic Research under grant no. 20-03-00909.

Keywords: Mixed-mode stationary phase, Ion chromatography, HILIC, RP HPLC

Nano-coating with Carbon-Like, π-Conjugated Organic Layer onto Porous Silica Surface for Ultra-selective Adsorption

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Graphene as a two-dimensional carbon in which π electrons are integrated on the same plane is attracting attention as a functional interface that produces high selectivity. On the other hand, in order to introduce the graphene structure into the porous carrier at high density and act as a functional interface, it is necessary to prepare nano-sized graphene and immobilize it on the porous interface. However, it's not easy and it's not a cost-effective approach. We report a bottom-up π-conjugated interface introduction method that can be immobilized on a porous carrier by surface polymerization using two low-molecular monomer systems and carbonization by a subsequent heat treatment process. In this report, we explain that the unique surface prepared by this method exhibits excellent stereoselectivity. In particular, it is also explained that very high selectivity can be achieved for geometric isomers.

Keywords: π-conjugated system, geometrical selectivity, adsorption, porous materials

Monolithic Silicas in High Performance Liquid Chromatography – Unique Material with Unique Benefits

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World of chromatography is still dominated by conventional packed-particle columns. But what if our analytical task requires something more special? Monolithic silica columns are ready to expand possibilities of your chromatographic separation. Monolithic silica columns are made of a single continuous-bed rod of high purity porous silica that is then bonded with C18, C8 and the other modifications therefore from column backbone and modification perspective it is still the same column as conventional silica-based column. However, monolithic columns remove backpressure issue as the primary consideration in method development and give back the flexibility of choices in flow rates for much higher throughput, column lengths for superior resolution, and solvent choices for optimum selectivity's that smaller and smaller sized packed-particle columns have slowly taken away over the decades. Because monolithic silica has no individual particles to shift or break over time, column performance is consistent over much longer lifetime. Their high permeability also makes them very forgiving of shortcuts for example easier to aggressively flush out to reequilibrate. The separation of matrix-rich samples, such as herbs, food, or biological samples tends to reduce the lifetime of particulate columns especially if insufficient sample preparation/cleanup is performed before chromatographic separation. Monolithic silica columns due to their bimodal pore structure allow the separation of matrix-rich samples with extended column lifetime, with no or very reduced sample preparation required, therefore significantly reducing the cost of operation.

A Tag-and-Count Approach for Quantifying Surface Silanol Densities using Atomic Layer Deposition and High-Sensitivity Low-Energy Ion Scattering

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Surface silanols (SiOH) are important moieties on glass surfaces, and the density of these surface silanols determines the functionalization and processing potential of many glass surfaces. Here we present a tag-and-count methodology to quantify surface silanol densities on fused silica by tagging surface silanols with Zn via atomic layer deposition (ALD), followed by detection of the zinc by high sensitivity-low energy ion scattering (HS-LEIS). Shards of fused silica were hydroxylated with aqueous hydrofluoric acid and heated to 200, 500, 700, or 900 °C, which increasingly condense and remove surface silanols. Two different precursors that both deposit Zn, dimethyl zinc (DMZ) and diethyl zinc (DEZ), were explored. Surfaces treated with DMZ or DEZ were cleaned in preparation before HS-LEIS. Peak areas of Zn were used to quantify the surface density of SiOH. Using this methodology, a value of 4.59 OH/nm^2 was found for fully hydroxylated fused silica, with decreasing values with increasing temperature treatments. These values agree with given literature values.

Keywords: ALD, Process Development, Silica, Tagging

Novel types of Stationary Phases for Gas Chromatography Based on Deep Eutectic Solvents (DESs)

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This paper presents results of studies on applicability of Deep Eutectic Solvents (DESs) as stationary phases (SPs) for gas chromatography (GC). A two developed SPs based on DES were compared. First ever (DES-SP1) was obtained from tetrabutylammonium chloride (TBAC) as a hydrogen bond acceptor (HBA) with heptadecanoic acid being a hydrogen bond donor (HBD) in a mole ratio of HBA:HBD equal to 1:2 [1], while second (DES-SP2) was made of L-proline (protonated with hydrochloric acid) as a hydrogen bond acceptor (HBA) and xylitol as a hydrogen bond donor (HBD) in a molar ratio of HBA:HBD 5:1 [2]. The DES-SPs where characterized in respect to several volatile organic compounds (VOCs) as well as using the Rohrschneider-McReynolds constants. Both phases revealed to provide good peak symmetry, long term stability and very good efficiency. DES-SP1 revealed to have characteristics of intermediate polarity while the latter was polar. In both cases unusual selectivity – uncommon for commercial stationary phases was reported. It follows from synergistic effect of different types of molecular interactions, including hydrogen bond interactions of functional groups responsible for HBA and HBD role as well as hydrophobic interactions with alkyl chains present in some of the DES components.

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Keywords: natural deep eutectic solvent (NADES), separation techniques, volatile organic compounds (VOCs), hydrogen bond, molecular interactions

Deposition of Thin-Films in Capillary Columns using Near-Atmospheric-Pressure Atomic Layer Deposition

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We describe a near-atmospheric-pressure flow-through ALD reactor capable of functionalizing the inner walls of 0.5 mm silica GC capillaries. Some capabilities of the reactor include temperature and flow control, adaptable computer automated programing, vacuum monitoring, and fast deposition. Unlike traditional ALD, this reactor operates at near-atmospheric-pressure. Applications of the reactor include surface functionalization and passivation using various precursors, including to produce thin alumina films in capillaries. Surface characterization of witness silicon wafers and the capillaries themselves has been performed with spectroscopic ellipsometry, contact angle goniometry, atomic layer deposition, and transmission electron microscopy. New applications and developments of this technology will also be discussed in this presentation.

New porous monodisperse particles for increasing resolution in Liquid Chromatography

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In this poster we discuss the use of a new range of stationary phase chemistries allied with a fully porous monodisperse silica particles.

One of the major challenges in LC and LC-MS is achieving full resolution of compounds especially when metabolites and or isomeric species are involved. By combining the high efficiency of a monodisperse silica with new diverse stationary phase ligands, we have the potential to gain more selectivity and resolution between analytes.

Whilst C18 and C8 alkyl chain stationary phases are the most common choice for starting method development, they cannot achieve all separations with the required resolution sufficient to provide accurate qualitative results. The use of orthogonal stationary phases containing halogenated, aromatic or polar character allows differing mechanisms other than just hydrophobicity to be employed.

We discuss the use of several mixed stationary phases which allows more mechanisms of interaction to be used in the separation process to gain more resolution.

We highlight applications where these mixed stationary phases can be utilised to alter selectivity of complex samples, gaining resolution over a traditional C18 bonded phase.

Keywords: HPLC, UHPLC, Monodisperse, Stationary Phase, Silica particles

New Porous Monodisperse HPLC particles

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In this poster we discuss the use of a new fully porous monodisperse silica particle and its application in HPLC and UHPLC. Silica particles have been the mainstay in HPLC for 50 years and various innovations have taken place in terms of particle size, particle composition and particle morphology with the goal of improving chromatographic stability and efficiency.

We look at the use of a fully porous monodisperse particle in terms of the increased efficiency that it can provide. We look at the advantages over UHPLC particles and core-shell particles, in particular sample loading, method scaling and column backpressure. We discuss the van-deemter curve and band dispersion in relation to the tighter d90/10 size distribution provided by a monodisperse particle.

Combining this monodisperse particle with a range of selectivity's can then provide the ultimate in terms of sample resolution and sensitivity when trying to develop a new and improved HPLC methods.

Keywords: HPLC, UHPLC, Monodisperse, Stationary Phase, Silica particles

Separation of Peptides and Protein Digests in Mixed-mode Chromatography

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Mixed-mode chromatography (MMC) is a promising tool for the separation and analysis of a variety of compounds. Traditional stationary phases (SPs) often exhibit single interaction mechanism. Better to say single interaction mechanism prevails. Additional types of interactions with the single mode SP were often viewed as detrimental. Mixed-mode stationary phases are types of SPs where multiple interactions between analytes and SPs (but also between mobile and stationary phases) play a significant role and at least two interaction mechanism types should be offered by the SP that both significantly contribute to the retention.

In this work we focused on the use of commercial mixed-mode columns which exhibit reversed-phase/anion-exchange properties. At first, retention characteristics and selectivity of tested MM columns for a set of model peptides were evaluated. The effect of mobile phase pH, type of organic modifier, buffer type and its concentration on retention of peptides on individual columns was tested. The retention and selectivity for protein digests under different mobile phase conditions were evaluated.

Authors greatly acknowledge the financial support from Czech Science Foundation [Project No. CSF 20-19655S].

Keywords: mixed-mode columns, ultra performance liquid chromatography, protein digests, , peptides, separation

Application of Fiber-enhanced Raman Spectroscopy to High Performance Liquid Chromatography for the Analysis of Proteins and Their Prosthetic Groups

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The aim of the study was to investigate the capability of fiber-enhanced Raman spectroscopy (FERS) using a liquid-core waveguide (LCW) for protein identification after chromatographic separation. Since the three-dimensional structure of proteins is crucial for their biological function, changes in conformation can lead to failure of biological functionality. Raman spectroscopy is a powerful technique to investigate the structure of proteins and their prosthetic groups, but it is sensitive to e. g. fluorescent impurities. In combination with size-exclusion chromatography (SEC) proteins are separated from matrix compounds in an aqueous mobile phase, thus maintaining their native structure. Different proteins have been investigated and can be identified by the online coupling of SEC and FERS. Separated proteins are excited coaxially flowing through the pathway of the LCW. The Raman scattered light is then detected in backscattering geometry. In particular, hemoproteins like hemoglobin exhibit strong Raman bands due to resonance Raman enhancement of characteristic modes of the heme prosthetic group. Hence different species of hemoglobin such as methemoglobin and oxyhemoglobin can be distinguished using FERS.

Keywords: Fiber-enhanced Raman spectroscopy (FERS), Size-exclusion chromatography (SEC), online coupling, hemoglobin

Analysis of Endocannabinoids and Polar Metabolites in Human Placenta using LC/MS

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Metabolomic analysis attempts the comprehensive qualitative and quantitative description of a metabolome, which could provide a key tool for understanding the development of various diseases. The crucial task is the development of quantitative analysis of low-molecular weight polar compounds such as neurotransmitters, biogenic amines, catecholamines and middle-polar endocannabinoids in complex biological matrices. For this purpose, optimization of chromatographic and mass spectrometry conditions for untargeted metabolomic analysis were performed. Ultrahigh-performance C18 column compatible with 100% aqueous mobile phase was selected for the best separation performance for both endocannabinoids and polar metabolites under the same chromatographic conditions except the gradient of mobile phase. Both developed and validated LC/MS methods were applied to the comprehensive analysis of metabolome in the human placentas obtained from preterm and term births. To identify significant differences in metabolite composition among both tested group of samples, the data were processed and evaluated using multivariate data analysis such as principal component analysis and orthogonal partial least square methods. Increased levels of some endocannabinoids isomers in placenta were observed for preterm birth.

This work was supported by project No. 22-13967S sponsored by the Czech Science Foundation.

Keywords: metabolomics, endocannabinoids, LC/MS, placenta, statistical analysis

Sample Preparation for the Metabolomic Analysis in Neurological Disorders

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Recently, metabolomics research has greatly improved due to the advances in the performance and reliability of analytical methodologies. Accurate measurements using sophisticated UHPLC/HRMS are used for large-scale studies including metabolomic profiling. Nevertheless, the pre-analytical stage is critical and not least deserves considerable attention. The metabolomic study of central nervous system (CNS) disorders involves the preparation of biofluids and low-mass tissue samples using laborious manual processes. Metabolite extraction is a key step that directly affects the quality of the obtained data. To address this, we compared different sample extraction procedures to eliminate interferences while preserving the nature of the metabolome, and, at best, to minimize sample manipulation steps. In this project we focus on RPUHPLC/HRMS analyses of structural lipids and low molecular weight neurotransmitters that are of importance in the context of CNS metabolism. Studied analytes have diverse physical and chemical properties that is considered in the preanalytical steps. Commonly used sample preparation approaches such as liquid-liquid extraction by Folch, Blight and Dyer, and Matyash methods are compared and superior modifications for extraction are introduced. We discuss pros and cons of methods for extraction of various structural lipid classes from porcine brain. Neurotransmitters including catecholamines, indolamines, amino acids and other metabolites were extracted from human plasma and preconcentrated using various solid-phase extraction (SPE) and micro-SPE protocols. Based on obtained results, we propose suitable workflow with optimized conditions of biological sample preparation for metabolomic analyses in neurological disorders.

This work was supported by project no. 22-13967S (Czech Science Foundation).

Keywords: metabolomics, lipids, neurotransmitters, extraction, brain, plasma

Chiral SFC/MS Method for the Direct Separation of Mono- and Diacylglycerol Isomers: Application to the Analysis of Hydrolysis Products of Triacylglycerols by Lipases

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Glycerolipids usually exist in biological systems as enantiomers and regioisomers that require separation before the quantification. The knowledge about the concentration of individual isomers is important due to their different biochemical properties given by the stereospecific environment in the organism. In recent years, supercritical fluid chromatography (SFC), especially coupled with mass spectrometry (MS), has become a promising alternative to traditional liquid chromatography methods for lipidomic analysis due to the development of robust and efficient commercial systems. However, chiral SFC separation of lipids has not been fully investigated. In this work, we focused on the development of a novel SFC/MS method for the direct regio-/enantioselective separation of mono- and diacylglycerols using polysaccharide-based stationary phases. The selected representatives of isomers were synthesized and used as standards for chiral SFC/MS analysis. The influence of main chromatographic parameters on the separation was evaluated in detail. The optimized conditions provided the baseline resolution of *sn* -1, *sn* -2 and *sn* -3 monoacylglycerols isomers, as well as *sn* -1,2, *sn* -2,3 and *sn* -1,3 diacylglycerols isomers. Finally, the developed SFC/MS method was applied for the analysis of the hydrolysis products of triacylglycerols by lipases.

This work was supported by project no. 20-12289S (Czech Science Foundation).

Keywords: chiral separation, diacylglycerol, hydrolysis, lipase, monoacylglycerol, SFC

UHPSFC/MS Lipidomic Analysis: Inter- and Intra-Class Separation of Lipids

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Extensive research has recently established ultrahigh-performance supercritical fluid chromatography – mass spectrometry (UHPSFC/MS) coupling as an excellent tool for the fast and comprehensive characterization of lipids. The goal of this work was to study UHPSFC separation of lipids according to the polarity (interclass) and fatty acyl composition (intraclass). The selectivity of chromatographic columns with different chemistry and the effect of particular chromatographic conditions on the lipid class and specie resolution were carefully assessed. Column chemistry was shown to have a significant effect on inter- and intra-class selectivity. The most pronounced influence on interclass separation was found for the solvent type that was used as a modifier, which enabled the lipid class resolution to be finely adjusted. When propan-2-ol was added to methanol, a positive effect was observed for the resolution of mainly polar lipid classes. The nature of the stationary phase showed the most prominent effect on intraclass selectivity, while other chromatographic conditions were used for partial improvement in resolution of lipid species. Special attention was paid to the long-term repeatability of UHPSFC retention times crucial for lipidomic analyses. Optimized UHPSFC/MS lipidomic method is based on a diol column and a gradient of methanol – propan-2-ol – water – ammonium formate modifier that provides the resolution of all lipid classes in brain samples within 7 min of analysis. This method has shown excellent retention times repeatability for the 555 injections in lipidomic study with the standard deviations below 0.012 min for all lipids. This demonstrates the ability of the method for highly selective and repeatable lipidomic analysis of biological samples.

This work was supported by project no. 20-12289S (Czech Science Foundation).

Keywords: SFC/MS, lipids, lipidomics, intraclass separation, interclass separation

Plant Based Products Intended for Authentication vs. Limited Set of Known Discriminating Metabolites: Hungarian Liquors in the Crosshair of Untargeted Metabolomics

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Non-administrative, that is, analytical validation of authentic products of protected origin and/or technology often faces the need for trade-off issues in terms of analytical methodology not only to fit financial frames but to select the groups of analytes intended for discrimination. In the case of plant derived products – where targeting secondary metabolites usually offers a straightforward way for characterization – the processing technology of raw materials determines whether volatile/non-volatile, hydrophilic/hydrophobic, low/medium/high molecular weight, inorganic/organic species and metabolites might be addressed in order to provide a qualitative/quantitative set of mostly molecular entities that can be applied for robust product discrimination and authentication. For example, the less gentle the extraction method of the plant biomass to arrive at a final product the more difficult it might be to detect and quantify either known or unknown analytes that are representative and selective for the given plant species. Additionally, the use of rare or exotic plants, or the application of plant tissues that are not used for general medicinal purposes (which are, accordingly, hardly covered by scientific reports) all make the analytical task relatively difficult.

The goal of our study was to carry out targeted (i.e., scientific databases related) and untargeted metabolomics analyses of Hungarian liquors produced with Hungarian distilled apple fruit spirit ("pálinka") from flowers of dandelions (*Taraxacum officinale*) and hawthorn fruits (*Crataegus monogyna*) to support the application for protected origin labelling. Taking into account that the flowers of *T. officinale* are not regarded as plant medicinal drugs and the extraction efficiency of "pálinka" spirit (with approx. 35-40 v/v% final ethanol concentration) towards flavonoids is too low to show up the most widely studied *T. officinale* and *C. monogyna* metabolites, ultra-performance (reversed phase + hydrophilic interaction) liquid chromatography - ion mobility assisted high resolution mass spectrometry was chosen as the ultimate analytical tool for untargeted metabolomics, backed by multivariate statistical analyses, to discover analytes with adequately high discrimination power. Facing the lack of usual flavonoids and their derivatives, plant and extraction technology dependent chlorophyll catabolites and phenolic acids seem to offer the mean for the reproducible and product specific characterization.

Keywords: UPLC-IMS-ESI-QTOF-MS, Vion, HILIC, product authentication, untargeted metabolomics, dandelion, hawthorn fruit, PLS, PCA

Targeted Metabolic Profiling Workflow for Urine, Plasma and Cell Extracts by Hydrophilic Interaction Liquid Chromatography Tandem Mass Spectrometry

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In this study, a versatile metabolic pathway-based targeted approach was developed for cellular, urinary and plasma metabolomic analysis using an UHPLC-QTrap-MS system operated in the multiple reaction monitoring (MRM) mode. MRM ion pairs were acquired from HeLa cell samples through untargeted analysis using SWATH-UHPLC-QTOF-MS, as well as by searching for metabolites in pathway databases. Two HILIC-MS methods employing a Waters Premier BEH Amide column were developed, utilizing two different chromatographic conditions (20 mM NH_4FA as buffer additive adjusted to a pH = 3.5 with formic acid in ESI^+ mode and 20 mM NH_4Ac adjusted to a pH = 7.5 with acetic acid in ESI^- mode. 161 metabolites were successfully detected in ESI+ mode, whereas 92 were detected in negative ionization mode, totaling to a number of 253 compounds in three different biological matrices covered by the analytical system employed. Both established HILIC methods were validated in accordance to the FDA guidelines for bioanalytical method development and validation based on 105 authentic chemical standards. Precision was determined on three different concentration levels and was below 15% for the entirety of the analytes. Matrix effects, process efficiency and recovery extraction were evaluated following the Matuszewski protocol with ^{13}C-labeled cell extract as internal standards. Four different cell extraction protocols were also further studied and compared based on an experiment series involving the calculation of individual metabolite recoveries (pre/post extraction spiking ^{13}C isotopic labeled standards), with a monophasic Methanol/Water extraction mixture (1:1) showing the best results. Eventually, the method has been utilized to quantify metabolites in HeLa cell extracts.

Keywords: metabolomics, HILIC, targeted, mass spectrometry, bioanalytical

Targeted Analysis of Sugar Mono-Phosphates by Liquid Chromatography Coupled with Tandem Mass Spectrometry

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The separation and quantification of sugar phosphates is an area of active investigation, due to their crucial roles in the biological system. In this study, liquid chromatography methods coupled to mass spectrometry detection were developed for sugar mono-phosphates. Different LC modes for sugar phosphates were explored, the mixed-mode HILIC/anion exchange chromatography (Shodex HILICpak VT-50 2D) stood out for unraveling the sugar monophosphates in glycolysis and pentose phosphate pathway. The LC conditions including column temperature, buffer concentration, pH value, and the gradient were optimized to gain a better separation of targeted compounds. The mixed-mode chromatography method resolved eleven biologically important mono-phosphorylated sugars and thereby enables simultaneous detection and quantification of those compounds, including glucose-1-phosphate, glucose-6-phosphate, fructose-6-phosphate, pyruvate, L-lactate, xylulose-5-phosphate, ribulose-5-phosphate, ribose-5-phosphate, ribose-1-phosphate, erythrose-4-phosphate, and sedoheptulose-7-phosphate.

Keywords: sugar phosphate, mixed-mode chromatography, HILIC, LC-MS, targeted metabolomics

UC/HILIC, a Novel Strategy to Improve the Metabolomics Coverage of Current Unified Chromatography

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Unified chromatography (UC), an advanced version of supercritical fluid chromatography (SFC), which applied gradient elution with mobile phase changing continuously from supercritical to subcritical and to liquid states, allows to further extend the SFC applications. Compared to SFC, the polarity working range of UC is much wider, however, many of highly hydrophilic compounds (e.g., phosphorylated metabolites, or multi carboxylic acids) still show very poor peak shapes or can't be eluted under UC conditions, thus hampering the methods' metabolome coverage. In this study, we proposed the first proof-of-concept of UC/HILIC, a novel strategy to extend the current UC metabolome coverage by employing a HILIC like gradient right after the UC gradient on a single column in a single measurement. The approach inherited the advantages of UC regarding the analysis of neutral and hydrophobic compounds, at the same time, utilizing the advantages of the HILIC regarding the analysis of hydrophilic compounds. The proposed UC/HILIC was able to not only improve ~10% higher metabolome coverage but also provide much better chromatographic performance (i.e., more symmetrical peaks, higher peak resolution, and narrower peak width) compared to the current UC in just one single analysis. Moreover, the optimized UC/HILIC coupled with mass spectrometry method (UC/HILIC-MS) showed comparable sensitivity and detection limits with other published studies utilizing GC, IC, or HILIC-MS.

Keywords: supercritical fluid chromatography, unified chromatography, hydrophilic interaction chromatography, mass spectrometry, UC/HILIC

Development and Optimization of Supercritical Fluid Extraction-Supercritical Fluid Chromatography Coupled to Mass Spectrometry Method (SFE-SFC-MS): Application to Cosmetics

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Nowadays, consumers are aware of the environmental impact that can have the product they consume. Thus, the cosmetics industry is adapting in order to respond to these environmental requirements. The introduction of the supercritical CO_2 in this field is in agreement with the precepts of green chemistry especially those related to eco-extraction. The purpose of this study is to demonstrate the feasibility and robustness of SFE-SFC coupling for the extraction of different lipids present in spinach for cosmetic applications. For this, a multi-loop system was used in order to study the composition of the different spinach extracts. Linoleic acid, a cheap lipid, was used to test the robustness of the method: sensitivity, repeatability and reproducibility.

The results obtained after injection of linoleic acid at 400 bar showed the feasibility of SFE-SFC coupling with MS detection. However, significant variation in area peaks from one loop to another (RSD % > 10 % for n=3) were obtained. In order to improve the variation in the injection, different pressure values (100 bar to 1000 bar) were tested. These experiments showed that the pressure of the system has no significant impact on the injected volumes and that the problem comes from the system of 12 loops. Despite the fact that the robustness of these loops could not be verified with linoleic acid, spinach extractions at 400 bar were realized to obtain trends. The results showed that the polar lipids of interest such as Mono- and Digalactosyldiacylglycerols (MGDG and DGDG) were successfully extracted and characterized. In perspective, pressurization tests of the 12 loops before injection will be done to improve the results.

Keywords: SFE, SFC, Green chemistry, Lipids, cosmetics

A Comparison of the Effects of Metal-free Columns and Bio-inert UHPLC Systems on Nucleotide Analysis

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The importance of bio-inert UHPLC systems has increased in recent years in order to improve the reliability and sensitivity of biomolecule analysis. One of the major concerns in the quantitative analysis of biomolecules is the adsorption of the molecules onto metal surfaces, which can lead to a dramatic decrease in sensitivity. [1,2] Therefore, it is essential to utilize a system with complete inertness in the sample flow path.

Different combinations of stainless-steel column, stainless-steel UHPLC, metal-free column, and inert UHPLC have been used for the separation of the oligonucleotides adenosine monophosphate (AMP), adenosine diphosphate (ADP), and adenosine triphosphate (ATP). In particular, ATP and ADP show strong adsorption on metal surfaces. Hence, these components are very suitable to assess the influence of the inert column and UHPLC system on the recovery.

By using metal-free components, the sensitivity of nucleotides could be significantly increased. While the metal-free column and the inert system led to a decrease in adsorption, the best results can be obtained by using both systems in combination.

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Keywords: metal-free, inert, UHPLC, oligonucleotides, columns

Pea Seed Metabolite Profiling Using Combined GC/MS and PyGC-MS Analysis

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We present a new method of metabolite profiling of hard-to process seed microsamples using combined GS/MS and PyGC-MS analysis. Certain types of sturdy plant microsamples frequently pose a significant challenge for metabolite profiling analysis. Generally, that is due to their resistance against standard sample preparation and metabolite extraction methods and due to the incomplete coverage of metabolite types the usable methods can provide. The advantage of the described method is the capitalization on the combined sample preparation protocol and the complementary information from both GC methods applied to different matrices produced during the extraction process and thus provide a more complete metabolic profile. The method is demonstrated on analysis of seed coat and hilum of two pea genotypes. It enabled the detection of numerous metabolite products both non-polar (wide range of short- and long-chain fatty acids and several phytosterols, namely β-sitosterol, campesterol, isofucosterol etc.) and polar (sugars, sugar alcohols, organic acids, amino acids, and few phenolic compounds such as epigallocatechin) from one piece of pea seed microsample. This protocol involves one-step reaction for preparing fatty acid methyl esters using rapid methanolysis/methylation procedure, which also substitutes the often costly or complex conventional additional derivatization steps otherwise required for GC/MS of fatty acids. The effects and dynamics of various degradation processes (such as ozonation or exposure to heat, UV rays and hydrogen peroxide) on the metabolic profile were also examined.

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Keywords: GC/MS, PyGC-MS, microsamples, degradation processes, pea seeds

Determination of pK_a of Analytes and pH of Buffers in Methanol-Water Mobile Phases by the Internal Standard – Capillary Electrophoresis Method

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The retention of acid-base compounds in liquid chromatography is strongly dependent on the degree of ionization of the compound, which in turn depends on the pK_a of the compound and the pH of the mobile phase. Optimization strategies for the chromatographic separation of this type of compounds require the knowledge of these parameters and their variation with the composition of the mobile phase.

The internal standard – capillary electrophoresis method (IS-CE) is a high-throughput method developed for a fast determination of acidity dissociation constants (pK_a) from the pK_a of the internal standards (IS), and thus, it does not require the potentiometric measurement of the pH of the solution.

In this work, the IS-CE method is extended to methanol-water mobile phases. The reference pK_a values of a set of 46 acid-base compounds (23 neutral acids and 23 neutral bases) have been accurately established from electrophoretic measurements in methanol-water. The pK_a values of 6 of the ISs were also potentiometrically measured in all mobile phases to anchor the electrophoretic pK_a scales.

The established ISs reference set allows to determine the pK_a of analytes and measure the pH of buffers in the range 2-11 (in water) for any methanol-water mobile phase. It has been tested determining the pH of some of the most common buffers.

Keywords: Capillary electrophoresis, Internal standard, Acidity constants, Methanol

Analysis of Intact Proteins with Capillary zone Electrophoresis Coupled to Mass Spectrometry using Uncoated and Coated Capillaries

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Proteins are the actuators of many vital biological processes. Understanding the structural characterization, identification of macromolecules in their intact states entails the studies of cell biology, disease prevention and treatment [1]. Top-down mass spectrometric (MS) technique is sensitive to enable the studies for structural and dynamical identification of intact proteins when coupled with capillary zone electrophoresis (CZE) [2]. However, a serious concern is the analyte adsorption on the bare fused silica (BFS) capillary surface, which necessitates the application of extreme pH or the use of coatings to minimize the analyte-wall interactions [3]. Our study involves the use of BFS capillaries employing the background electrolytes with very low pH and compares the analytical performance with those coated with polybrene as a dynamic and linear polyacrylamide (LPA) as a static coating material. The work demonstrates the differences in the ideal operating conditions (optimal pH, proper capillary conditioning etc.) of each capillary.

The results suggested that the analysis in BFS capillaries with BGE of very low pH (pH=1.8) resulted in good precision (0.56-0.78 RSD% and 1.7-6.5 RSD% for migration times and peak areas respectively) and efficiency values with minimum adsorption into the capillary surface. Coated capillaries showed higher resolving power for the separation of different forms (subunits of hemoglobin) of the protein. However, the separation performance in LPA coated capillary distinguished from others based on their stability, reproducibility over 25 runs and shorter analysis time in less than 10 min. The applicability of the proposed methods was also supported by the analysis of protein rich samples (e.g., snake venom). Hereby, the application of BFS capillaries for the analysis of intact protein mixtures would be considered also an efficient choice compared to coated capillaries when ideal conditions are applied.

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Keywords: capillary electrophoresis, mass spectrometry, intact protein, top-down

Development of Capillary Electrophoresis Method for the Simultaneous Separation of Boswellic Acids and Nonsteroidal Anti-Inflammatory Drugs

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This presentation describes the development of a capillary electrophoresis (CE) method for the simultaneous separation of boswellic acids and nonsteroidal anti-inflammatory drugs (NSAID), which can be used as adulterants in dietary supplements containing *Boswellia serrata* extract. The method was optimized for the mixture of *Boswellia serrata* extract and 15 NSAIDs. To make the method MS-compatible, only volatile background electrolytes (BGEs) were tested. The influence of BGE composition including buffer type and its concentration and pH, and the type and amount of organic modifier on separation selectivity was studied. The separation was carried out in 40/48.5 cm and 88/96.5 cm fused silica capillaries, 50 µm i.d. with the applied voltage of + 30 kV and at the temperature of 25 °C. The analytes were detected at 200 and 250 nm.

The best resolution for the individual NSAID (15 compounds resolved into 12 peaks) was achieved when using the acidic BGE (50 mmol/l acetic acid (pH 4.5) : MeOH : ACN, 10:3:12), the boswellic acids, however, migrated unresolved with EOF. The use of the alkaline buffers decreased the separation selectivity for the NSAIDs, but it allowed to improve the resolution of boswellic acids. The *Boswellia serrata* extract components no longer migrated at the rate of EOF and were separated into 4 peaks when using 40 mmol/l ammonium acetate (pH 8.5) : MeOH : ACN, 5:1:4 buffer, and into two peaks when using 40 mmol/l ammonium bicarbonate (pH 8.5) : MeOH : ACN, 5:1:4 buffer. The mixture of 15 NSAID was resolved into 9 peaks in both cases. The separations developed with UV detection were transferred to CE coupled to mass spectrometer with electrospray ionisation and triple quadrupole detection.

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Keywords: capillary electrophoresis, mass spectrometry, NSAID, boswellic acids

Optimization of CZE-UV Method for Simultaneous Analysis of Seven Biological Substances

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Seven biological substances – epinephrine, norepinephrine, dopamine, serotonin, tyramine, thiamine, and pyridoxine, were analyzed simultaneously by simple capillary zone electrophoresis with UV detection. The mixture comprises two B vitamins and five amines associated with inflammatory bowel diseases. CZE-UV method was performed in hydrodynamically closed mode – separation capillary (300 µm ID, 160 mm length) is ended with semipermeable membrane to prevent hydrodynamic flow and has higher sample load capacity than a conventional hydrodynamically open system, what results in lower LOD. Simultaneous separation of chemically similar structures is challenging and requires a thorough optimization process. As BGE, 25 mM GABA + 50 mM HAc + 0,1 m-HEC was chosen, but resolution between analytes was not sufficient (serotonin and dopamine comigrated in one peak). Various additives of organic solvents – methanol, isopropanol, acetonitrile, tetrahydrofuran, and β - cyclodextrins were tested as modifiers of the separation environment. A combination of additives - 5% THF and 10% IP was chosen as the best variant. Linear range was 0.5-50 µg/mL with great linearity ($r^2 > 0.99$). Achieved LODs ranged from 0.15-1.25 µg/mL.

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Keywords: capillary zone electrophoresis, UV detection, inflammation, organic solvents, β-cyclodextrins

Hyphenation of 2D-CE with MS/MS Detection for Determination of Serotonin in Clinical Urine Samples

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Crohn´s disease is one of the most increasing diseases worldwide that affects people in productive age. It represents a type of inflammatory bowel disease affecting any part of the gastrointestinal tract. Serotonin has many physiological functions in the central nervous system and also in the periphery. Over 90% of the total amount of this biogenic amine is presented in enterochromaffin cells in the gut. Serotonin can play a significant role in inflammatory bowel disease pathophysiology and diagnostics. A novel 2D-CE method based on a combination of isotachophoresis (ITP) and capillary zone electrophoresis (CZE) coupled with tandem mass spectrometry (MS/MS) detection was developed for analysis serotonin in clinical human urine samples. The column coupling arrangement enabled sample preconcentration and purification in the ITP step and own electrophoretic analysis in the second CZE step. The developed approach was characterized by favorable performance and validation parameters. The limit of detection (LOD) value was predicted at ultra-trace concentration level – 33.97 pg/mL. Finally, the method was applied for determination of serotonin levels in human urine samples of three patients suffering from Crohn's disease and one healthy individual. The measured concentrations of serotonin in human urine samples were normalized on creatinine levels. The normalized serotonin concentration levels were in the range of 6.88 – 18.03 ng/mmol.

Acknowledgments: This work was supported by the projects UK/21/2022, VEGA 1/0514/22, APVV-15-0585, KEGA 027UK-4/2020 and carried out in the Toxicological and Antidoping Center at the Faculty of Pharmacy Comenius University.

Keywords: capillary electrophoresis, isotachophoresis, mass spectrometry, serotonin, Crohn's disease, clinical samples

Development of a CZE-UV Method with Repeated Sample Injection in Hydrodynamically Closed System for Determination of Ibuprofen

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The speed of analysis and sample throughput are very important parameters that determine the real application potential of the developed method in real practice. Capillary electrophoresis represents a powerful tool for the separation of a wide variety of substances in different sample types. Various approaches are used to improve sample throughput in the CE environment, such as multiple injection or multisegment injection strategies. All of them were implemented only into the hydrodynamically open CE systems. In the present work, a CZE-UV method with repeated sample injection within one run in hydrodynamically closed separation system was developed and optimized. The non-steroidal anti-inflammatory drug ibuprofen was selected as the model analyte. Separation was performed in a background electrolyte composed of 10 mM MOPS with 20 mM TRIS and 0.05% m-HEC. The time period between repeated sample injections was optimized, and the value of 100 s was selected as the optimal one. Under these conditions, it was possible to analyze three samples in one run. Furthermore, the developed method was characterized by excellent validation parameters, such as limit of detection at the 0.3 µg/mL concentration level.

The work was supported by the projects VEGA 1/0514/22, KEGA 027UK-4/2020, APVV-15-0585 and FaF/2/2022, and was carried out in the Toxicological and Antidoping Center at the Faculty of Pharmacy, Comenius University in Bratislava.

Keywords: capillary electrophoresis, hydrodynamically closed system, repeated sample injection, sample throughput, ibuprofen

Dynamic Microfluidic Device for Rapid Determination of Octanol/Water Partition

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The determination of partition coefficient (*P*) is a widespread approach to predict the transport and accumulation of bioactive molecules in biological and natural environment. Traditionally, the evaluation of this coefficient is done by the determination of the partition of a species between the octanol and the water phases (O/W). The standard method so-called "shake-flask" is well known for its simplicity. However, it is a static and time-consuming method that requires large quantities of solvents and reagents.

Herein, we introduce a dynamic microfluidic plate for the determination of octanol-water partition. The design consists in a 2 separated microfluidic channels distributed in a 3D structure, where the octanol channel flows over and perpendicularly through the water channel. The continuous and bi-directional perfusion of the W/O solution is induced by tilting the device. Since the reservoirs are connected by microfluidic channel, flow is achieved by placing the plate under an angle on an interval rocker that inverts the angle at regular intervals.

Working at such small scale under dynamic conditions we can reach the equilibrium faster and reduce time and costs of the analysis. Full compatibility with standard equipment and its user-friendly operation makes this O/W partition platform readily applicable in routine laboratories.

Keywords: octanol-water partition, microfluidics, pharmacokinetics, 3D printing, two-phase flow

Effect of Viscosity on the Determination of the Retention Factor in Electrokinetic Chromatography

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The chromatographic retention factor (*k*) of a compound in a given system is a parameter not only related to its chromatographic behavior, but also used to estimate relevant physicochemical or biological properties of the compound. Therefore, the correct determination of *k* is of main importance for many applications.

There are several ways to determine the retention factor. However, when the compounds are ionized, the mobility of the compound in plain buffer (μ_0) is subtracted from the mobility in the micellar or microemulsion system at the same pH (μ). We have noticed that the addition to the buffer of the components needed to create the micelles and especially microemulsions, may create significant differences in viscosity in the two compared mediums which cause important deviations in the determined *k* values.

In this work we evaluate the retention profiles along pH of several ionizable compounds in microemulsion electrokinetic chromatography (MEEKC). To overcome the problem of the different viscosity we propose a simple correction factor based on the mobility ratio (μ/μ_0) of ionized compounds that do not interact with the microemulsion, such as benzoate anion or ephedrine cation. Important changes in the profiles are observed after the correction, especially when the compounds are partially or totally ionized.

Keywords: retention factor, microemulsion, MEEKC, viscosity, ionizable compound

Modeling the Enantiomeric Separation of a Mixture of Drugs by Cyclodextrin Electrokinetic Chromatography

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Capillary Electrophoresis (CE) has shown a great potential in the field of chiral separations due to some advantages such as the low consumption of reagents and samples, high efficiency, and simplicity since the chiral selector can be added to the separation medium in the Electrokinetic Chromatography mode. Cyclodextrins (CDs) are the most used chiral selectors employed in chiral EKC due to their high discrimination power against a big variety of compounds. However, the use of a CD as the sole chiral selector sometimes does not originate the desired stereoselective separation. Therefore, systems based on the use of CDs mixtures are used.

The objective of this work was to model the enantiomeric separation of a mixture of drugs by EKC using a combination of CDs. Dubsky´s model was employed for this purpose. By performing a small number of individual experiments separately with each CD, Dubsky's model enabled to foresee the results that could be obtained for any possible combination of concentrations and relative proportions of CDs in the mixture. A good agreement between the experimental results obtained and those predicted by the model was observed. Some antagonistic and synergistic interesting effects relative to the use of the mixture of CDs were also predicted by the model and experimentally corroborated. In addition, the model predicted the reversal in the elution order of some compounds when changing the total CDs concentration according to the experimental observations.

Keywords: capillary electrophoresis, Dubsky's model, drugs, simultaneous enantiomeric separation

Highly Efficient Analysis of 18 PAHs with Shim-C18-PAH

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Polycyclic aromatic hydrocarbons (PAHs) are a risk for the human health as many of them are carcinogenic, mutagenic and toxic for reproduction. They are also hardly degradable in the environment. Therefore, PAH analysis is crucially important. Shim-C18-PAH is a new and specially designed column for the challenging PAH analysis. Shim-C18-PAH delivers perfect resolution and fast analysis for PAHs even at ppb level.

Keywords: Polycyclic aromatic hydrocarbons, PAHs, HPLC, Fluorescence

Improvements in the Chromatographic Analysis of Antiviral Compounds Utilizing a New Hybrid Organic-Inorganic Surface Technology

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Antiviral pharmaceutical compounds are under development for treatment of mild, moderate, and severe critical illnesses, such as COVID-19. It is important that routine chromatographic methods developed to analyze these pharmaceutical compounds accurately distinguish the active pharmaceutical ingredient (API) and low level-impurities well before they reach alert/action levels.

To meet this requirement, parameters such as retention, peak shape, and sensitivity are critically important. Free metal-ions released from column materials and instrument corrosion can interact with analytes through complexation, oxidation, and epimerization reactions. This interaction can result in poor, unacceptable peak shape, and possibly the complete loss of the target analyte, particularly at low concentrations.

In this poster, we compare the reversed-phase separation of antiviral compounds investigated for treatment of COVID-19 generated using conventional chromatographic materials to a separation achieved using materials embedded with a new hybrid organic-inorganic surface technology.

Keywords: antiviral compounds, non-specific adsorption, metal interactions

Characterization of vaccine antigen/adjuvant interactions by Capillary Zone Electrophoresis: Application to polio virus/aluminum oxyhydroxyde

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Characterization is a very important part in new vaccine development to provide information required to manage the process and ensure the quality of final product. Among characterizations, the study of antigen-adjuvant interactions in the final vaccine formulations is required as interaction competitions may take place between the different antigens in combination vaccines. Work presented in this poster concern development of a capillary zone electrophoresis (CZE) methodology for antigen-adjuvant interactions study. CZE is firstly optimized on six model proteins. The developed methodology is then used to separate three strains from inactivated polio virus, each strain being a whole virus composed of copies of 4 viral proteins and study their interaction with aluminum oxyhydroxide used as adjuvant. The antigen-adjuvant interactions could be modulated by addition of phosphate ions playing the role of competitors for the poliovirus.

FUNDING: This work was partly funded by Sanofi under a Cooperative Research and Development Agreement with the University of Montpellier and CNRS. CM, JFC, and JT are Sanofi employees and may hold shares and/or stock options in the company. LL and HC declare no competing financial interest.

Keywords: vaccines, capillary zone electrophoresis, virus, aluminum oxyhydroxyde

Analytical Study of the Separation Behaviour of Oligonucleotides on IP-RPLC

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Ion-pair reversed phase liquid chromatography (IP-RPLC) using a mobile phase containing TEA (triethylamine) as IP reagent and HFIP (1,1,1,3,3,3-hexafluoro-2-propanol) as acidic modifier has been widely used for oligonucleotide analyses. In this study, we investigate the effect of analytical conditions such as pH, types and concentration of IP reagent (TEA and other alkylamines) and HFIP, and column temperature on the retention and peak shape of oligonucleotides. We also compare the separation behaviour of 20 and 21mer oligo-RNAs which contain phosphorothioate modifications at every linkage (all PS RNAs) to phosphodiester RNAs (all PO RNAs) with the same nucleotide sequence.

The pH of mobile phase was increased by adding alkylamine and the retention of oligonucleotides was enhanced up to pH 8 and then decreased as the pH approached 9. Therefore, the effects of alkylamine types and column temperature on the separation of PO RNAs and PS RNAs were compared by simultaneously varying the concentrations of alkylamine and HFIP maintaining a pH around 8. Increasing the column temperature resulted in better peak shapes for PO RNAs. However peak fronting was observed at 90°C for PS RNAs. Changing the IP reagent from TEA to a different alkylamine resulted in different effects of retention and peak shape, and in some cases, the resolution of 20 and 21mer was improved.

In addition, we demonstrate the effect of column hardware on oligonucleotide separation and sample conditioning. Standard column hardware is compared to the recently introduced YMC-Accura Triart columns with bioinert coated hardware.

Keywords: oligonucleotides, phosphorothioate oligonucleotides, IP-RPLC, ion-pairing, bioinert column hardware

High-Throughput Analysis of Oligonucleotides using a Single Quadrupole Mass Spectrometer for Quality Control

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Since the advents of Alexander Todd and his group's research of the oligonucleotide synthesis in the early 1950's and the introduction of the solid phase phosphoramidite synthesis in the early 1980s, the fields of immunology, virology, and RNA based therapeutics utilizing custom designed oligonucleotides has only increased in public interest. Subsequently, the commercial demand has amplified dramatically especially with the emergence of the COVID-19 pandemic. To give perspective, single stranded DNA has been a pioneering research tool for therapeutics for over 20 years providing insight into precursor (pre)-mRNA splicing, gene expression, as well as immuno-pathways. As of 2020 there are more than 50 antisense oligonucleotide therapeutics in various stages, 25 of which are in advanced stages (Phase II or III), and the US FDA (United States Food and Drug Administration) currently has approved a total of 11.

Laboratories producing large arrays of customized DNA need to support this elevated throughput via increased automation and accuracy using intact mass determination for quality control. With this workflow from robotic DNA synthesis all the way through a confident pass/fail outcome for the expected sequence. Determination of the intact oligonucleotide mass uses a single quad LCMS method with minor method optimizations provide cost savings and the reduction of 1,1,1-3,3,3-hexa-fluoro-iso-propanol (HFIP) and sodium adduct abundancy.

Keywords: Single quadrupole mass spectrometer, intact mass deconvolution, DNA, RNA, oligonucleotide analysis, quality control

Characterisation of Polyethylene Fibres as a Reference Hydrophobic Material for Chromatographic Purification of Peptides

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Peptide purification is generally performed through reversed-phase liquid chromatography (RPLC) in silica-based resins as a sole or final polishing step. This technique is particularly preferred as most impurities from chemical synthesis are very similar to the target peptide in size and charge, but their hydrophobicity is sufficiently different to be resolved by RPLC. Although the efficiency of purification is fully dependent on the hydrophobic properties of both the peptides and the resin, these intrinsic hydrophobic properties are not determined for method development. Instead, purification is adjusted based on empirical separation performance in arbitrary chromatographic columns by changing mobile phase conditions. In this work, we propose the use of a reference hydrophobic material to estimate the intrinsic hydrophobic parameters of peptides and resins independently as tools for rational separation method design. Surface characterisation of polyethylene fibres was performed using nitrogen adsorption, contact angle and scanning electron microscopy. The chromatographic separation performance of these fibres was assessed by inverse liquid chromatography using hydrophobic probes and standard peptides. Despite the low specific surface area of polyethylene fibres (<1 m^2/g) compared to that of fully porous silica (ca. 400 m^2/g), a selectivity value of 2 was still obtained between the most hydrophobic peptide standards. Additionally, the minimum concentration of acetonitrile required to elute each peptide standards are highly correlated (r^2=0.967) to their Kyte & Doolittle hydrophobicity values, offering the use of these fibres as a rapid chromatographic hydrophobicity test that is not affected by external factors such as pore size distribution or classic silanol-associated electrostatic interactions.

Keywords: Peptides, peptide hydrophobicity/hydrophilicity, hydrophobicity scale, polyethylene fibres, reversed-phase liquid chromatography (RPLC)

Simultaneous Reversed-phase and Anion-exchange Method Scouting for mRNA Impurity Determination

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mRNA is typically produced by transcribing a plasmid DNA template via what is called *in vitro* transcription (IVT), mimicking the process that takes place in the cell (*in vivo*). After synthesis, the mRNA must be purified from the remaining reaction byproducts. Purification is important because some impurities may trigger immune responses and decrease mRNA translation. While some impurities will remain from the raw materials, others will be more dependent on the mRNA sequence. Among the most common impurities are nucleotides, enzymes, DNA templates and fragments, abortive transcript fragments, double stranded RNA (dsRNA), primers. HPLC with UV detection is ideally suited for estimating mRNA purification efficiency. Ion-pairing reversed phase (IP-RP) and anion exchange chromatography (AEX) are the separation modes typically used for this purpose. Several ion-pairing agents are available for mRNA analysis by reversed-phase. Similarly, AEX can be run with different salt systems. Mobile phase pH, buffer concentration, column temperature etc. all significantly impact the method, but exponentially increase the number of possible combinations of chromatographic settings and, therefore, the complexity of method development. The right conditions for each modality depend on the length and conformation of the mRNA, and are not easily predicted. Therefore, screening a variety of mobile phase and chromatographic conditions is recommended to maximize the chances of developing a fit-for-purpose method. However, this approach comes with a considerable time investment. To mitigate the time burden, we scouted IP-RP and AEX methods in parallel using a dual-UHPLC system. The dual system consisted of two ternary pumps capable of managing up to 12 different solvents each. The autosampler consisted of two independent injectors and shared sample vessels. The temperature of the reversed-phase and anion exchange columns were controlled independently. An extensive number of conditions were scouted and analyzed within 2 days using a 2500 nt mRNA. Using this approach methods for IP-RP and AEX were developed with limited time investment. The most suitable gradient conditions for the IP-RP method were obtained using 25 mM hexyl ammonium acetate at pH 7, and column at 50 °C. For the AEX method, the best conditions were obtained with 40 mM TRIS at pH 9, and a salt gradient with $NaClO_4$; 10% MeCN was added as organic modifier, and the column temperature was set at 80 °C.

Analysis of Lipid Nanoparticle Composition

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Lipid nanoparticles (LNPs) have emerged as promising delivery vehicles for nucleic acids in the pharmaceutical industry. To ensure safety and efficacy of the final drug product, the lipid components need analytical characterization of composition, ratio, and degradation. During this research work, liquid chromatographic method development for the analysis of the lipid components of patisaran (trade name Onpattro and first LNP-encapsuled RNA drug approved by the FDA and the EMA) is shown in a quaternary setup. A method-combining methanol (MeOH) and acetonitrile (ACN) resulted in optimal separation of the four LNP components with excellent peak shapes, precision, and sensitivity. The Agilent 1290 Infinity II Bio LC with Agilent 1290 Infinity II ELSD enables universal detection of the lipid components lacking a UV chromophore. In addition, the high dynamic range of the Agilent 1290 Infinity II ELSD allows the detection of all four lipids in the patisaran-like sample.

Keywords: Lipid nanoparticles, ELSD, Bio LC, pharmaceutical industry

Quantification of Dopamine and Serotonin Metabolites in Cerebrospinal Fluid: Comparison of a UHPLC-MS/MS Method to a UHPLC coupled to Fluorescence Detection Method

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Inborn errors of dopamine and serotonin metabolism are rare diseases characterized by mutations on the genes coding for enzymes involved in these metabolic pathways or for neurotransmitter transporters. To diagnose these inborn diseases, biomarkers have been identified and validated in cerebrospinal fluid (CSF), namely 5-hydroxy-tryptophane, 5-hydroxy-indol-acetic acid, 3-ortho-methyl-DOPA, homovanillic acid and 3-methoxy-4-hydroxyphenylglycol. The diagnosis is based on CSF analysis by UHPLC coupled whether to MS/MS or to fluorescence detection (FD) depending on the desired budget and practicality. The present work aims at comparing a UHPLC-MS/MS method to a UHPLC-FD method for the quantification of these biomarkers.

The UHPLC-MS/MS and UHPLC-FD methods were validated in terms of accuracy, linearity, precision and matrix effect. The lower limits of quantification (LOQ) were ranging between 0.5 nM and 10 nM and between 1 and 5 nM for the UHPLC-MS/MS method and the UHPLC-FD one, respectively. We verified both methods' applicability by analyzing 30 CSF samples. The two methods allowed to distinguish pathological samples from healthy ones, hence, their suitability for the diagnosis of inborn errors of neurotransmitter metabolism. Concerning the comparison of both methods, MS/MS allowed to reach higher specificity than FD. Moreover, UHPLC-MS/MS being more selective, it allowed faster analysis with 6 minutes *per* run versus 10 minutes for the UHPLC-FD method.

Keywords: Biomarkers, Cerebrospinal fluid, UHPLC-MS/MS, Fluorescence detection

Cation-Exchange Chromatography for the Separation of Monoclonal Antibodies

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Cation exchange chromatography (CEX) is a widely used non-denaturing approach for the characterization of acidic and basic variants of therapeutic monoclonal antibodies (mAb). Beside the classical salt gradient separation mAbs can be eluted applying pH gradient or salt-mediated pH gradient. In order to achieve good separation the combination of the mobile and the stationary phases can be an important aspect.

The goal of our work was to find alternative mobile phases for the cation exchange separation of monoclonal antibodies. Different biologically applied buffer compounds were used for studying the generated and the actually emerging pH response. It was found that the MES/DAP buffer system provides a linear pH response in the range of $5 \leq pH \leq 10$ in pH and in salt-mediated pH gradient modes, while the pH response of citric acid/CHES + NaOH is linear in $2.5 \leq pH \leq 10$ range, but the observed peak shape is only acceptable, when the pH gradient is combined with a salt gradient.

It was also demonstrated that the stationary phase has an impact on the pH gradient forming in the columns. Therefore during method development for characterization of mAbs not only the mobile phase but the stationary phase has to be taken into consideration.

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Keywords: mAb, CEX, pH-gradient

Identification of Transformation Products and Metabolites of Selected Bisphenols

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Bisphenol A (BPA) is used in Polycarbonate (PC), Polyacrylic resins (PAR), Polysulfones (PSU), Epoxy resins (ER), and Polyetherimides (PEI). It is also used in recycled Polyvinyl chloride (PVC) [1–3]. These BPA-containing materials have a wide area of application, especially outside. All outdoor applications are exposed to a variety of environmental impacts, like temperature, solar radiation (physical influences), chemical influences (rain or ozone), biological influences (microorganisms), and mechanical influences (hail, sand). These impacts are damaging and aging the material which can be followed by leaching or migration of pollutants like Bisphenol A into the environment. Understanding the fate and behavior of the released pollutants is very important. Therefore, different transformation products of selected Bisphenols will be generated and analyzed:

- Generation of technical transformation products with oxidizing agents that are used in water treatment plants. Simulation of Industrial wastewater treatment with the Fenton reaction, an advanced oxidation process.

- Production of transformation products by global radiation to study the environmental behavior of released pollutants.

- Simulation of the phase I metabolism by an electrochemical cell coupled to mass spectrometry.

All metabolites and transformation products will be analyzed by a variety of gas and liquid chromatographic techniques coupled to mass spectrometry and 1H/13C-NMR experiments.

Keywords: transformation products, bisphenols, metabolites, wastewater, environment

Challenges in Designer Drug Analysis, Through the 4F-MDMB-BICA Synthetic Cannabinoid

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Synthetic cannabinoids (SCs) are the largest group of designer drugs that bind to the same CB1 and CB2 receptors to which THC-Δ9 attach. However, SCs can be highly toxic thanks to their full agonist activity and high affinity for cannabinoid receptors. Their appearance on the black market changes rapidly, so their identification, establishment of consumption, and clinical treatment are essential tasks in forensic toxicology. The main problem with confirming SC intake is the available short time for analysis of parent molecules after their consumption in biological samples due to their rapid metabolism. Therefore it is crucial to analyse not just the parent molecules but also their characteristic metabolites. The 4F-MDMB-BICA is a new dangerous illicit SC because several deaths have been attributed to its consumption in Hungary. In our work, 4F-MDMB-BICA were incubated with human liver microsome in order to identify the most abundant and characteristic *in vitro* metabolites using UHPLC-HRMS/MS method. The parent molecule and the ester hydrolysis primary *in vivo* metabolite were quantitatively confirmed in authentic positive blood and urine samples with a validated routine LC-MS/MS method.

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Keywords: LC-MS/MS, metabolites, synthetic cannabinoid, human liver microsome incubation, human blood and urine

Determination of Endocannabinoids from Human Serum with LC-MS/MS Method

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Recent studies identifies five endogenous substances so called endocannabinoids (ECs) acting on CB1 and CB2 cannabinoid receptors. These ECs are anandamide (AEA), 2-arachidonoyl glycerol (2AG), arachidonoyl ethanolamine, (O-AEA), 2-arachidonyl glycerol ether (2AGe) and arachidonoyl dopamide (NADA). CB1 and CB2 receptors not only occur in the central nervous system, but they are widely distributed in various organs and tissues. Recent studies shows that endocannabinoids can also act on other receptors including transient receptor potential vanilloid type-1 (TRPV1) and orphan G-coupled receptors GPR55. This indicates that ECs can play different physiological roles both in central and in peripheral tissues. There are several structurally similar compounds like, oleic acid ethanol amide (OEA), palmitic acid ethanol amide (PEA), n-oleoyldopamine (OLDA) and stearic acid ethanol amide (SEA) that are not acting on CB receptors, but can affect EC actions.

Due to LC-MS/MS method allows the quantitative analysis of lipophilic and apolar metabolites with short analysis time and minimal sample preparation (no derivatization is needed), and can differentiate between similar compounds even from a complex matrix, it is widely used for the analysis of ECs.

In our study a quantitative method has been developed using triple quadrupole mass spectrometer coupled to reverse phased liquid chromatography, for the simultaneous determination of AEA, OEA, NADA, SEA, PEA and OLDA from human serum. The samples were taken from patients after surgical intervention involving deep anesthesia. The aim of the clinical study is the investigation of the correlation between the EC level expressed in the human body and the anesthetic method used during the intervention.

Targeted Metabolomics of Tryptophan and its Metabolites in Neurological Diseases

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The kynurenine pathway is the main degradation pathway of the essential amino acid tryptophan, leading to the production of both neuroprotective and neurotoxic compounds. Observed changes in metabolite concentrations may serve as potential biomarkers and/or therapeutic targets.

Liquid chromatography coupled to mass spectrometry (LC-MS) is an excellent option for the simultaneous, fast and accurate determination of several low-concentration analytes. During our work, we developed a new, short and robust UHPLC-MS/MS method for the quantification of tryptophan and its eleven metabolites (3-hydroxyanthranilic acid, 3-hydroxykynurenine, 5-hydroxyindoleacetic acid, anthranilic acid, kynurenine, kynurenic acid, quinolinic acid, melatonin, picolinic acid, serotonin and xanthurenic acid). After the validation of the method, the concentration of the 12 compounds was examined in the body fluids of multiple sclerosis or migraine patients to interpret the metabolic pathways characteristic of the diseases.

A significantly higher quinolinic acid/kynurenic acid ratio was found in multiple sclerosis patients compared to the control group. The increased picolinic acid/quinolinic acid ratio may result from the ability of the neuroprotective picolinic acid to antagonize the neurotoxicity of quinolinic acid or reflect the inflammatory process of the disease.

The results of our migraine-related research show that the entire metabolic pathway is depressed during the interictal (attack-free) period, but increased metabolite concentrations can be observed during the ictal interval.

Keywords: Tryptophan metabolism, Validation, Multiple sclerosis, Migraine

Development and Clinical Application of a Novel DLLME-HPLC-DAD-FLD Method for the Determination of CDK4/6 Inhibitors in Patient Plasma Samples

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Cyclin D dependent kinase 4 and 6 (CDK4/6) inhibitors palbociclib (PAL), ribociclib (RIB) and abemaciclib (ABE) are novel drugs used in the treatment of HR+, HER2- breast cancer. They are undergoing additional monitoring to gain further insight into their security profiles, as well as the inter-patient pharmacokinetic variabilities. In this work, a novel, cost-effective and eco-friendly dispersive liquid-liquid microextraction (DLLME) sample preparation procedure and a HPLC-DAD-FLD method were developed for the analysis of these drugs in human plasma. The samples (100 µL) were extracted with 150 µL of the mixture of isopropanol and chloroform (1:2, v/v) as the dispersive and extracting solvents, respectively. High extraction yields (above 90%) and good sample clean-up were achieved. The chromatographic conditions include an XBridge phenyl column (150 x 4.6 mm, 3.5 µm) with methanol and water containing 0.1% formic acid in gradient elution. At a 1 mL/min flow rate and a 30 °C column temperature, the analytes eluted within 6 min. The method proved linear in the ranges of 250–6000 ng/mL for RIB, 150–4000 for PAL, and 100–2500 for ABE, thus enabling their determination in real patient plasma samples. Mean precision (% CV) of quality control samples was less than 6.7% and the accuracy (% bias) was less than 1.5% for all analytes. The method was successfully applied for the quantitation of the drugs of interest in real patient plasma samples. In comparison to other previously published methods, this approach utilises a novel plasma sample preparation procedure and ensures adequate quantitation limits using easily accessible detectors.

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Keywords: palbociclib, ribociclib, abemaciclib, DLLME, TDM

Column Hardware Modified with a Hydrophilic Hybrid Surface for Aqueous Biomolecule Separations

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Large biomolecules carry hydrophobic moieties and are often multiply charged under conditions applied in liquid chromatography. Therefore, there is potential for both hydrophobic and ionic secondary (non-specific) interactions to occur with chromatographic hardware (column frit and inner wall) to the detriment of peak recovery, peak shape and the overall sensitivity of the LC analysis. To mitigate these types of non-specific interactions, MP35N, titanium and PEEK are often used in place of stainless-steel (SS) materials. In many cases, these modifications improve recovery and peak shape to a certain extent, but in some cases, the above alternatives have not entirely addressed all problems related to non-specific adsorption.

Despite all these efforts, some samples have remained a challenge in terms of minimizing analyte to column hardware interactions. To decrease such undesired adsorption and secondary interactions, a new hydrophilically modified hybrid surface (h-HST) has been developed which incorporates an additional hydrophilic layer on its surface. As a result, it exhibits reduced electrostatic properties and a hydrophilicity that facilitates challenging aqueous separations. This new material has been characterized and found to be superior in many respects.

This hydrophilic surface was used in the form of size-exclusion chromatographic (SEC) column hardware and compared to unmodified HST and SS hardware with a challenging separation condition entailing the use of low ionic strength volatile buffer. The h-HST material yielded improved monomer and aggregate recovery, higher plate numbers and more symmetrical peaks for challenging monoclonal antibodies (mAbs) and antibody-drug-conjugates (ADCs).

Ion-exchange (IEX) columns can also benefit from the new h-HST hardware. MAb and nucleic acid (intact mRNA) samples showed improved recovery and separation quality through the use of h-HST column hardware, where it was seen that h-HST provided higher sensitivity and more repeatable peak areas from injection-to-injection.

The new h-HST chromatographic hardware shows significant benefits for SEC and IEX separations of biomolecules. The hydrophilic modification limits undesired hydrophobic interactions during aqueous separations while hybrid polymer of the h-HST surface mitigates electrostatic interactions that can occur between metals and analytes. As such, there is significant potential in the use of h-HST chromatographic hardware to facilitate more robust, more sensitive analyses for a multitude of challenging separations and analytes.

Keywords: secondary interactions, hydrophilic surface, size-exclusion, ion-exchange, column hardware

MLC Technique and QRARS/QSARS Models to Predict Different Properties of Potential Pesticides

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Newly synthesized carbamic acid and phenoxyacetic acid derivatives considered as potential pesticides were investigated using micellar liquid chromatography technique (MLC). Chromatographic parameters were used as lipophilicity descriptors and applied in QRARs and QSARs method to predict different parameters characterizing bioactivity of compounds tested. In QSARs models the numbers of hydrogen bond donors (HBD), acceptors (HBA), and rotatable bonds (NBR) were considered as independent variables. All the derived QSAR equations were evaluated statistically and validated as being very good. The results showed the effect of lipophilicity, acidity and/or basicity as well as flexibility on compounds bioacitvity and allowed to identify those that are the most promising as potential pesticides. The investigations highlighted the significance and possibilities of combined chromatographic techniques and QRARs/QSARs methods in modeling important properties of potentially active organic compounds and reducing unethical animal testing.

Keywords: micellar liquid chromatography, QRARs, QSARs, bioacticity, pesticides

Automating chromatographic data batch-processing in Drug Discovery with MGears

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The processing and review of chromatographic data is often a labour-intensive bottleneck in the Drug Discovery process. In recent years, the advent of lab robotics and HTE (High Throughput Experimentation) coupled to faster instrumentation has been driving up the volume of analytical data generated exponentially. Software solutions to automate those manual review and decision-making steps offer the potential to free up significant analysts' time, speed up DMTA (Design-Make-Test-Analyse) cycles and enhance both the quality and consistency of our science. At AstraZeneca, we have recently been evaluating MGears, an automation engine plugin in the Mnova software from Mestrelab Research, with views to assist multiple internal processes involving large batches of chromatographic data. MGears GUI helps define the desired input (identifying / extracting the relevant raw data), the desired output (whether it's publishing results in a pdf report, in a csv file or write them to a database) and the specific processing steps and tasks that needs to happen in between. Once set up, automation workflows can be saved, shared, and run either on-demand (i.e. manually triggered by a user), on a time schedule, or in real-time mode (active listening for new data and on-the-fly processing). We will present four different application examples of this analytics tool at AstraZeneca. Those will be of particular relevance to Pharmaceutical R&D, but also of interest to the wider Analytical Science community.

Keywords: LC-MS, automation, data processing, drug discovery, software tools

Deep Learning Approach for HAP Retention Time Prediction of New Crown Ether HPLC Column

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A novel mesogenic crown ether stationary phase was obtained by coupling between Lichrospher Si 100 NH_2 and the mesogenic carboxylic crown ether acid liquid crystal CELC. Characterization of CELC was made with proton NMR, and the nematic state was determined by DSC. Thermal study of the new material exhibit transitions in Vant' Hoff plots indicating changes of the structure of the phase during heating. Analytical chromatographic behaviors of the new stationary phase BLCSP were investigated by reversed phase LC. Separation of polyaromatic hydrocarbons (PAHs) was obtained using high water content mobile phase. Bonded materials exhibit a liquid crystal-like behavior and molecular shape recognition toward planar and non-planar solutes probably due to the mesogenic state [1,2].

QSRR technique is becoming a key component allowing the prediction of analytes retention time, and providing insight into the mechanisms of separation.

The main objectives of this work are, on the one hand, to develop Deep Learning-based prediction software, equipped with a friendly graphical user interface that shortens the development process of the liquid chromatography separation method in general.

On the other hand, several training operations have been performed on a dataset of HAP. Sequence from the new reversed-phase chromatographic column which is primarily used for building a model that relates defined characteristics of the analytes in the training set to their t_r and LnK values, secondly in order to test the efficiency of the code and to validate the implemented model. The evaluation metrics have showed good values.

According to obtained results, the evaluation metrics indicates that the crown ether-RP column can be recommended for the prediction of t_R and Lnk of retention of a target compound of known chemical structure.

Keywords: HPLC, Crown ether, QSRR, Deep learning

Automated UHPLC Method Development for Mebendazole and Related Impurities, from Method Scouting to Robustness Testing

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Mebendazole (methyl(5-benzoyl-1Hbenzimidazol-2-yl) carbamate) belongs to a class of anthelmintic drugs and is widely used in the treatment of nematode infestations such as hookworm, roundworm, whipworm, pinworm, and threadworm. It functions by blocking tubulin formation within parasitic intestinal cells, which disrupts glucose uptake, digestion, reproduction and eventually leads to parasite death.

To ensure safety and efficacy of drugs like mebendazole, it is essential to monitor product and process-related impurities throughout the drug lifecycle, from initial screening to quality control and quality assurance. According to the International Council for Harmonisation (ICH) guidelines, e.g. in quantitative tests for impurities' content, the active pharmaceutical ingredient (API) and related impurities must be well resolved for accurate quantification.

HPLC method development typically consists of two steps: method scouting and method optimization. Specifically, key chromatographic parameters such as column, mobile phase composition and pH, and organic solvent type are first screened during method scouting. Once a viable set of starting conditions is identified, method optimization occurs in which chromatographic parameters (e.g. gradient profile, flow rate, and column temperature) are iteratively adjusted with the ultimate goal of providing a fit-for-purpose method. To ensure long-term method stability and facilitate future method transfer between instruments, robustness testing explores the effects of method parameter variation on a method's reliability. Robustness is generally evaluated during late stage method development or early stage method validation.

Due to the labor and resource-intensive nature of HPLC method development, process automation and acceleration are areas of constant interest. In this work, we present an automated method development workflow utilizing a Thermo Scientific™ Vanquish™ Flex UHPLC system combined with ChromSword Chromeleon Connect. The automated method development workflow includes method scouting, optimization, and robustness testing. A fast, robust UHPLC method was developed to quantify mebendazole and related impurities, which highlights the utility of Vanquish Method Development systems for streamlining method development and minimizing manual user-instrument interaction.

Keywords: automated method development, software-assisted method development, robustness test, UHPLC, mebendazole, impurity analysis

Applying Quality by Design Principles to the Migration of a Compendial Method between Multiple HPLC Systems

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Within the pharmaceutical industry, compendial LC methods are used to assess product safety and efficacy. Analytical laboratories are often required to migrate these methods to different laboratories or different models of LC instrumentation. The methods may be migrated without revalidation, however equivalent performance must be demonstrated. Method migration can be challenging. Differences across HPLC systems can impact method performance. A plan designed to identify and control how method performance is affected by differences in instrumentation is a valuable tool in obtaining a successful outcome.

Quality by Design (QbD) Principles were incorporated in the development of a plan to migrate the USP Ibuprofen Tablets Organic Impurities method from a legacy HPLC system (originator system) to two HPLC systems from different vendors (receiver systems). Development of the plan was a three-step process. The first step involved a review of each the systems to understand their similarities and differences. In the second step, identified system differences were examined for their risk to method migration. This assessment assigned a risk level to each identified parameter. In step 3 of the process, a control strategy was devised for the parameters assigned a high level of risk. This control strategy was implemented on both receiver systems, and the analysis performed. The three-step process identified the following parameters as having the highest risk to the migration of the Ibuprofen Tablets Organic Impurities method: injector carryover; detector noise; and tubing dimensions. The control strategy included defining the needle wash composition and number of washes to control carryover; ensuring an adequate lamp warm up time; defining the sampling rate and degassing the mobile phase to reduce noise; and adhering to the vendor recommended tubing dimensions for each system. With the control strategy in place, the method was run on both receiver systems. Each of the systems met the pre-defined acceptance criteria for successful method migration, specifically the USP system suitability requirements for relative standard deviation, resolution, and signal to noise ratio. In addition to meeting the acceptance criteria, the two receiver systems showed improved peak area precision (%RSD) and increased sensitivity (higher signal to noise value) than the legacy HPLC system.

Quality by Design principles were utilized for the development and implementation of a plan for the migration of the USP Ibuprofen Tablets Organic Impurities method between a legacy HPLC system and two HPLC systems from different vendors. The approach identified each system's performance capabilities and provided an understanding of how instrument parameters potentially affect method performance. Using this information,

potential risks to method migration were identified, and a control strategy devised and implemented. The results obtained on the receiving systems met acceptance criteria thus highlighting the Quality by Design approach as proactive and successful strategy for method migration.

Keywords: Quality-by-Design, compendial method, metho migration

MOREPEAKS, Optimization Software for Multidimensional Liquid Chromatography

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Method development in one- and, especially, two-dimensional liquid chromatography (LC×LC) can be very time consuming. Our group has aimed to aid in this cumbersome task. By utilizing retention modelling, *i.e.* modelling the elution time of compounds as a function of modifier content, thousands of theoretical chromatograms can be simulated within minutes. Thereafter, an optimal separation can be chosen from a Pareto optimality plot. In recent years, PIOTR has been reworked into the free-access Multivariate Optimization and Refinement Program for Efficient Analysis of Key Separations (MOREPEAKS) [1].

In this presentation, the algorithms that have been incorporated to aid with optimization will be presented. These include peak-detection and peak-tracking tools for LC-MS and LC×LC-MS data. In addition, retention models for more-complicated retention mechanisms, such as hydrophilic-interaction liquid chromatography and supercritical fluid chromatography have been investigated and will be addressed.

[1] Stef R.A. Molenaar, Peter J. Schoenmakers, & Bob W.J. Pirok. (2021). MOREPEAKS. *Zenodo*. doi.org/10.5281/zenodo.5710442

Keywords: Optimization, Software, Liquid chromatography

Development of a Data Quality Score to Characterize the Reliability of Features Generated by Processing of Data from Non-target Analysis with HPLC-HRMS

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In the past years, non-target analysis (NTA) with HPLC-HRMS has been established as a complementary approach to established target analysis. NTA raw datasets are extensive and contain multiple dimensions, and therefore it is not unexpected that data processing of these data is a critical step that should be taken carefully. Various processing tools are available for this purpose, each with different principles and combinations of mathematical operations. However, all programs have in common that their outputs are connected to significant data reduction. As a result of these algorithms, a feature list can be obtained that is the starting point for subsequent identification, prioritization, and comparative analyses. However, the feature lists highly depend on the algorithms used, as each processing tool has specific parameters to be set. Furthermore, there is no guarantee that every entry in those lists indeed stems from chemical substances due to false positives. Unfortunately, the reliability of detecting analytes as features is not constant but differs from case to case and is often unknown. To that end, this study develops a new feature detection algorithm that quantifies the individual feature reliabilities, which improves identifying false positives as unreliable features. We investigated the whole data processing workflow from raw data to feature list creation. Processing steps that reduce data lead to relevant loss of information on former data quality. Our new approach stores this data quality information within a parameter called Data Quality Score (DQS). The DQS contains information for each data processing step (centroiding, mass binning, feature detection). For its estimation, we use regression analysis and apply the laws of error propagation. The DQS characterizes the reliability of each feature and, therefore, is additionally added to retention time, m/z, and intensity in the feature list. Our new NTA feature detection algorithm increases the transparency in feature list creation, significantly improves false-positive detection, and enhances the overall interpretability of feature lists in NTA.

From Robustness to Uncertainty of Measurement in Chromatographic Assay Methods Development with a Single Software

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The announcement of the future ICH Q14 guideline on Analytical Methods Development has highlighted the need to develop chromatographic assay method following Analytical Quality by Design concept and rules.

NeoLiCy® software for statistical assessment of analytical methods' life cycle have been designed to provide the analysts with all necessary statistical tools and data integrity level required for this task. This software allows validation and robustness design, calculations and reporting according to the regulatory bodies' requirements.

Robustness is assessed in **NeoLiCy®** by means of Design of Experiments and associated statistical analysis. An original tool, based on matrix experimental results prediction interval, for interpretation of method robustness, has been implemented in the software and we demonstrate the interest of this original feature in concluding on method robustness.

Using chromatographic assay methods results, we show how such a software package can take the best from classical validation designs data and provide the analyst with prediction interval and uncertainty estimation, even in the case of unbalanced validation designs.

In the case of balanced validation designs, combined validation of precision and trueness (accuracy) is available in the software and we show the interest of producing accuracy profiles (by means of prediction or content tolerance intervals) and uncertainty profiles, covering the whole studied concentration range, according to the latest recommendations for analytical methods validation.

Keywords: Analytical Quality by Design, Robustness, Accuracy Profiles, Uncertainty of Measurement, Uncertainty Profiles

The Best of Both Worlds – Tailored Analysis of Affinity Selection MS Data with a Generic Automation Engine

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Affinity selection mass spectrometry *(ASMS)* has proven to be a powerful technique for early discovery in pharmaceutical research. Large libraries can quickly be screened using multiplexed samples and high throughput MS automation, but this can create a bottleneck for data analysis. One solution to this would be to use home grown or commercial software highly tailored to this analysis, but often such software imposes restrictions in that it must be used in the way the designers envisaged and offers little flexibility. Alternatively, one could use instrument software, but this may make connection to other systems and 'bookkeeping' in the process difficult. Our solution tries to get the best of both worlds with a customizable automation engine, which employs a core ASMS analysis feature. We have developed a customizable, generic automation engine, which is common across analytical techniques such as LCMS and NMR. In addition, we have developed an ASMS plugin for this engine to run the core of the analysis. This plugin in combination with the low/no code automation engine allows workflows to be implemented flexibly to fit specific user requirements and integrations into the wider IT environment.

Keywords: affinity selection, data analysis, pharmaceutical, compound libraries, automated processing

Automated LC-MS Data Analysis to Determine Physicochemical Properties of Lead Compounds in Early Drug Discovery

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Physicochemical properties of lead compounds such as solubility, Log P/Log D and stability are important criteria in early drug discovery, where numerous compounds are synthesized and routinely measured with LCMS-based analysis. Manually processing, analyzing, and reporting data is time consuming and labor intensive. To solve this problem, we have developed software tools to fully automate the entire process: sorting data from different vendors, verifying molecules, performing quantitative analysis, and reporting results. The software will initially sort data according to experimental design; match the expected molecular ion from isotope peaks of ionic molecules in mass spectrometry, then quantitatively determine Log P/Log D and solubility at given wavelengths of UV absorption. Interactive reports are automatically generated and are associated with a color-coded well-plate viewer, where Log P/Log D, solubility, purity, as well as customer defined values can be compared and graphically viewed. Additionally, the data point present in the well-plate viewer is associated with individual compound, and the interactive reports out of LCMS analysis of compounds dissolved in different solvent. This allows users to quickly compare results and accelerates the decision-making process.

Keywords: physicochemical properties, data analysis, pharmaceutical, compound libraries, automated processing

Intelligent Peak and Spectrum Deconvolution Using Photodiode Array Detector

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The fast and efficient separation of complex mixture consisting of multiple compounds including impurities as well as major drug substances still remains as a challenging application for liquid chromatography in the field of pharmaceutical analysis. In some cases, complete separation of those complicated compounds is difficult even after a method optimization for improving separation. A novel data analysis technique named i-PDeA II (Intelligent Peak Deconvolution Analysis II) was developed for extracting two or more target peaks from unseparated peaks using the obtained data from three-dimensional photodiode array detector with the multivariate curve resolution alternating least squares technique. It also employed an expectation maximization algorithm with a bidirectional exponentially modified Gaussian model function as a constraint for chromatograms and numerous PDA spectra aligned with time axis. The i-PDeA II can automatically extract respective peak profiles and absorption spectra from unseparated elution band by simply specifying the wavelength range and elution time interval. Obtained results can be directly used for spectrum identification and quantitative determination. The i-PDeA II can also be used to separate and quantitate peaks of co-eluted isomers that show normally similar retention times and hard to be separated chromatographically but a little bit spectral difference there is. We performed a model separation of isomers of three components using the i-PDeA II and evaluated the spectral identification and quantitative determination for three components system of isomers. The i-PDeA II provided practically satisfactory error between true and simulated peak area values at resolution (Rs) of 0.6, relative concentration of 1:1:1. Errors at different relative concentrations were confirmed as well. Through these investigations, it has been confirmed that the i-PDeA II provides fast and robust separation analysis even when method development efforts fail to achieve complete separation of the target peaks. Additionally, this approach is potentially applicable to quantitative peak deconvolution analysis of co-eluted compounds having the same accurate mass. This is a complementary to a quantitative analysis of co-eluted compounds using LC-MS to differentiate the proportion of response attributable to each compound.

Keywords: Peak deconvolution, Photodiode array detector, Spectral deconvolution

Investigation of the Retention Mechanisms of Chlorinated Cellulose-based Chiral HPLC Stationary Phases

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The use of chiral liquid chromatographic stationary phases is one of the most important analytical methods for enantiomer purity control and purification of racemic mixtures. Among the different types of chiral stationary phases, polysaccharide-based ones are well known for their versatility. Although traditionally used in normal phase chromatographic systems, more and more reverse phase applications are being developed.

In liquid chromatography, knowledge of the nonlinear equilibrium isotherms is essential for characterizing the processes occurring in the mobile phase and on the stationary phase surface. The retention mechanism can be investigated more reliably by nonlinear chromatography methods than by processing retention data from high dilution samples.

Cellulose-based stationary phases were overloaded by high-volume injection of N-heterocyclic pesticides. Measurements were carried out with aqueous polar solvent in several mobile phase compositions over a wide temperature range.

The inverse method was used to determine the parameters of a pre-selected isotherm taking into account the heterogeneous surface area of the stationary phase. The method provides an estimate of the model parameters of the selected isotherm for both enantiomers by minimizing the differences between the elution bands calculated by the inverse method and the measured chromatographic peaks.

The estimated isotherm parameters can be used to optimize the composition of the mobile phase to overload the stationary phases.

Keywords: retention, liquid chromatography, chiral chromatography, nonlinear chromatography, invers method

Cyclodextrin Complexation Study of the Unstable Acetoxychavicol-acetate, a Promising Component of Galangal (*Alpinia galanga*)

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Herbal medicines are becoming increasingly popular in developed countries. However, physicochemical characterization, and pharmacokinetic testing of potential herbal active ingredients is essential for effective and safe (phyto)therapy and optional drug development. 1-Acetoxychavicol acetate (ACA) is a promising constituent of greater galangal (*Alpinia galanga* (L.) Willd), with a variety of biological activities, but its poor stability and water solubility may limit clinical applications. Cyclodextrins (CDs) are an excellent tool to improve the bioavailability, as their inner cavity can accommodate apolar guest molecules through inclusion complexation.

The aim of this work is to optimize the pharmacokinetic properties of ACA by CD inclusion complexation. The complex stability constants were determined by the environmentally friendly, low sample consuming affinity capillary electrophoresis, applying various CD derivatives. Both the positively and negatively charged derivatives were able to form complexes with the uncharged ACA, and the cavity size, the substituent type and the degree of substitution also influenced the complex formation. The negatively charged sufoalkylated-beta-CD analogs were able to form the most stable complexes, exceeding 1000 M^{-1}. Based on our results, the "ideal excipient" was selected for a CD-based preparation, solubility, stability, and permeability studies.

Keywords: affinity capillary electrophoresis, cyclodextrins, inclusion complexation, bioavailability enhancement, galangal acetoxychavicol acetate

Chromatographic Self-Recognition of Chiral Zwitterion Ion Exchange Stationary Phases

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Separation of enantiomers on chiral stationary phases (CSPs) is recognized as the most common first-choice approach to obtain optically pure substances. Currently, there is a great number of CSPs, which are routinely used in research laboratories and industry, and allow for chiral separation of almost any racemic mixture of choice. The developed CSPs are mostly optimized to have the broadest possible scope of application for compounds possessing various structures, however, the chiral self-recognition of the novel selectors is usually much less studied.

The aim of this study is to compare the chiral self-recognition capability of *Cinchona* alkaloid-based CSPs, namely, commercially available chiral zwitterion ion exchangers (Chiralpak ZWIX (+) and Chiralpak ZWIX (–)) and some of their recently synthesized analogues. We have used the selectors themselves and their precursors as analytes, and evaluated the chromatographic performance of the commercial and experimental columns in high-performance liquid chromatography using a buffered polar organic mobile phase. We have observed that the commercial pseudoentiomeric columns usually provide the reversal of elution order for the majority of analytes. For the modified selectors, the elution order has been primarily driven by the *Cinchona* part of the selector, but several exceptions to this rule have also been identified.

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Keywords: self-recognition, chiral stationary phases, chiral ion exchangers

Examination of Chemoselectivity Gained from a Chiral HPLC Method Screening Sequence in Polar Organic Solvent Mode

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Polar organic (PO) mode generally provides good solubility and short retention times, enabling short analysis times which facilitate automation. To exploit the full selectivity space on one of the most often applied chiral stationary phases, i. e. amylose tris(3,5-dimethylphenylcarbamate) (ADMPC) in PO mode, consideration of the hysteresis phenomenon proved to be fruitful for extension of the selectivity space [1]. Hysteresis means that the state of a system depends on its history. In chiral chromatography, the retentions in mixtures of methanol and 2-propanol, and ethanol and 2-propanol may depend on which neat alcohol was used previously on the ADMPC column [1, 2]. The stable ones among the history dependent states had already been identified, and a new chiral method screening sequence arose from these [1]. The effectiveness of this sequence has been evaluated in terms of important aspects in the pharmaceutical industry. Chemoselectivity is such an aspect which is rather difficult to assess, because it does not refer to a specific compound, but to the relation between one enantiomer and all non-enantiomeric impurities with a wide range of possible structural differences. In the poster presentation a novel evaluation of chemoselectivity is shown using orthogonality plots of retention times of 31 different chemical entities for all the eluent pairs of the proposed sequence. The results show that if a method screening strategy provides a set of orthogonal hits rather than a single hit, chemoselectivity can be sucessfully addressed.

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Keywords: chiral HPLC, system orthogonality, hysteresis, amylose tris(3,5-dimethylphenylcarbamate), history dependency

Evaluation of Cyclodextrin- and Cyclofructan-based Chiral Selectors for the Enantioseparation of Psychoactive Substances in Capillary Electrophoresis

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The rapid appearance of New Psychoactive Substances (NPS), and mainly synthetic analogues of cathinone, in global drug market, raised the concern of worldwide public health. These analogues are purchased on online platforms and synthesized by more or less simple modifications of molecular structures of previously banned psychoactive substances in order to circumvent law. As many pharmaceutical compounds, illicit drugs, including NPS, are chiral and the pharmacological and neurotoxic potential of the pure enantiomers could differ. Therefore, analytical methods, capable of differentiating enantiomers, are of great importance. During this study, a simple and an easy-to-prepare electrophoretic method was developed for the enantioseparation of 10 NPS derivatives. Different types of β-cyclodextrin- and cyclofructan- based chiral selectors, both native and derivatized, were used and evaluated, and the most effective ones were determined in regard to resolution and analysis time. In addition, several electrophoretic parameters, such as the concentration of the chiral selector, the concentration and the pH of the background electrolyte, were examined in order to optimize the separation conditions. A 1-mM sulphated cyclofructan-6 (SCF-6) for amphetamine derivatives, a 1-mM sulphated cyclofructan-7 (SCF-7) for cathinone derivatives in a 20-mM sodium phosphate buffer at pH=2.5, a temperature of 25 °C and an applied voltage of 25 kV proved to be the optimum electrophoretic and operating conditions. In addition, the method was validated by estimating the intra- and inter-day precision.

Keywords: Capillary Electrophoresis / Cyclodextrin / Cyclofructan / Psychoactive Substances

Adsorption Dynamics of Leucyl-Leucine Stereoisomers in a Chirobiotic V Column

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Mass transfer of L-Leu-L-Leu and D-Leu-D-Leu in a Chirobitic V (250 x 4.6 mm) column was studied by means of the measurement of van Deemter curves and molecular diffusivity of the dipeptides in the mobile phase H_2O/MeOH (90/10, v/v). It was shown that mass transfer had an enantioselective character, with the stronger retained stereoisomer, D-Leu-D-Leu, demonstrating larger peak broadening. The Van Deemter plots of the Leu-Leu stereoisomers had an unusual convex-upward shape. Interestingly, that not only the kinetic *C*-term but also the eddy dispersion *A*-term were different for enantiomers. This is a theoretically unexpected but occasionally found in chromatographic studies situation [1, 2]. Probable reasons causing such a behavior are discussed as well as the mechanism of interaction between the Leu-Leu stereoisomers and the grafted chiral selector (vancomycin), resulting in enantioselective adsorption/desorption kinetics. A conclusion is derived that the stereoisomers with the D-leucyl residue at the C-terminus require a slow steric adjustment to the chiral cavity of the selector that leads to slow but strong adsorption, while the stereoisomers with a C-terminal L-leucine do not enter the chiral cavity, being retained at the external surface of the selector. Thus their adsorption is fast but weak.

This study was supported by the Russian Foundation for Basic Research (Grant No. 20-53-26007).

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Keywords: dipeptides, mass transfer, van Deemter equation, chiral stationary phase, Chirobiotic

High-Performance Liquid Chromatographic Separations in Polar Organic and Normal Phase Mode Utilizing Polysaccharide-based Chiral Stationary Phases

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The enantioseparation of *β*-aminolactone and *β*-aminoamide analogs with *N*-azole and *N*-benzoazole functional groups was investigated using polysaccharide-based chiral stationary phases. The influence of mobile phase composition on the enantioseparations were studied in both normal phase and polar organic mode. Acid and base additives were found to affect enantiorecognitions markedly in both modes. Special attention has been paid to the enantiomer elution order and examples were found for the reversal of the enantiomer elution order. Relationships between the structure of selector and selectand and the chromatographic parameters were evaluated to reveal mechanistic details of chiral recognition.

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Keywords: enantioselective separation; polysaccharide-based chiral stationary phases; normal phase mode; polar organic mode;

Chiral Analysis of Indacaterol in Enantiomerically Pure Pharmaceutical Formulations by Cyclodextrin Electrokinetic Chromatography

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In the pharmaceutical field, the inherent chirality of a wide variety of compounds plays an important role because both enantiomers may have different mechanisms of action, pharmacodynamics, and pharmacokinetics. In this line, the chiral separation and quality control of new single enantiomer drugs acquire a high relevance. Indacaterol (IND), a bronchodilator used for the long-term treatment of airway obstruction in adults with chronic obstructive pulmonary disease, is marketed as R-enantiomer. The main aim of this work was the development of a chiral methodology for the separation of RS-IND by Electrokinetic Chromatography. After a screening of cyclodextrins (CDs) and the evaluation of the pH effect on the enantioseparation, carboxymethyl-α-CD in formic acid at pH 4.0 was selected as the most adequate background electrolyte. Subsequently, Box-Behnken designs were performed to optimize CD concentration, temperature, and applied voltage using both short-end and long-end injections. Under the optimal separation conditions and using the short-end injection, IND enantiomers were separated in less than 5 min with a good resolution. Then, the methodology was validated and applied to the quality control of R-IND in pharmaceutical formulations.

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Keywords: Cyclodextrin-Electrokinetic Chromatography, Enantiomeric Separation, Indacaterol, Short-end Injection

Enantiomeric Separation of Licarbazepine by Electrokinetic Chromatography with Carboxyethylated derivatized Cyclodextrins. Study of the Enantiomer-Selector Interactions by NMR

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Capillary Electrophoresis, in the mode of Electrokinetic Chromatography (EKC), is one of the most powerful analytical techniques in the field of chiral separations. The study of the enantiomer-chiral selector interactions taking place in EKC has a high interest to investigate chiral recognition. NMR is particularly suitable in this kind of studies.

The enantiomeric separation of Licarbazepine by EKC using two carboxyethylated cyclodextrins (CDs) was achieved in this work. A reversal in the enantiomer migration order was observed depending on the CD. Construction of the corresponding Job plots from ^{1}H NMR titrations and intermolecular NOE interactions between some of licarbazepine's aromatic protons and the internal hydrogens of both CDs permitted to conclude in the formation of 1:1 inclusion complexes in both cases, even though the structure of the enantiomer-CD complexes was slightly different for the two CDs. Apparent association constants for enantiomer-CD complexes were determined for both CDs by the Scott method and no differences between enantiomers were found.

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Keywords: Electrokinetic chromatography, enantiomeric separation, NMR, cyclodextrin, licarbazepine.

Liquid Chromatographic Enantioseparation of Fluorinated *β*-Phenylalanine Analogs Utilizing Superficially Porous Particles

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The enantioseparation of nonfluorinated *α*- and *β*-phenylalanines and fluorinated *β*-phenylalanine analogs have been investigated utilizing chiral stationary phases. The employed chiral selectors include macrocyclic antibiotics, such as vancomycin, teicoplanin, and teicoplanin aglycone, isopropyl carbamate functionalized cyclofructan-6, and Cinchona alkaloid-based *tert*.-butyl carbamate quinine. All of the selectors were covalently bonded to 2.7 µm superficially porous silica particles. In these studies, we used columns with both 3.0 mm and 2.1 mm internal diameters. The influence of mobile phase composition on the enantioseparations is discussed. Both thermodynamic and kinetic studies were performed to characterize the utilized chiral stationary phases.

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Keywords: enantioselective separation; macrocyclic glycopeptide-based chiral stationary phases; cyclofructan-6-based chiral stationary phases;

Enantioseparation of 4-Phenyl-substituted Pyrrolidin-2-one Derivatives on Immobilized Cellulose-based Phase Derivatized with 3,5-Dichlorophenylcarbamate under Multimodal Elution

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The discovery of pharmaceuticals based on the pyrrolidin-2-one pharmacophore remains still an active research field of medicinal chemistry. Introduction of a substituent into the heterocycle of pyrrolidin-2-one forms a chiral center, therefore a search for effective analytical methods is necessary for optical purity control of 4C-substituted pyrrolidin-2-one derivatives.

Among the available liquid chromatography methods for enantiomeric separation, direct approach using chiral stationary phases (CSPs) is currently the method of choice due to its relative simplicity. Our previous studies have shown that immobilized cellulose-based CSP derivatized with 3,5-dichlorophenylcarbamate, together with mobile phases consisting of ethanol and *n*-hexane mixtures, is effective for separation of racemic 4C-substituted pyrrolidine-2-one derivatives.

It is well known that mobile phase is not just a passive carrier of chiral analytes through the column, but rather an important component of the chiral recognition mechanism. Polysaccharide-based CSPs are multimodal and can be used under normal-phase, polar organic and reversed-phase conditions. Multimodal screening of a small number of CSPs with broad enantiorecognition abilities has been recognized as the best strategy to achieve rapid and reliable separations of chiral compounds. Therefore, the aim of this study was to compare the enantioseparation of three racemic 4-phenyl-substituted pyrrolidine-2-one derivatives on the above-mentioned CSP under normal-phase, polar organic and reversed-phase HPLC conditions.

Keywords: 4-Phenyl-substituted pyrrolidin-2-one derivative; Separation selectivity; Cellulose tris(3,5-dichlorophenylcarbamate); Multimodal elution

Chiral Recognition Mechanism Studies of Tyr-Arg-Phe-Lys-NH_2 Tetrapeptide on Crown Ether Based Chiral Stationary Phases

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Crown ether chiral stationary phases (CSP) have been successfully used for separating enantiomers of various racemic compounds containing primary amino groups. Although chiral recognition mechanism for crown ether CSPs is generally understood, on a molecular level, the exact chiral recognition mechanisms, especially for the resolution of short peptides are still unclear.

Initially, by using μ-opioid receptor agonist tetrapeptide Tyr-Arg-Phe-Lys-NH_2 as a model compound, we have previously reported on application of crown-ether based CSPs in tetrapeptide enantiomer and stereoisomer separations [1]. Following, an in-depth study between peptide chemical structure and tetrapeptide retention behaviour indicated the N-terminal Tyr residue as the potential interaction site, responsible for retention and chiral recognition in Tyr-Arg-Phe-Lys-NH_2 tetrapeptide [2].

By employing known physical methods for structure determination, as well as *in silico* studies, the aim of this research is to support our previous studies by further investigating intermolecular interactions driving the chiral recognition of Tyr-Arg-Phe-Lys-NH_2 on crown ether CSPs.

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Keywords: Tetrapeptide, Chiral recognition, Crown ether chiral stationary phases, Enantioseparations

Speciation and Validation of Metal Compounds in Environmental Water Samples

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By using capillary zone electrophoresis (CZE), a method for separating and determining selenium and arsenic compounds was developed. By stacking huge quantities of samples injected onto the column with the field-amplified injection approach, selenium and arsenic enrichment was achieved on-column. Within 4 minutes, selenium and arsenic compounds could be preconcentrated over 100-fold. This method was used to determine the concentrations of selenium and arsenic in real water samples. The detection limits were less than 25 mg/L. The relative standard deviations for selenium and arsenic species determination in water samples ranged from 4.0 to 15.6 %. Subsequently, high performance liquid chromatography (HPLC) coupled with inductively coupled plasma tandem mass spectrometry (ICP–MS/MS) was used to validate the speciation results obtained using the electro-separation method.

Keywords: speciation, chromium, High Performance Liquid Chromatography Tandem Mass Spectrometry, environmental water samples

Historical Assessment of Atmospheric POP Depositions in Muntinu Glacial Lake, Southern Romanian Carpathians, Based on Radionuclide-Dated Sediments

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The aim of this work is to evaluate the history of atmospheric deposition of persistent organic pollutants (POPs) in Muntinu Glacial Lake, Southern Carpathian Mountains, in Romania, using sediments dated with ^{210}Pb and ^{137}Cs radionuclides. Alpha and gamma spectrometry were used for the entire sediment extraction device (carota) to measure the amount of 137Cs. To determine the age of each sediment layer, the 210Pb dating method was applied [1].

The extraction of POPs was performed by ultrasound-assisted method, followed by purification of the extract by open-column chromatography to isolate the target compounds from sediment samples.

Sixteen polycyclic aromatic hydrocarbons (PAHs), twenty organochlorine pesticides (OCPs) and twelve polychlorinated biphenyls (PCBs) were determined in each dated sediment layer by gas chromatography coupled with mass spectrometry (GC-MS) for PAHs, and with electron capture detector (GC-ECD) for OCPs and PCBs [2].

The results of this study show that in the last 100 years, the POP concentrations measured in sediment samples ranged from 2.53 to 156.27 ng/g for PAHs, from 1.78 to 71.12 ng/g for OCPs, and from ND to 76.03 ng/g for PCBs, being well correlated with the location and the main events (wars, industrial revolution etc.) of the last century. The diagnostic ratio between the sum of all PAHs with low molecular weight and the sum of all PAHs with high molecular weight ($\Sigma_{LMW}/\Sigma_{HMW}$) shows that, in the analyzed sediments, the main source of PAHs is a pyrogenic one, being represented by the atmospheric deposition.

In conclusion, in the absence of monitoring data, the analysis of POP residues in sediments could be a suitable method to reconstruct the history of surface water pollution.

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Evaluation of the History of POPs Pollution in Știucilor Lake, Cluj County, Romania, based on radionuclide-dated sediments and GC analyses

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The aim of this work is to assess the history of POP pollution in Știucilor Lake, Cluj County, Romania, based on radionuclides dated sediments. The ages of each sediment layer was determined by measuring the amount of ^{137}Cs and ^{210}Pb using alpha and gamma spectrometry. A number of 16 Polycyclic aromatic hydrocarbons (PAHs), 20 Organochlorine Pesticides (OCPs) and 12 Polychlorinated biphenyls (PCBs), were determined in each dated sediment layer by gas chromatography (GC) coupled with mass spectrometry (SIM mode) for PAHs, and electron capture detector (ECD) for OCPs and PCBs. Ultrasound assisted extraction (USAE) followed by extract purification on open column chromatography was used in order to isolate and to purify the target compounds in sediment matrices, prior to their GC analysis process. Deuterated compounds were used as internal standards for quantitative analyses and also for recovery/extraction efficiency. PAH diagnostic ratios, HCH isomer ration, and sum of DDT, Chlordane metabolites is used as a tool for identification and assessment of the emission sources of pollution. The dating and analysis methods were applied for two sediment cores, and with the obtained results, an assessment was made to evaluate the history of POPs pollution in the last 100 years.

Keywords: History of pollution, Știucilor Lake, Polycyclic aromatic hydrocarbon (PAH), Organochlorine Pesticide (OCP), Polychlorinated biphenyl (PCB).

The Analysis of Semi-volatile Additives in Wines by Vacuum-assisted Headspace Solid-Phase Microextraction method

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The purpose of this study is to develop and improve of the Vac-HS-SPME method and determining semi-volatile components for analyzing of alcohol containing products, with a focus on wine analysis. The adoption of an effective GC-MS technique of analysis is offered as a solution. Furthermore, the emerging process adheres to "green" chemical concepts.

Vac-HS-SPME was effectively used to detect diverse classes of semi-volatile compounds in food samples; the reason is it provides superior extraction. HS-SPME is a more suited and effective approach for identifying chemicals in wine. In SPME, volatile analytes were always extracted quicker than semi-volatiles, therefore analysis required a higher temperature. Vacuum-assisted HS-SPME was also shown to consistently provide excellent extraction efficiencies and sensitivities in short sample durations and at low temperatures.

Wine quality is determined by semi-volatile organic components, which determine fragrance and varietal characteristics. The yeasts manufacture some of the volatile molecules that give wine its flavor from semi-volatile chemicals during fermentation. Propylene glycol, sorbic and benzoic acids, as the main wine's semi-volatile additives were identified by GC-MS combined with Vac-HS-SPME. The preliminary optimized method has been approved for identification of these and other supplement preservatives in various types of wine by fiber coating, which has the following specified parameters: evacuation tense (t = 2 min), extraction time (t = 30 min), temperature of extraction (T = 60 ^{O}C), without pre-incubation time.

The data of screening results of different wines were received by using the parameter optimized Vac-HS-SPME method and analysis on GC-MS. Concentration of each analytes was determined by the standard addition method. LOQs of Propylene glycol, Sorbic acid, and Benzoic acid were 0.01–150 mg/L, 0.1–1500 mg/L and 1–100 mg/L respectively.

Acknowledgement: This work was conducted under the project AP08857501 «Improvement and development of highly sensitive methods for ensuring food safety in Kazakhstan» funded by the Ministry of Education and Science of Kazakhstan from 2020 to 2022.

Keywords:Food Science, Wine quality, Semi-volatile additives, Gas Chromatography-Mass Spectrometry, Vacuum-assisted Headspace Solid-Phase Microextraction

Justicia spicigera Dye Purification: Optimisation and Scale-up of an Offline 2D LC Separation Based on pH Change

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The characterisation study of the colouring materials of the *Codex Borbonicus*, a 16th century Aztec manuscript, revealed the use of a traditional Mesoamerican textile dye extracted from *Justicia spicigera* plant to produce brown paint layers. The unique record of this dye in a Mesoamerican codex, to the authors' knowledge, and the scarcity of information regarding the chemical composition of the coloured extract motivated its analysis in laboratory. Preliminary chromatographic characterisation of the aqueous extract of *J. spicigera* leaves pointed out the existence of two major compounds responsible for the colour of the extract. Thus, with a view to their complete structural characterisation, the development of a chromatographic separation method for the purification of these target compounds by preparative liquid chromatography was carried and is presented is the work herein.

The conditions of chromatographic separation were optimised on an RP-LC analytical set-up with UV-Vis and MS detection. The performances of five different C18 stationary phases (available for preparative chromatography) into four elution systems (different organic modifier under different pH conditions) were compared concerning the separation of the two compounds of interest and their neighbouring peaks. The richness of the extract and the limited choice of stationary phases not giving satisfactory separation in a mono-dimensional mode, the isolation of the target compounds in an offline bidimensional separation on the same stationary phase was considered. The complementarity of the elution systems provided by pH modification allowed to increase the purity of the collected fractions. The optimised conditions for each separation dimension were then transferred to the preparative system allowing the purification the target compounds. Structural analyses techniques (IR, Raman, ^{1}H and ^{13}C NMR spectroscopies and direct infusion HR-MS spectrometry) are still in progress to characterise the isolated coloured compounds of interest.

Effect-directed Isolation of Bioactive Compounds from Giant Goldenrod (*Solidago gigantea* Ait.) Leaf

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Natural products obtained from plants possess a broad structural diversity exhibiting versatile bioactivity, thus they are of significant importance as potential candidates in drug discovery. Nowadays, the overpopulation of invasive species is a threatening phenomenon resulting in severe ecological and environmental damage. *Solidago gigantea* Ait. (giant goldenrod) is a plant native to North America, but currently it is considered a highly invasive weed species in Central Europe. Because of its favorable pharmacological effects, it is also recognized as a medicinal plant. It contains a wide variety of secondary metabolites, e. g. flavonoids, phenolic acids, and mono-, di- and triterpenoids.

This research aimed to screen the *n*-hexane extract of *S. gigantea* leaf for bioactive compounds such as antibacterials. The *in situ* detection of biological activity was performed by high-performance thin-layer chromatography (HPTLC) separation coupled with various bioassays (e. g. HPTLC–*Bacillus subtilis*). Fractionation and purification of the sample as well as the isolation of bioactive natural products were carried out by successive preparative normal-phase and reversed-phase flash column liquid chromatography. The isolated compounds are characterized comprehensively by mass spectrometry (HPTLC–ESI–MS, HPTLC–IT–MS^n) and a microdilution method to determine their IC_{50} values. Their structure is planned to be elucidated by 1D and 2D NMR techniques.

Based on the preliminary results, their chromatographic properties and their behavior upon derivatization with *p*-anisaldehyde sulfuric acid reagent imply that they might be diterpenes. However, this hypothesis must be supported by further experiments to unambiguously identify the isolates.

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Keywords: high-performance thin-layer chromatography hyphenations, preparative liquid chromatography, bioassay-guided isolation, natural products, giant goldenrod (Solidago gigantea Ait.)

Evaluation of the Stability of NMN in Yeast Culture Fluid and its Effect on Human Skin Model

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Skin aging progresses due to intrinsic factors including chronological alteration, and extrinsic factors including UV, smoking, and air pollution. The phenotypes of skin aging include wrinkles and pigmentation. One of the mechanisms of aging is to decrease NAD^+ levels in the body, since it is noted that suppressing the decrease of NAD^+ leads to anti-aging for the skin. Therefore, with focusing on nicotinamide mononucleotide (NMN), the precursor of NAD+, anti-aging of human skin was investigated through evaluation of the stability of NMN and elucidation of the effects on the skin.

NMN was dissolved and a resulting NMN solution was stored at high temperatures to evaluate NMN stability. The amount of NMN was quantified by UHPLC at regular intervals, and the degradation product was identified. As a result, nicotinamide was identified as an NMN degradation product.

In addition, we investigated the NMN permeation into the skin using a skin mimic membrane and the effects of NMN on collagen production of fibroblast. The results indicated that NMN penetrated into the membrane and enhanced collagen production.

These results suggest that NMN is degraded mainly by hydrolysis reaction and that NMN penetrates into the skin and enhances collagen production.

Keywords: Nicotinamide mononucleotide, Stability, Skin permeation,

Determination of Carbonyl Compounds in Infant Formulas by GDME-HPLC-DAD

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Infant formulas present in their composition proteins, fatty carbohydrates, vitamins and minerals necessary for nutrition in the early stages of human development. They must undergo ultra-high temperature (UHT) processing to guarantee their safety. However, these conditions promote nutrients degradation reactions such as lipid peroxidation and Maillard´s reaction. These reactions give lead to highly reactive carbonyl compounds that can interact with proteins and nucleic acids, so they are considered potentially toxic. This implies their monitoring in food is of special interest for the control of food quality and risk for consumers.

For this purpose, a fast, sensitive and environmentally friendly method was proposed for simultaneous determination of five carbony compounds in infant formula by gas-diffusion microextraction (GDME) with simultaneous derivatization using o-phenylenediamine (oPDA) combined with high-performance liquid chromatography with diode-array detection (HPLC-DAD). The method was validated according to FDA guidelines, obtaining satisfactory results. After validation, the method was applied to the analysis of 28 infant formulas.

Keywords: carbonyl compounds, GDME, HPLC, infant formulas, food quality control

Evaluation of Soxhlet, SPE and UAE Extraction Procedures for the Quantification of Seven Major Cannabinoids in Eight Cannabis Infused Edibles by LC-MS

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As cannabis edibles poised to expand into several markets, the analysis of of cannabinoids in food and food supplements represents a critical issue. Therefore, in this study, a quantitative LC-MS method was developed and validated for the analysis of seven major cannabinoids in cannabis beverages and goods. The proposed approach was confirmed by the good correlation coefficient, recovery, carryover effect and reproducibility. Stability of the samples through various storage time periods was also evaluated. In particular, the samples were stored in the freezer and in the refrigerator for three, seven and fifteen days. In addition, three different extraction techniques (SPE: Solid Phase Extraction, Soxhlet and UAE: Ultrasound Assisted Extraction) were compared in order to highlight the one with the best efficiency. In regard to the concentration and the recoveries of the detected cannabinoids, UAE proved to be a much better extraction procedure than Soxhlet, while the results obtained by use of SPE in beverages where similar to the ones obtained by use of UAE. It was also observed that CBD and CBG were the most commonly occurring cannabinoids. The quantification though of CBG in many food edibles was not possible due to coelution with other substances in the matrix. According to results of this research, CBD oil contains the highest concentration of CBD and Δ^9-THC, followed by cannabis tea and cannabis hemp seeds. Δ^9-THC was found in five out of eight cannabis-infused edibles, while, according to the suppliers, all products were "THC-free". Lastly, it was observed that all edibles were positive for Δ^9-THC, except from cannabis beer and energy drink. In cannabis hemp seeds, Δ^9-THC was found to be within the legal concentration limits.

Keywords: Cannabis infused edibles; Liquid Chromatography - Mass spectrometry; Solid Phase Extraction; Soxhlet; Ultrasound Assisted Extraction

HPLC-HRMS and Chemometric Analysis of Carobs Polyphenols – Parameters Affecting their Phenolic Composition

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Polyphenols in carobs have recently attracted great attention due to their wide range of biological and health-promoting effects. In the present work, a comprehensive study was conducted to find an optimum method for the extraction, purification, and characterization of these valuable bioactive substances in carobs and their derived products. Under this framework, the ultrasound-assisted extraction of polyphenols was optimized by the maximization of the yield in total phenolics using response surface methodology. In particular, the effects of solid-solvent ratio, solvent concentration, extraction time, sonication amplitude, and sonication mode were investigated and optimized using a complete experimental design.

The phenolic profiles of carobs and carob-based products were, then, analyzed by the use of high-performance liquid chromatography – high-resolution mass spectrometry (HPLC-HRMS). In particular, the effects of the ripening stage, processing method, and geographical origin on carobs' phenolic composition were investigated through the analysis of carobs and derived products by the use of HPLC-HRMS, in combination with complete statistical analysis. In addition, the distribution of secondary metabolites across the fruit was also established through the characterization of the phenolic pattern of different carob parts. In regard to the effect of geographical origin, a multivariate statistical approach was employed to study the relationship between the phenolic composition of carobs and their growing regions.

Keywords: Ceratonia siliqua L., polyphenols, ultrasound-assisted extraction, HPLC-HRMS, chemometrics

Prevalence of Some Organic Contaminants in Vegetables and Drinking Water Samples Consumed by Rural Roma Communities in Transylvania

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The aim of this research is to assess the prevalence of some organic contaminants in vegetables and drinking water in 25 rural Roma communities in Transylvania, Romania, in order to assess human exposure.

20 organochlorine pesticides (OCPs), 13 organophosphorus pesticides (OPPs), 12 polychlorinated biphenyls (PCBs), 16 polycyclic aromatic hydrocarbons (PAHs), 4 pyrethroids, glyphosate (Gly), and aminomethylphosphonic acid (AMPA) were analyzed as follows: OPPs, PAHs, and pyrethroids by GC-MS; halogenated compounds (OCPs and PCBs) by GC-ECD; Gly- and AMPA-derivatized compounds and PAHs by HPLC-FLD. These compounds were isolated from drinking water samples by solvent extraction (OCPs, PCBs, and PAHs in hexane; OPPs and pyrethroids in dichloromethane), while from vegetable samples by QuEChERS method.

A total of 57 water samples and 50 vegetables (potatoes, carrots, parsley, celery, onions, lettuce, spinach) from 25 rural Roma communities in Transylvania were analyzed.

The research leading to these results has received funding from the Norway Grants 2014-2021 under Project contract no. 23 / 2020 (RO-NO-2019-0463).

Keywords: pesticides, polychlorinated biphenyls, polycyclic aromatic hydrocarbons, vegetables, drinking water.

Quantitative Analysis of Human Milk Oligosaccharides in Breast Milk, Infant Formula and Food Supplements by HPLC-MS/MS

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Human milk is the gold standard for the nutrition of infants, and its nutritional value is mainly attributed to human milk oligosaccharides (HMOs). These complex sugars with unique structural diversity serve not only as prebiotics but they exert a protective role against some neonatal pathologies, they reduce allergies and autoimmune diseases' incidence, play a crucial role in brain development and in the gut barrier's maturation. The most abundant HMOs are now commercially available and have already been approved by authorities to be added with different combinations and contents not just in infant formulas but in dietary supplements and functional food also. Hence, it is essential to develop rapid and reliable techniques to quantify HMOs for quality control and proper assessment of their functionality in food and other products. However, analytical characterization of HMOs has proven to be challenging. The combination of their monosaccharide composition and connectivity, the configuration of the anomeric centres and branching together led to a vast number of potential regio- and stereoisomers, often indistinguishable by conventional analytical methods.

Herein we report a fast and robust HPLC-ESI-MS method for the separation and quantitation of three isomeric pairs of HMOs (2'-fucosyllactose and 3-fucosyllactose, lacto-*N*-tetraose and lacto-*N*-neotetraose, 3'-sialyllactose and 6'-sialyllactose) in different matrices: infant formula, breast milk, and food supplements. Compounds were separated as their alditol forms by a porous graphitic carbon column, and quantified in negative MRM mode using maltotriose as internal standard. The developed bioanalytical method was validated in terms of selectivity, accuracy, precision, recovery, carry-over, matrix effects and stability. The main advantages of this method are the simple and fast sample preparation, the low volume of sample required and the simultaneous analysis of neutral and acidic HMOs within the same run.

Keywords: human milk oligosaccharide, HPLC-MS/MS, porous graphitic carbon, infant formula

Identification of 3,5-dimethylpyrazole Aquifer Contamination as the Origin of Odor Events in a Drinking Water Supply System

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A study on the organic compounds causing recurrent odor events in a local drinking water supply system (Catalonia, NE Spain) has been conducted. The water system provides water to more than around 10.000 people and supplies completely from groundwater after disinfection by chlorine addition.

Closed-loop stripping analysis (CLSA), Sensory GC and Liquid-Liquid Extraction (LLE), all followed by Gas Cromatography coupled to Mass Spectrometry (GC-MS) analysis, have been used as analytical methodologies to identify the compounds responsible for the odor events. They allowed the initial identification of 4-Bromo-3,5-dimethylpyrazole (BrDMP) as the probable compound causing the odor in treated water. BrDMP is formed by the chlorination of water that contains 3,5-dimethylpyrazole (DMP), which could be detected in the source water.

Once the compound responsible was identified, the determination of its organoleptic characteristics was carried out by trained and untrained panelists. Solvent, phenol and disinfectant were the main descriptors associated with these off-flavors in treated water.

Afterwards, an online SPE-Liquid Chromatography- High Resolution Mass Spectrometry (online SPE-LC-HRMS) method was developed in order to confirm the identification and to monitor the presence of DMP in different points of the aquifer.

Concentration levels of DMP in the source water were up to 70 µg/L. BrDMP reached 30 µg /L in the chlorinated water during odor episodes.

In this work, we explained the strategies of identification and quantification, the determination of its organoleptic properties and the study to eliminate the DMP from raw water.

Keywords: drinking water, odor, chlorine, 4-Bromo-3,5-dimethylpyrazole, mass spectrometry

Mid- and Long-Term Stability Study of Cyclic Diarylheptanoids from *Carpinus Betulus*

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Cyclic diarylheptanoids showing antioxidative, anti-inflammatory, and cytotoxic properties have been isolated from the bark of *Carpinus betulus* (European hornbeam, Betulaceae). The constituents being present in higher quantities include carpinontriols A (**1**) and B (**2**), giffonin X (**3**), and 3,12,17-trihydroxytricyclo[12.3.1.1^{2,6}]nonadeca-1(18),2(19),3,5,14,16-hexaene-8,11-dione (**4**).

The aim of our work was to determine the mid-term (12 week) and long-term (23 week) stability of the four major diarylheptanoids by evaluating the effects of storage time and temperature as well as that of the medium. Aqueous and methanol solutions of the isolated compounds together with hornbeam bark extracts prepared with ethyl-acetate and methanol were stored at -15, 5, and 22 °C. The quantities of the analytes of interest were determined using a validated UHPLC-DAD method, the degradation products were tentatively characterized by UHPLC-Orbitrap-MS.

Compounds **2** and **4** were considered to be stable. Quantities of compounds **1** and **3** significantly decreased after 12 and 23 weeks, as compared with their initial concentrations ($p < 0.05$). In the long-term studies, the best storage temperature was -15 °C for all samples. The complex media of the extracts provided significantly larger stability in the medium term at all studied temperatures for both **1** and **3**, as compared to their solutions. However, in the long-term studies, the solvents did not influence the stability of the compounds at -15 °C. The degradation products of both **1** and **3** were generated by a neutral loss of 18 Da indicating the elimination of a water molecule.

Keywords: diarylheptanoid, stability, decomposition, hornbeam, UHPLC-MS

Determination of Phenolic Compounds using Capillary Zone Electrophoresis Coupled to Mass Spectrometry

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Phenolic compounds are molecules having at least one hydroxylated aromatic rings. These compounds can be divided into several groups, such as simple phenols, phenolic acids, flavonoids, tannins, coumarins, etc. Phenolic compounds as phytochemicals can be found in most plant tissues.

The antioxidant activity and the human health beneficial effects of phenolic compounds in plant foods, red wine, honey or tea is well-known and intensively studied [1].

The phenolic compounds are generally analyzed using GC or HPLC, however CE can be an alternative or complement method to chromatographic separations. The coupling of capillary electrophoresis with mass spectrometry via electrospray ionization combines the fast and efficient separation of CE with the selectivity and sensitivity of MS. Thus it counts an important tool in the characterization of phenolic compounds in samples including complex matrix materials.

In our work 15 phenolic compounds were separated by capillary zone electrophoresis. As a simpliest choice and MS compatible electrolyte a 0.5 M NH_4OH solution was applied as background electrolyte. At this high pH of the electrolyte (pH=11) all phenolic compounds were ionized and completely separated. The electrolyte of high pH also generated a strong electroosmotic flow, which resulted in fast separation. A further advantage of the highly basic running electrolyte was that it provided proper basic conditions (after its merging with isopropanol:water (1:1) sheath liquid) for the negative mode MS detection. The separation and the MS detection parameters were optimized and the analytical performance data were determined. The linear detection ranges for the analyses of the components were generally between 1-200 μg/mL.

A optimized CZE-MS method was applied for the determination of several phenolic compounds in honey. The MS detection revealed several constitutional isomers of the studied phenolic compound, which could be separated from each others.

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Simultaneous Determination of Compounds with Laxative Action in Aloe Vera Plants Under Different Conditions and in Aloe Vera Based Products by a Simple HPLC-DAD Method

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The bioactive compounds of latex in *aloe vera* are associated with several of its medicinal properties. The purgative effect of latex is ascribed to aloe emodin and aloins A and B. In this study, a simple HPLC method was developed and validated for the determination of these compounds. The optimized method was then applied to both aloe leaves and aloe commercial products. It was observed that the concentrations of aloins in plants follow a specific pattern based on the position of the leaves. Two aloe leaves from a plant, under normal conditions, demonstrated the highest amount of aloins. It was also observed that climatic and environmental conditions have a significant impact on the concentrations of aloins A and B. Finally, it was examined whether the content of total aloins in aloe-based products was under the maximum levels set by EU legislation. It was observed that, in only two out of thirteen products, detection and quantification of aloins were achieved. The total content, which was 1,32 μg/ mL and 7,48 μg/ mL, exceed the maximum levels set by EU legislation.

Keywords: Aloe Barbadensis Miller, aloe products, aloe emodin, aloins, HPLC

Antioxidant Activity of Yellow Mealworm (*Tenebrio Molitor* Larvae) and Effect Directed Isolation of the Antioxidative Fraction

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Famine, global warming, land degradation and habitat destruction are only some of the burning problems the world is facing nowadays. With the world population growing, food demand is rising as well and with that, these problems are only expected to keep growing. Discovery of novel food sources that are nutrient dense, simple, cost-effective and environmentally friendly to produce is therefore of great importance. Yellow mealworm (*Tenebrio molitor* larvae) fits these requirements well, which is supported by the fact that this insect species was the first approved by the European Food Safety Authority as a novel food. Apart from high protein content, edible insects also possess a good lipid composition and even some biologically active compounds. In our study, different solvents and solvent mixtures were used to prepare extracts of yellow mealworm obtained from two different sources. The extracts were tested for antioxidant activity by the 2,2-diphenyl-1-picrylhydrazyl radical (DPPH) spectrophotometric assay and the results were compared. The most promising extract determined in the previous step was further fractionated by high-performance thin-layer chromatography (HPTLC) by using the so-called effect-directed fractionation approach guided by *in situ* DPPH derivatization. Finally, the method was transferred to preparative TLC and the antioxidant fraction in the selected extract was isolated and submitted for characterization. Hyphenated chromatographic techniques were used to identify the compounds in the isolated fraction. The results from the antioxidant spectrophotometric and *in situ* assays, as well as the effect-directed isolation and characterization of the antioxidant fraction will be presented and discussed.

Keywords: yellow mealworm, DPPH, antioxidants, high-performance thin-layer chromatography, effect-directed isolation

The Determination of Cannabinoids Content within Gummy Based Confectionary

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The growth of the Cannabinoid industry has led to a wide variety of nutraceutical products being commercially available. The leading products within this range are confectionary in nature, typically gummy based sweets. This work looks at the extraction method from the confectionary utilizing dedicated cannabinoid method for the analytical analysis.

The extraction method is tested using standard spiking addition techniques using non cannabinoid containing gummy confectionary, as well as using commercially available CBD gummy products. This combination of testing procedures ensured a robust and accurate extraction method was developed for a variety of gummy based confectionary products. The standard spiking tested out the methods precision, accuracy across differing spiking levels and specificity. Investigations were also carried out to ensure the method is suitable for gummy based confectionary that is suitable for vegans, which does not contain gelatin.

Keywords: Cannabinoids, HPLC, gummy based confectionary

Analysis of Organic Acids in Beer by Ion-Exclusion Chromatography and Post-Column pH-Buffering Conductivity Detection

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Yeast generates during fermentation numerous chemical compounds including ethanol, carbon dioxide, aldehydes, alcohols, fatty acids and organic acids, among others. The last compounds (mainly acetic, citric, formic, lactic, malic, succinic, and pyruvic acids) can influence besides the flavor (sour, bitter or salty) also the pH of beer. The presence of the acids can also contribute to inhibition the growth of some bacteria helping to improve shelf-life of beer. Therefore, the control of content of organic acids in beer is important.

Ion-exchange chromatography with gradient elution or ion-exclusion chromatography used in isocratic mode are established liquid chromatography methods for analysis of organic acids. The acidic eluent usually applied for ion-exclusion chromatography improves separation of organic acids, but the sensitive conductivity detection is affected by low-ionization grade of the analytes at low pH.

This poster presents a method for analysis of organic acids in beer based on ion-exclusion chromatography and pH-buffered conductivity. The method involves the successive addition of a pH-buffering reagent after column separation, to adjust the pH level to close to neutral. This not only reduces background noise, but also dissociates organic acids from the substance being analyzed. Consequently, electrical conductivity detection can then detect these organic acids with high sensitivity and selectivity.

Keywords: organic acids in beer, ion-exclusion chromatography, pH-buffered conductivity detection

Scalable Separation of Ecdysteroids using Centrifugal Partition Chromatography (CPC)

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Ecdysteroids are a group of bioactive, structurally diverse, non-toxic compounds known as analogues of the insect molting hormone, furthermore, they are widely consumed as dietary supplements for their biological effects. Their rapidly increasing use in both consumer and research areas requires the development of efficient and large-scale preparative purification methods.

Following the LC-MS analysis of a *Cyanotis arachnoidea* root extract purchased from Chinese source, we targeted the purification of 5 minor ecdysteroids in addition to the selective isolation of the major component 20-hydroxyecdysone (20E). Partition behavior of the crude extract was tested in more than 30 biphasic liquid-liquid chromatographic solvent systems (SS). In the evaluation of the partition, solubility, and selectivity data, special emphasis was made on sustainability of the SSs. Fractionation was performed in several ternary systems in ascending or descending mode using a laboratory-scale CPC device (250 mL rotor volume), and a pilot-scale CPC (2100 mL rotor volume). The composition of the fractions was monitored by HPLC. The autoxidation of purified 20E yielded calonysterone, which was the first to be purified up to industrial scale-up using our CPC method.

In conclusion, 20E can be isolated from plant extracts at the targeted quality via a cost-effective and scalable method, and several valuable minor ecdysteroids can be enriched in separate fractions, such as dacryhainansterone and calonysterone.

Acknowledgement: this work was funded by the NKFIH, K-134704, KDP 1005968.

Keywords: ecdysteroid, centrifugal partition chromatography, plant extract, autoxidation

Evaluating the Content of Heavy Metals in Aloe Vera Gel Samples through Microwave-assisted Digestion Followed by Graphite Furnace Atomic Absorption Spectroscopy

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There is a growing interest and popularity linked to aloe vera in the cosmetic industry due to its wide variety of therapeutic benefits. Among these qualities, anticancer, anti-inflammatory, antibacterial, antiviral, antipsoriaticy, and healing properties, can be highlighted, most of them derived from the biological activity of a set of phytochemical compounds present in aloe gel.

However, there are other compounds in aloe vera gel, including aloins and certain heavy metals, that are undesired given their health hazard. In this sense, the European Union Regulation (CE) nº 1223/2009 of November 30, 2009 prohibits in the Annex II the presence of heavy metals in cosmetic products, including those products derived from aloe vera. Besides, heavy metals are also classified as contaminants in aloe vera and its derivatives for the International Aloe Science Council (IASC).

In the present study, the presence of heavy metals in different aloe vera cosmetics is evaluated by setting up an analytical method requiring first a microwave-assisted digestion, optimized for aloe gel, followed by graphite furnace atomic absorption spectroscopy (GFAAS).

Keywords: Cosmetics, Aloe vera, Heavy metals, Microwave-assisted digestion, GFAAS

Optimization of the Method of Forming Nano-Emulsions and Their Characterization

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Citrus essential oils are complex mixtures of volatile compounds with manyfold possibilities to be used as active antioxidant and antimicrobial ingredients in food, cosmetics or pharmaceutical products. These uses are limited by their susceptibility to external factors such as: light, temperature, pH, oxygen, humidity. In order to enhance the physical-chemical stability of citrus essential oils, they were encapsulated into nano-emulsions. In this study, nano-emulsions were prepared through the ultrasonication method, using citrus oils as lipidic phase and Tween 80 and ethanol as surfactant, and co-surfactant respectively. Five types of citrus oil nano-emulsions were prepared by mixing 8% (v/v) of oil phase (bergamot, tangerine, orange, pomelo and lemon essential oils) with 1% (v/v) of Tween 80, 1% (v/v) of ethanol and 90% of deionized water using a magnetic stirrer and sonication at 72 amplitudes for 15 minutes. The PDI, turbidity, morphology, volatile profile and bioactive properties were investigated and their stability was monitored under different environmental conditions (storage at room temperature, at 37°C, refrigeration, freezing). Each emulsion exhibited different degrees of gravitational separation, the one stored at 37°C being the most unstable, showing coalescence. Gas chromatography mass spectrometry (GC-MS) coupled with headspace solid phase micro-extraction (HS-SPME) was used to characterize the volatile fingerprint of nano-emulsions. Based on the results obtained from the chromatographic analysis, the main compounds present in all studied was D-limonene with a concentration varying between 103.804 ± 8.112 mg/kg and 172.962 ± 25.012 mg/kg. In addition, other aroma compounds specific to citrus essential oils were identified, from the class of aldehydes, terpenes and terpenoids, but in lower concentrations.

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Keywords: nano-emulsions, citrus essential oils, optimization method, nanotechnology, chromatography

SPME-GC/MS for Pesticides Analysis: Palm Wine Case of Study

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Palm wine is a widely consumed product on the African continent. It is produced by fermenting the spontaneous sap of palm trees and is a nutritious drink (1) which is consumed without any treatment. A previous study (2) focused on the aromatic profiles of palm wines and five main families of VOCs were highlighted by HS-SPME-GC/MS, esters being predominant. The aim of the present study is to check the presence of pesticides in palm wines from the Ivory Coast. Indeed, pesticides are used around the world to provide maximum agricultural production to feed ever-growing populations. However, it presents a real danger to humans above a certain concentration. To successfully analyze pesticides potentially present in palm wines, a HS-SPME-GC/MS method was developed. SPME can be automatized and does not require solvent, is easy to do, relatively low cost and fast. Different fibers (PDMS, PA, DVB/Carbon WR/PDMS) were tested and the best conditions were determined by experimental design with 48 trials (equilibration time, extraction time, temperature, salt, desorption time and temperature). Various pesticides (organochlorines, organophosphates, methylparathion, terbutylazine, diazinon) were separated by a ZB5MS + column following a gradient program. The method was validated in terms of linearity, limit of detection and quantification, accuracy and repeatability, and allowed the quantification of 36 pesticides in 43 min. Among the 32 samples analyzed in triplicate, 7 pesticides have been detected in 10 samples (dichlorvos, methyl parathion, pirimiphos methyl, p,p'-DDE, p,p'-DDD, 4,4'-DDT, endrine ketone). Dichlorvos was the only one detected at levels above the European maximal limits.

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HPLC Determination of Biogenic Amines in Beer by AQC Derivatization

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Biogenic amines are naturally occurring biotoxins that are formed or degraded by the normal and physiological metabolism of microorganisms, plants, and animals. In food, they may occur naturally in small amounts, but are more commonly formed by bacterial degradation. If this decomposition is intentional, it is usually referred to as fermentation.

Analysis of biogenic amines can be performed by HPLC. Like their biological precursors, i.e. amino acids, most of biogenic amines lack a suitable chromophore, and cannot be detected by UV absorption. To overcome this limitation, a typical approach is to chemically label the amines with fluorescent molecules. 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC) is widely used due to the stability of AQC-derivatized compounds and the reproducibility of the derivatization reaction with both primary and secondary amines. The AQC fluorescent derivatives are more hydrophobic than the origin molecules, therefore enabling reversed phase mode separation for the otherwise highly hydrophilic amines.

In this work, the AQC-labeling approach was applied to biogenic amines in beer. The reaction yield of nine biogenic amines was evaluated at different reaction times. Evaluation of the reaction kinetics was used to identify the optimum reaction time and assess the robustness of the method against small changes of reaction time. Amine content in ten beers from different brands was investigated. Limit of quantitation at ppb level was obtained for all amines. Polyamines had in general higher LOQ compared to mono-amines; this contradicted the expectation that multiple reaction sited for the AQC would result in higher sensitivity.

Keywords: biogenic amines, food safety, fluorescence, derivatization, beer

Improvement of NMN stability and inhibitory effects of polyphenol components on NMN degradation

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In recent years, the aging population in Japan has increased, and the extension of healthy life expectancy has become increasingly important. As one of the causes of aging, reduced levels of nicotinamide adenine dinucleotide (NAD^+) in the body have been reported. Recently, nicotinamide mononucleotide (NMN) intake has attracted attention as a means of inhibiting NAD^+ decrease. NMN is the immediate precursor to NAD^+, and its ingestion has been reported to increase NAD^+ levels in the body and produce various anti-aging effects. However, NMN is easily degraded in an aqueous solution. In this study, NMN stability and promotion of cellular uptake of NMN through inhibiting enzyme activities were analyzed while investigating the interaction with polyphenol solutions.

NMN was dissolved and stored at several temperatures in a high-content polyphenol solution, and the stability of NMN was analyzed by reversed-phase liquid chromatography. NMN was also added to HepG2 cells and cultured, then intracellular NAD^+ levels were measured using LC-MS/MS.

NMN stability increased approximately 1.3-fold in the polyphenol solution compared to the control. Short-term storage tests revealed that the degradation reaction of NMN was a first-order reaction, with an activation energy of 107.1 kJ/mol. Furthermore, NAD^+ levels increased in HepG2 cells treated with the polyphenol solution. The results suggest that CD38, an enzyme in HepG2 cells, is responsible for increased NAD^+ levels.

Keywords: NMN, NAD+, LC-MS/MS

Screening of *Akebia quinata* D. Bioactivity via Effect Directed Analysis Based on HPTLC Hyphenated HRMS

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Akebia quinata Decaisne from the Lardizabalaceae family is an important plant in Chinese Tradition Medicine. The different parts of the plant are used to treat urinary tract infections, lack of menstruation, and improve lactation [1]. Lately, plants have played an essential role in discovering and developing new pharmaceuticals and clinically useful drugs. The evaluation and isolation of bioactive compounds are relevant because of the increasing occurrence of opportunistic infections and multidrug resistance. Hence, the present study aimed to evaluate bioactive compounds of Akebia using high-performance thin-layer chromatography (HPTLC) combined with bioassays and high-resolution mass spectrometry (HRMS) [2]. The antioxidant assay (DPPH reagent), antibacterial bioassays (*Bacillus subtilis* and *Avilibrio fischeri*), enzyme inhibition bioassays (α-amylase, tyrosinase, α/β-glucosidase, β-glucuronidase, butyrylcholinesterase, and acetylcholinesterase), effective hormone assays (pYAVAS and pYAVES) and genotoxic assay (Genotox) of plants were detected. The discovered active zones were further characterized using straightforward HRMS.

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Keywords: HPTLC, HPTLC-MS, Akebia quinata, effect directed analysis

Effect Directed Analysis of *Clitoria ternatea* L. Flower Based on HPTLC-MS

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Clitoria ternatea L. (*C. ternatea*) from the Fabaceae family, also known as the butterfly pea, is a well-known plant used in Ayurvedic medicine. In traditional Ayurvedic medicine, *C. ternatea* has been used as a brain tonic to treat stress and depression and enhance memory and intelligence [1]. The aim of our study is the identification and determination of chemical constituents of *C. ternatea* flower using a streamlined combination of high-performance thin-layer chromatography (HPTLC) with direct bioautography (DB) assays [2]. Antibacterial, antioxidant, enzyme inhibition, hormone effective, and genotoxic assays were selected to evaluate and determine biological properties and identify active zones using mass spectrometry.

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Keywords: HPTLC, HPTLC-MS, Clitoria ternatea, effect directed analysis

Green Synthesis of Colloidal Gold Nanoparticles using Citrus Essential Oils - Morphology, Physico-Chemical Characterization and Microbiological Properties

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Essential Oils (EOs) have a strong focus in the scientific world these days and citrus essential oils began to show increased interest due to their antioxidant and antimicrobial properties (Bora, Kamle et al. 2020, Brahmi, Mokhtari et al. 2021). Also, nanostructures are intensely studied and exploited in various fields including the agri-food sector. Particularly, combining EOs and nanostructures can be an efficient way to improve the EOs stability and properties.

This study aims to produce colloidal gold nanoparticles by reduction of Au^{3+} ions with citrus EOs. The syntheses were carried out using lime, lemon, orange, tangerine and grapefruit EOs, at boiling and pH between 8 and 9. The morphology of the nanoparticles was characterized by transmission electron microscopy (TEM) and dynamic light scattering (DLS), while their stability was assessed by UV-Vis spectroscopy and Zeta potential measurements. Their antioxidant activity (DPPH method) and antimicrobial properties were also studied.

The nanoparticles showed characteristic UV-Vis absorption maxima between 521-547 nm, depending on the EO used as reducing agent. The diameter of nanoparticles was in the range of 13- 23 nm. Zeta potential values indicated a strong stability in time of the colloid and this has been also confirmed by UV-Vis spectroscopy at 6 months after production.

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Keywords: gold nanoparticles, colloidal gold, citrus essential oils, DLS.

LC-Q-TOF: An Excellent Choice for Confirmatory Analysis of Doping Substances in Dietary Supplements

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The dinamically growing and poorly controlled market of dietary supplements, including sports supplements could cause several problems to the consumers (eg.: health risk, inadvertent doping) because of the possible presence of banned and harmful substances (anabolic agents, stimulants, synthetic drugs etc.). The determinations of trace amounts of these compounds require accurate and highly sensitive analytical procedures. In this respect, the widespread used liquid chromatography tandem mass spectrometry (LC-MS/MS) technique itself is not sufficient for unique and exact compound identification. In additon, the matrices of the sports supplements are quite complex as they might contain high amount of proteins, minerals, mineral oils and herbal products. Although sample purification steps play crucial role in LC-MS/MS analysis, sometimes verification measurements are needed by an independent instrument/method to confirm the primary MRM data obtained by LC-MS/MS, these measurements are confirmed by LC coupled with quadrupole time-of-flight mass spectrometer (LC-Q-TOF). In this work we present our in-house developed and validated methodology for the identification and quantification of a number of banned substances (LOQ between the range of 0,2-10 ng/g) from dietary supplements. We show examples for dietary supplements containing banned and dangerous substances, such as ephedrine, strychnine, methamphetamine and yohimbine. There are also examples for false positive cases, when LC-Q-TOF measurements did not prove the presence of the compound found in the sample beforehand.

Keywords Q-TOF, dietary supplements, banned substances, verification measurements

HPLC-FLD Method for the Detection of Sulfonamides in Natural and Organic Fertilizers Collected from Poland

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Antibacterial substances such as sulfonamides are used all around the world for treatment in human therapy and veterinary medicine. After the administration of antibiotics, between 30% and 90% of the initial dose given is excreted. Thus, poultry droppings, pig feces and liquid manure, used as fertilizer for agricultural land, are often contaminated with antibiotics. Little is known about concentrations and the fate of antibiotics in manure and soil. These parameters are of great importance when evaluating the role of contaminated manure in the spread of antibiotic agents into the environment, and to assess the risk of water and food contamination through this pathway. Nevertheless, manure is the source of a significant part of veterinary drug pollution in the environment and is currently not active monitored. Therefore, a chormatographic method with a fluorescence detector was developed for the determination of sulfonamides (sulfaguanidine, sulfadiazine, sulfamerazine, sulfamethazine and sulfamethoxazole) in natural and organic fertilizers in order to determine the frequency of these antibacterial substances and their quantification in poultry and pig feces, slurry and digestates. The method was validated according to EU requirements. Using the HPLC-FLD method for all analyzed matrices, recoveries were satisfactory (77.00–121.16%). Limit of detection and limit of quantification were 13.53–23.30 and 26.02 –40.38 µg/kg, respectively, depending on the analyte. The forty-four samples of natural and organic fertilizers were analyzed, in four samples showed sulfamethoxazole in the amount from range 158 to 11070 µg/kg. Our results showed that sulfonamides in animal feces samples were not detected frequently, but the concentrations are comparable to published research results by other scientists around the world. The results may also indicate that other antibiotics are used more often than sulfonamides in Poland, e.g., tetracycline antibiotics. The results of the research indicate that the presence of antimicrobial substances in natural and organic fertilizers should be monitored before introducing them to farmland. The amounts of sulfamethoxazole found in the present study could have an ecotoxic effect on the microbiota inhabiting land and surface waters, and could be absorbed by crops.

Keywords: sulfonamides, HPLC-FLD, pig feces, poultry, feces, slurry, digestate, environmantal contamination

Bioactive Compounds from Citrus Peel Waste Evaluated by Supercritical Fluid Chromatography: Study of Stationary and Mobile Phase Composition

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Citrus peel wastes from the juice industry are a source of a diversity of bioactive compounds, such as terpenoids, coumarins, psoralens, flavones, and flavanones. Among the technologies required to add value to these residues, separation techniques prevail to identify and quantify the bioactive compounds. Terpenoids are evaluated by gas chromatography and the others are usually analyzed by reversed-phase liquid chromatography (RPLC). Supercritical Fluid Chromatography (SFC) has emerged in the analytical scenario as a competitive technique for analyzing compounds of different polarities. This aspect is due to the possibility of tuning the CO_2-based mobile phase by the addition of organic solvents. This work explored the ability of SFC to separate the abovementioned compounds divided in four subgroups: terpenoids (TERP), coumarins and psoralens (COUM), polymethoxylated flavones (PMF), and flavonoids glycosides (FLAV) with photodiode array detection. Twelve stationary phases of different chemistries and dimensions (used for both RPLC and NPLC modes and designed for SFC itself) were employed in a Waters Ultra-Performance Convergence Chromatography (UPC²) system with CO_2-methanol mobile phase starting at 98:2 (% v/v) at 25 °C and 150 bar of backpressure. For a fair comparison, other chromatographic conditions were adjusted based on the column dimensions. Extracts from tangerine and Tahitian lime peels plus orange and lime essential oils were mixed and used as a model for compounds usually found in these matrices. A criterion based on desirability was chosen to evaluate each class individually and globally. Overall, Torus 1-Aminoanthracene presented the best performance, although other stationary phases were more suited for specific classes. Following that, the composition of the modifier added to CO_2 was studied. A blend of methanol and acetonitrile (1:1) with 5 % of water and 0,1 % of methanesulfonic acid was found to give an optimal overall resolution. Interestingly, acetonitrile provided a better resolution between critical pairs and helped to decrease the system pressure, while water and acidic additive improved peak shape of FLAV. Peel extracts from different species of Citrus and commercial essential oils were evaluated by the developed method.

Keywords: Supercritical fluid chromatography, Citrus, Flavonoids, Coumarins, Psoralens

Evaluation of the Volatile Profile of Passion Fruit Juices using Headspace Solid-Phase Microextraction and Gas Chromatography - Mass Spectrometry

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Canary Islands (Spain) offer a unique climate for tropical fruits' grow, with current interest on passion fruit due to its worldwide consumption demand. In this context, it is important to obtain, evaluate, characterize, and select the plant material that is best adapted to the agro-environmental conditions of the archipelago, while also fulfilling the demands of the international market. One of the most important characteristics to determine passion fruit' quality is the aroma (combination of odor and flavor) of the fruit, properties that can be attributed to the type of volatile and semi-volatile organic compounds present in the fruit, and their contents.

This study reports a headspace solid-phase microextraction (HS-SPME) method in combination with gas chromatography-mass spectrometry (GC-MS) for the qualitative analysis of the volatile profile of passion fruit juices harvested in Canary Islands, as a screening test to compare with those from other sites.

Keywords: Headspace solid-phase microextraction, Gas chromatography-mass spectromery, Qualitative analysis, Passion fruit, Volatile profile

Grain Sorghum Crops: Use of Green Manure as Biofumigant and to Increase Soil Fertility

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Cyanogenic glycosides are phytochemicals involved in plant defence against herbivores by virtue of their ability to release toxic hydrogen cyanide (HCN) upon tissue disruption. In addition, endogenous turnover of cyanogenic glycosides without the liberation of HCN may offer plants an important source of reduced nitrogen at specific developmental stages [1]. Dhurrin is the major cyanogenic glycoside in Sorghum, its level is higher in young sorghum seedlings and it declines with age, although nitrogen fertilization of older plants is known to increase dhurrin synthesis by transcriptional regulation. In this work the effects of green manuring on the soil, in terms of bio-fumigation activity towards the elaterids that can damage potato crops and the soil ferility, were evaluated. In particular, five soil treatments were carried out in different parcels of a cultivation field, then potatoes were planted and cultivated. The ground land of the present study is located in a countryside near Bologna (Italy), where representative samples of soil and sorghum leaves were collected to evaluate the release of organic matter and humic acids, and durrin content, respectively. To evaluate the influence of sorghum green manure treatment on soil fertility, the humification index was calculated as the ratio between the carbon concentration of humic acids (HA) and fulvic acids (FA). The quantification of dhurrin content in leaves was carried out by solid-liquid extraction followed by HPLC / MS analysis and reported as mg of dhurrin per gram of dry matter. The results showed that an improvement in humic acid content in soil was verified after sorghum green manuring and how different soil treatments can affect dhurrin synthesis in sorghum leaves.

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Keywords: Biofumigation, cyanogenic glycosides, dhurrin

Grape seed powder inhibits lipid digestibility in various foods. An *in vitro* study

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A variety of fat-containing foods were chosen as model matrices and co-digested with grape seed powder (GSP) to study lipid digestibility. Digestion simulation was carried out with the Infogest static in vitro consensus method. Lipolysis of triacylglycerides – the main lipid fraction of the tested foods – were assessed based on determining the free fatty acid content from the digesta using standard derivatization methods and GC-FID. Effect of GSP on lipid digestion was evaluated during in vitro digestion simulation using cream, baked carp and baked beef samples.

Dose dependency of GSP was tested first with cream (30% fat), as a high fat dairy product with the addition of 5, 10 and 15 w/w% GSP to 0.5g cream. No dose dependent effect of GSP on lipolysis was observed in the studied range, however the addition of 5% GSP was enough to inhibit lipolysis by 14.5% from 69.2±6.1% to 59.4±0.6%. Baked carp (BC) and baked beef (BB) with 14.4% and 16.1% fat content (w/w % as consumed) respectively were also tested. Bioaccessible fatty acid content of BC decreased from 71.3±1.3% to 51.7±8.7%, and that of BB from 70.5±2.6% to 56.2±3.9%. Our results derived from digestion simulation experiments with a variety of real foods unanimously show that grape seed – associated with a number of health-protective effects including anti-obesity related effects such as weight loss or decrease free fatty acids and triglycerides in blood and white adipose tissue – is partly explained by the GSP on digestive enzymes.

Keywords: grape seed powder, lipid digestion, Infogest, beef, carp, cream, obesity

Characterisation of Delta-8 THC Distillates using HPLC with PDA and Mass Spectrometry Detection

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The use of delta-8 THC in consumer products has caused safety concerns in the United States (US). Though delta-9 THC is the main pharmacologically active component in the cannabis plant, its psychoactive isomer, delta-8 THC, naturally occurs in the plant at low levels. Bulk delta 8-THC is typically produced from hemp derived CBD (<0.3% delta-9 THC) which many producers consider legal under the 2018 US Farm Bill. The regulations governing the use of synthetic components derived from hemp are not clearly addressed which has created a growing market for delta-8 THC production and use. The conversion of CBD to delta-8 THC requires harsh conditions leading to multiple reaction byproducts which need to be characterized to enhance understanding of the chemical components produced and their potential risks to consumer safety.

Distillate samples were dissolved in acetonitrile and analysed using HPLC with both photodiode Array, and single quadrupole mass spectrometry detection. A C18 column, 4.6 x 100 mm, 2.7 µm maintained at 25 ^{0}C was used to separate the cannabinoids using isocratic elution with a mobile phase consisting of 0.1% formic acid in water and acetonitrile. Several known cannabinoids were identified in the samples based on retention time and UV spectra, including delta-8 THC and delta-9 THC. However, several unidentified peaks were also detected in the UV data. The UV spectra indicated that there may be structural similarities between the unknown components and the primary compound in the distillates, delta-8 THC. In the MS analysis, the software highlighted *m/z* 315 as the base peak for several unknowns. Cannabinoid spectral libraries were generated using authentic standards, the lambda max and retention times, were used to aid in confirming the identity of the target components and highlighting potential structurally related unknowns by spectral matching.

LC/MS Study of Ergoline Alkaloids Isolated from *Ipomoea tricolor* (Convolvulaceae)

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Ipomoea tricolor (Convolvulaceae) seeds contain ergoline alkaloids derived from indole, such as ergine (LSA), ergometrine, ergometrinine, among others [1]. Some of them are well known for their psychotropic effects and for generating the ergotism condition. Ergolinic alkaloids have been used as precursors for the synthesis of various compounds for the treatment of Parkinson's disease, vasoconstriction, *etc* [2]. *I. tricolor* seeds (1 g) collected from CENIVAM research experimental plots (Bucaramanga, Colombia) was used to obtain the extracts, using the matrix solid-phase dispersion technique (MSPD) with modified silica gel C18 as adsorbent and acetone as an elution solvent. The chemical composition of the extracts was analyzed by UHPLC-ESI/*Orbitrap*, the product ions were formed at 10, 20, 30, 40 eV, in the high-collision cell (HCD). The following identification criteria were employed: (1) determination of the exact masses of the protonated molecule and the elemental composition, (2) the study of the fragmentation pattern, (3) the study of the isotopic distribution and the comparison of the mass spectra with those reported in the literature. The extraction yield obtained was 3.00 %, two ergoline alkaloids were isolated from *I. tricolor*, *i.e.*, ergine $C_{16}H_{17}N_3O$ $[(M+H)^+$, *m/z* 268.14392] and ergometrine $C_{19}H_{23}N_3O_2$ $[(M+H)^+$, *m/z* 326.18467]. Characteristic product ions were formed at *m/z* 223.12262 $[(M+H)\text{-}CH_2NO]^+$ corresponding to the alpha cleavage of the amide group and *m/z* 251.11737 $[(M+H)\text{-}NH_2]^+$, resulting from the loss of the amino group in ergine. *Ipomoea tricolor* seeds can be a valuable source of ergoline alkaloids, starting material for the synthesis of various drugs and bioproducts.

Keywords: Ipomoea tricolor, alkaloid, ergolines, UHPLC-ESI/Orbitrap.

Analysis of Amino Acid Contents in Aronia Juice

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Aronia berries and their products have beneficial effects on human health through preventing lifestyle-related diseases. For instance, in animal models, aronia juice (AJ) reduces blood glucose levels of mice suffering from type 2 diabetes and obesity. AJ inhibits the enzymatic activity of dipeptidyl peptidase IV. Until recently, we have focused on polyphenols in AJ to elucidate the mechanism of these physiological functions of AJ. In this study, the amino acids in AJ were quantified to examine the beneficial function of AJ that does not depend on polyphenols.

Known amounts of standard amino acids were added to six aliquots of the juice, and then amino acids were extracted by adding four volumes of methanol. The amino acids were labelled with dabsyl chloride (4-dimethylaminoazobenen-4'-sulfonyl chloride), and the labelled amino acids were cleaned up by micro-scale solid phase extraction using MonoSpin C-18. A $^{13}C_6$-dabsyl amino acids mixture was added to each cleaned sample as internal standards, and then the sample was subjected to reverse-phase chromatography on an InertSustain C18 column coupled with ESI-MS.

AJ contained 5.9 mM asparagine (Asn) as the most abundant amino acids. AJ contained 1.5 mM γ-aminobutyric acid (GABA) and 1.6 mM aspartic acid, and the other proteinogenic amino acids were found in the range from 20 to 500 μM except methionine, which was not detected. Although many popular fruits contain Asn, the Asn content of AJ is highest among these fruits such as apples, pears, and oranges.

Keywords: Aronia, Asparagine, GABA

Direct Analysis of Valuable By-products in Cork Wastewater

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Cork sector plays an important economic role in several Mediterranean regions and the manufacturing process involves several stages with waste generation, mainly industrial water or cork boiling wastewater (CBW). This effluent presents interesting compounds for recovery but the characterization is long, difficult and expensive due to the high organic load. This work studied a direct analysis of the main chemical compounds which can be found in CBWs, through a sequential process.

In a first step, a wide range of phenolic compounds were analyzed in CBWs with a proposed HPLC-DAD methodology. Furthermore, the phenolic fraction was separated by green techniques and the supernatant studied to detect and quantify remained by-products, mainly low molecular weight polyphenols. In order to achieve a complete valorization, the carbohydrate composition in the clarified extracts was also studied by ionic HPLC, finding significant concentrations of monosaccharides such as glucose, galactose and arabinose among others. The results were in accordance to other authors who used laborious and complex pretreatments prior to the analysis of CBWs.

In conclusion, the proposed methodology offers a quick and efficient way to analyze the most important by-products in cork boiling wastewater for future design of recovery processes and/or depuration techniques.

Keywords: cork, wastewater, by-products, chromatography, analysis.

Investigation of Phenolic Compounds in Hemp

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Among hemp (Cannabis sativa L.) secondary metabolites, cannabinoids are certainly the most distinctive, and furthermore, hemp is well known for its terpene content. On the other hand, the content of phenolic compounds in hemp (e.g. flavonoids and phenolic acids) is also not negligible. These compounds are interesting from their biological activity point of view, for example they often possess antioxidant properties. In our research, extraction procedure for phenolic compounds from hemp plant material was optimised, 50% EtOH(aq) was selected as the best extraction solvent. By use of the optimised extraction procedure, extracts from different types of hemp grown in the Marche region (central Italy) were obtained and then used for study of the phenolic compounds. In addition, alternative potential approach for extraction of phenolic compounds from hemp was tested - the aqueous solution that remains after steam distillation of plant material (the part that is not distilled) was lyophilized and the phenolic compounds in the solid residues were determined. Spectrophotometric determination of total phenolic content, total flavonoid content, and DPPH antioxidant test were performed for both types of extracts. Further on, an HPLC method for determination of phenolic compounds in hemp was developed, followed by mass-spectrometric identification of individual main flavonoids in the extracts. The content of representative phenolic compounds in hemp was also quantitatively assessed.

Keywords: hemp, phenolic compounds, flavonoids, HPLC, LC-MS

Analysis of Flavonoids in Georgian Wine Waste Products by HPLC/DAD

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In recent years, special attention has been paid to the protection of natural resources in the world, and the creation of technological-organizational systems for the conservation and secondary use of plant raw materials is especially relevant. At the same time, it is known that natural products of plant origin have been used in medicine since ancient times and the demand for them is growing day by day in the world. Consequently, identifying a waste product as a new, natural resource as a source of biologically active substances is relevant and innovative issue.

This application note details a strategy for the extraction and determination of flavonoids of wine lees from different type of Georgian wine. Chromatographic detection was achieved using an Agilent Technologies 1290 liquid chromatograph Infinity with DAD detector. Separation was performed on Zorbax Eclipse plus C18 (250×4.6 mm, 5µm) column. The mobile phases consisting of 0.1 % water solution of formic acid : 0.1 % acetonitrile solution of formic acid with gradient elation. Flow rate - 0.8 mL/min., Detection took place at a wavelengths of 254 nm, 270 nm, 235 nm.

The research confirmed the content of flavonoids in Georgian wine (Rkatsiteli, Kisi, Saperavi) production waste - wine lees.

Keywords: chromatography, flavonoids, wine, waste, lees

Application of Micellar Mobile Phase for Quantification of Sulfonamides in Medicated Feeds by HPLC-DAD

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In recent years, a technique known as micellar liquid chromatography (MLC) has been used as an alternative method to conventional liquid chromatography. Micellar liquid chromatography is one of the modes of reversed phase liquid chromatography (RPLC) in which the mobile phases are aqueous solutios of a surfactant at a concentration above the critical micelle concentration.

The aim of this work was to optimize the isocratic mobile phase based on MLC along with using SDS as the modifier agent for simultaneous isolation and quantification of sulfonamides: sulfaguanidine, sulfadiazine, sulfamerazine, sulfametazine, and sulfamthoxazole in medicated feeds. To our knowledge, the stability indicating green reverse phase HPLC (RP-HPLC) method using environmentally benign eluents (propan-2-ol) has not been reported in the literature for the analysis of sulfonamides in medicated feed.

Satisfactory separation of sulfonamides from medicated feeds was achieved using a Zorbax Eclipse XDB C18 column (4.6 x 150 mm, 5 µm particle size) with a micellar mobile phase consisting of 0.05 M sodium dodecyl sulphate, 0.02 M phosphate buffer, and 6% propan-2-ol (pH 3). UV quantitation was set at 260 nm. Application of the proposed method to the analysis of five pharmaceuticals gave recoveries between 72.7% to 94.7% and coefficients of variations for repeatability and reproducibility between 2.9% to 9.8% respectively, in the range of 200 to 2000 mg/kg sulfonamides in feeds. Limit of detection and limit of quantification were 32.7–56.3 and 54.8–98.4 mg/kg, respectively, depending on the analyte.

The proposed procedure for the quantification of sulfonamides is simple, rapid, sensitive, free from interferences and suitable for the routine control of feeds. In the world literature, we did not find the described method of quantitative determination of sulfonamides in medicated feeds with the use of micellar liquid chromatography. the use of micellar liquid chromatography technique is advantageous because it protects analysts from the exposure to volatile organic solvents during chromatographic analysis. In addition, the selected mobile phase is cheaper and less toxic than those used in conventional RPLC. The proposed chromatographic procedure is useful for routine quantification analysis of the sulfonamides in medicated feeds for pigs and poultry.

Keywords: micellar liquid chromatography; sulfonamides; diode array detector; medicated feeds

Towards Usefulness of Gas Chromatography (GC) for Research on Advanced Chemical Treatment Processes

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Advanced Chemical Treatment Processes are currently a rapidly developing solutions in environmental and chemical engineering. This term relates to Advanced Oxidation Processes (AOPs) and Advanced Reduction Processes (ARPs) which can be alternatively used depending on type of target pollutants that should be degraded in water, wastewater as well as process streams having organic matrix. Effectiveness of AOPs and ARPs is obtained thank to radical species formed in the treated stream, which are responsible for rapid and high quantitative degradation of organic compounds [1]. Detailed analysis of process pathways, control of degradation effectiveness as well as formation of by-products can be effectively performed by high resolution gas chromatography (GC) with universal as well as selective detection, including hyphenation with mass spectrometry (MS) [2]. Dedicated fit-to-the-purpose procedures are needed. This paper presents recently developed procedures based on gas chromatography and examples of their application for detailed analysis of processes used for water and wastewater treatment as well as desulfurization of fuels. Sample preparation was based on dispersive liquid-liquid microextraction (DLLME), including application of application of deep eutectic solvents (DES). GC with flame ionization detector (FID), flame photometric detector (FPD), nitrogen-phosphorous detector (NPD) and MS were used depending on application.

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Keywords: separation techniques, volatile organic compounds (VOCs), wastewater treatment, desulfurization of fuels, deep eutectic solvents (DESs).

Optimization of a Novel Analytical Method for Total Phenanthrene Quantification

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The polycyclic aromatic hydrocarbons (PAHs) are organic pollutants generated by anthropogenic activities associated with industrialization, urbanization and other natural human activities. Phenanthrene is a typical low-molecular-weight PAH with dangerous ecotoxicity, bioaccumulation and long-term persistence in the environment. The aim of this study was to develop and optimize a fast and efficient sample processing method compared to commonly available extraction methods followed by short and sensitive analysis based on liquid chromatography coupled with DAD detection. The method was developed and optimized to monitor the levels of phenanthrene in different bacterial cultivation samples. Based on linearity (r^2= 0.9994, 25- 1000 µg/mL) and constant wavelength detection (254 nm) by DAD detector, the limit of detection and quantification of the novel phenanthrene method were determined to be 1.59 µg/mL and 4.83 µg/mL, respectively. Some of these bacteriae are capable of metabolizing phenanthrene molecules. The future possible application may include bioremediation in a PAHs – contaminated environment and its subsequent quantification analysis.

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Keywords: Phenanthrene, Liquid Chromatography, DAD detection, Processing Method

Development of a GC-FID Method for Determination of Short Chain Fatty Acids in Human Plasma

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Short chain fatty acids (SCFA) represent fatty acids produced by the gut microbiota. They are produced naturally within the colon by fermentation of carbohydrates, both dietary and endogenous, and protein that are accessible to the microbiota. It has been demonstrated that SCFA can affect the progress of various diseases, such as inflammatory bowel disease, diabetes, atherosclerosis, or colorectal cancer. Therefore, determination of SCFA levels in biological fluids (especially plasma) can be an effective tool for monitoring of the disease state or response to treatment. Here, we developed a new approach based on gas chromatography (GC) coupled with flame ionization detector (FID) for determination of SCFA in plasma samples obtained from healthy volunteers. The whole sample preparation procedure deals with a simple protein precipitation step (methanol as a precipitation agent was used). The separation step was performed on a SolGel-Wax (0.25 µm x 30 m x 0.25 mm) GC column. The developed method was applied for quantification of acetic acid, propionic acid and butyric acid in real human plasma samples. The determined concentrations of the investigated SCFA were as follows: acetic acid was in the range 2.92 – 178.70 µmol/L, propionic acid in the range 7.16 – 23.71 µmol/L and butyric acid in the range 1.74 – 165.14 µmol/L.

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Keywords: short chain fatty acids, gas chromatography, flame ionization detector, plasma, bioanalysis

Response of Flame Ionization Detector for Organic Compounds Containing Heteroatoms

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The Effective Carbon Number (ECN) concept introduced in the 1960s quantifies the flame ionization detector response for organic compounds to be analyzed. For hydrocarbons, the dependence of ECN on the specific carbon number approximates the linear relationship well. However, the response of hydrocarbons containing heteroatoms differs from this signal production mechanism.

The signal modifying effect of the heteroatom is a function of the quality of the heteroatom and of the bonding in which the heteroatom participates. In the literature, various factors are published about the effect of the different functional groups. These data can serve as a starting point for our measurements in cases if there is no possibility determine the response signal experimentally. Although, in our earlier papers we demonstrated that these functional-group-specific factors are not independent of the different chromatographic measurement conditions [1,2].

In our work, we determined the effective carbon number of some heteroatom-containing (oxygen, nitrogen, silicon) organic compounds and compared the outcomes with data published in the literature.

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Keywords: flame ionization detector, effective carbon number, detector response

Smart Micro Gas Chromatograph for Online Detection and Analysis of Malodorous Substances

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Odorous gas emissions represent a pressing environmental problem, which has attracted considerable attention in recent years. Municipal solid waste degradation in landfills is one of the major sources of offensive odors, conceivably creating nuisance in adjacent communities. This study aims to develop a prototype of an innovative portable analytical module intended for industrial-scale production, capable of monitoring the presence and evolution of odorous compounds. The innovative technology of this analytical module comprises an advanced micro gas chromatograph (μGC) based on MEMS (Micro Electro-Mechanical Systems) technology (pre-concentrator, separation column, and detector) combined with Artificial Intelligence and machine learning algorithms in an effort to map potential chromatographic patterns. Furthermore, a key factor of innovation lies in using a μGC system with filtered ambient air as the carrier gas, increasing portability and reducing frequent maintenance of the module. To the best of our knowledge, an analytical module with such characteristics has not been reported. The stationary phase used for this preliminary investigation is packed in a MEMS separation column. A series of qualitative tests were performed to evaluate the feasibility of using this analytical module. As a first step, a framework was developed upon which a broad variety of odorous candidates generally found in landfill sites was assessed for their suitability with the system. These compounds are very diverse, such as VOCs, halogens, esters, ketones, aldehydes, amines, and sulfur compounds. As a second step, some of these compounds were selected for preliminary tests, considering their boiling point, response to the detector (PID 10,6 eV), and abundance in landfill sites. Finally, the measurement conditions were tailored to optimize the separation and detection of the analytes. The preliminary results show that this analytical system seems to be a promising option, as the overlap of the individual chromatograms exhibits good resolution. The next steps of this work will consist of a quantitative individual analysis of the selected analytes, to detect a proportional signal-concentration correlation. Then, a quantitative analysis of different mixtures of these compounds will be performed to exclude synergistic effects that can affect the measurement's quality.

Faster GC-MS Analysis of the Priority PAH Pollutants with a Short Narrow-Bore Column and Method Translation

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Environmental samples throughout the world are monitored for 16 priority polycyclic aromatic hydrocarbons due to their elevated toxicity and carcinogenicity. These compounds range from naphthalene (2-rings, MW=128) to benzo[ghi]perylene (6 rings, MW=276). The standard analytical column is a 30 m x 0.25 mm x 0.25 µm 5% di-phenyl-type silarylene column, such as the Rxi-SVOCms. Analysis times can exceed 20 minutes once the column cleaning bake out is added to the end of the GC oven program. Migrating the analysis to a smaller format while maintaining the same phase ratio would preserve the same elution profile and optimized separations while taking a fraction of the time. The GC Accelerator kit provides a simple way to speed up sample analysis. By reducing oven volume, these inserts allow faster ramp rates to be attained, which reduces oven cycle time and allows for increased sample throughput and more capacity to process rush samples. When faster ramp rates are used, existing methods can be translated to smaller, high-efficiency, narrow-bore columns using Restek's EZGC Method Translator. With a scaled-down column, such as a 20 m x 0.15 mm x 0.15 µm, a properly translated method, and a GC Accelerator kit, one can obtain the same chromatographic separation seen on the 30 m x 0.25 mm x 0.25 µm column. Often with greater sensitivity and in approximately 2/3 the time, increasing instrument capacity by more than 30 percent.

Keywords: PAH, EZGC, Rxi-SVOCms, GC Accelerator

Development of an *in-situ* Measurement System for the Simultaneous Characterization of VOCs and SVOCs in Ambient Aerosols

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In this poster, we present the concept and the workflow of a novel mobile measuring system for the simultaneous analysis of gaseous and condensed/bound volatile organic compounds (VOCs) and semi volatile organic compounds (SVOCs) in ambient aerosols. The concept allows stand-alone, fully-automated and parallel sampling of the particulate matter (PM) and gaseous phase and its subsequently and successive analysis by thermal desorption combined to gas chromatography coupled to mass spectrometry (GC-MS). Collected PM and gas phase compounds are desorbed on individual units. The PM desorption unit is designed for an on-line derivatization of polar organic compounds before desorption. A PAL based robotic unit is used for handling of collected samples and the instrument is able to operate autonomously and remotely for at least 24 h with up to hourly time resolution. The possibility to automatically exchange between the different modules together with the high-resolution time combined with sensitive analytics make the system a useful tool for long-term field measurements of ambient air.

Keywords: aerosols analysis, automation, at-line measurements, GC-MS, on-line derivatization

Selection of Hold-up Volume Determination Methods and Markers in Hydrophilic Interaction Liquid Chromatography

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Retention mechanisms in Hydrophilic Interaction Liquid Chromatography (HILIC) are complex, mainly based on the partition of analytes between the eluent and a stationary phase consisting of water-rich layers partially immobilized on the chromatographic support or the bonded phase (underivatized silica, zwitterions, polar functional with relatively short alkyl spacers...). Resembling reversed-phase liquid chromatography (RPLC), mobile phases are normally made up hydroorganic eluents, mainly acetonitrile and an aqueous buffer.

In this work, common methods for hold-up time and volume determination in RPLC have been tested in HILIC. Small hydrophobic compounds as toluene, commonly used as hold-up marker in HILIC, are in fact retained and its use is discouraged. Therefore, it is proposed a homologous series method allowing to determine, in addition to hold-up volumes, the ranges of HILIC and RPLC column behavior, and a pycnometric approach, based on differences in column weight when filled with water or organic solvent, that allows the measurement of the volume of mobile phase (hold-up volume) plus the volume of eluent sorbed on the packing of the column.

Finally, from the results of both the homologous series and the pycnometric methods, the feasibility of using individual neutral compounds, inorganic salts and solvent peak disturbance as hold-up volume markers is tested too.

Keywords: HILIC, Hold-up volume, Homologous series, Pycnometry

Study of the Fundamental Band Broadening Properties of HILIC Using the Peak Parking Method

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Hydrophilic Interaction Liquid Chromatography (HILIC) has become a powerful technique for the separation of polar and ionizable compounds which employs a polar mixture of organic solvent and water (or buffer) as mobile phase in combination with a polar stationary phase. Because of the high affinity between both phases, some water is adsorbed on the polar surface of the stationary phase followed by several semi-adsorbed water-rich transition layers that actually act as the real stationary phase in HILIC.

In the present work, the peak parking method was tested in order to measure the band broadening under HILIC conditions. Accurate values of the effective diffusion of the solutes inside the particles are critical when determining the contributions to brand broadening in a chromatographic column which is a fundamental parameter in column kinetic performance studies.

Peak parking measurements were performed on a bare silica column and for a wide range of acetonitrile/water percentages since the volume and the composition of the water-rich layers depends on the water content of the eluent.

Keywords: HILIC, Band Broadening, Peak Parking, Effective Diffusion

Water Absorption in Hydrophilic Interaction Liquid Chromatography Columns. Influence of Mobile and Stationary Phases

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Over the last years, Hydrophilic Interaction Liquid Chromatography (HILIC) has become increasingly popular for the separation of polar and ionizable compounds. HILIC employs mixtures of water-miscible organic solvents and aqueous buffers as mobile phase in combination with a polar bonded phase (or even bare silica). Water from the eluent is preferentially adsorbed on the polar phase creating immobilized and/or semi-immobilized water-rich layers which function as stationary phase. The composition of these layers and amount of adsorbed water depends on both the composition of the mobile phase and the composition of the polar bonded phase and support.

In this presentation, the water absorption and the composition of the transition layers are studied for several HILIC columns with different bonded phases (zwitterionic sulfobetaine, zwitterionic phosphorylcholine, aminopropyl, pentafluorophenyl, polyvinyl alcohol, 1,2-dihydroxypropyl, cyanopropyl, and underivatized silica) with acetonitrile/water and methanol/water mobile phases. Combination of pycnometric and chromatographic (homologous series) methods are used to determine the excess of adsorbed water and the volume of the transition stationary layers, and hence the composition of these layers.

Results shows that zwitterionic columns adsorb more water than the rest of the columns.

Water adsorption is much larger for acetonitrile than for methanol mobile phases too.

Keywords: HILIC, Water absorption, Pycnometry, Homologous series

Acetonitrile/Aqueous Buffered Mobile Phases in Hydrophilic Interaction Liquid Chromatography: Not a Piece of Cake

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Hydrophilic interaction liquid chromatography (HILIC) is a suitable technique for the separation of hydrophilic and polar analytes, consisting of columns with polar bonded phases (or even underivatized silica) and hydroorganic buffered eluents, commonly containing a high proportion of acetonitrile (> 70% in volume). In such mobile phases, solubility, pH and buffer capacity can be very different from the values we all know for aqueous solutions.

Firstly, in order to define the buffer playing field, the variation of the pH stability range of a column with the acetonitrile content of the eluent was examined up to 90% of organic modifier. Then, the pH and buffer capacity variation of some of the most common buffering systems used in HILIC (acetic acid, formic acid, ammonia...) was examined, with particular attention to buffer solutions prepared from ammonium acetate and ammonium formate.

Secondly, the chromatographic behavior of some compounds with acid/base properties was assessed at 90% acetonitrile in a zwitterionic ZIC-HILIC column using different buffering systems. The obtained results revealed that not only the pH, but also the buffer nature, play a major role in the chromatographic retention.

Keywords: Buffer, HILIC, pH, solubility

Manipulating the Performance of Novel Zwitter-Ionic Stationary Phases for HILIC

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Hydrophilic interaction liquid chromatography (HILIC) remains a widely used method allowing to solve various problems of determination of polar analytes in complex real samples. Zwitterionic stationary phases are known to provide high separation selectivity for many classes of substances but still are represented by a limited set of commercially available columns. However, a variety of spacers and zwitterionic compounds could allow one to manipulate the selectivity of such stationary phases. Revealing the influence of each structure fragment on the chromatographic performance of the adsorbents could help to facilitate the creation of new stationary phases with desired properties.

In the present work factors affecting selectivity of zwitterionic adsorbents were investigated including the structure of the spacer and the zwitter-ion, the properties of the functional groups and the way of their attachment to the substrate, the functional layer branching. 3-Glycidoxypropyltriethoxysilane and 1,4-butanediol diglycidyl ether were used as linkers for silica modification. Glycine, N-methylglycine, taurine, N-methyltaurine, aspartic acid, and iminodiacetic acid were used for creating zwitter-ionic functionality in the adsorbents.

It was established that using 1,4-butanediol diglycidyl ether as a spacer led to an increase in hydrophilicity, selectivity and efficiency of the phases toward all classes of the test polar substances. Introducing glycine zwitter-ion allowed to keep selectivity while increasing material's hydrophilicity an efficiency toward neutral analytes. Increasing substrate coverage degree resulted in decreasing retention factors and selectivity for acidic analytes while keeping anion-exchange selectivity. The obtained results revealed the possibility of regulating the interaction of zwitterionic stationary phase with target analytes including ion-exchange selectivity.

This work was supported by the Russian Science Foundation through the grant №20-13-00140.

Keywords: Hydrophilic interaction liquid chromatography, zwitterionic stationary phase, separation of polar compounds

Novel Adsorbents for HILIC Obtained via the Ugi Multicomponent Reaction Varying Isocyanide and Carbonyl Compound

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Selectivity and efficiency in HILIC generally depend on the structure of the stationary phase, therefore, the actual direction in the method development is the synthesis of novel adsorbents and the study of their properties. Modification of silica substrate is not only to increase its hydrophilicity, but also opens up a versatile method to synthesize adsorbents with enhanced selectivity for diverse analytical tasks.

The aim of this work was to use flexibility of combinatorial Ugi reaction with using different commercially available compounds to manipulate the selectivity and increase efficiency of the resulting phases. A series of novel stationary phases was obtained by conducting the Ugi reaction directly on 3-aminopropyl silica substrate surface varying the structure of the carbonyl compound (acetone, 2-acetylfuran, 2-acetylpyrrole, and acetaldehyde) and isocyanide (*tert*-butyl isocyanide, ethyl isocyanacetate, 2-morpholinoethyl isocyanide, p-toluenesulfonylmethyl isocyanide, and diethyl isocyanomethylphosphonate). To increase the yield of the heterogenous reaction a synthesis time, a catalyst, and a solvent nature was varied.

Varying components in the Ugi reaction had a significant influence on the selectivity of the adsorbents toward sugars, amino acids, nucleobases and nucleosides, organic acids, and water-soluble vitamins. A 15–50% increase in the obtained phases efficiency (up to 60000 N/m) for different polar analytes as compared to the substrate confirmed the prospects for the formation of the proposed functional layers.

This work was supported by the Russian Science Foundation through the grant №20-13-00140.

Keywords: Hydrophilic interaction liquid chromatography, stationary phase, Ugi reaction, separation of polar compounds

Zwitterionic Polymer-Grafted Porous Silica Particles for HILIC Mode Stationary Phase

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Recently, hydrophilic interaction liquid chromatography (HILIC) has been attracting much attention because of its applicability for the separation of hydrophilic molecules.

Zwitterionic groups are useful for the hydrophilic organic phase of a HILIC stationary phase, and the zwitterionic group-grafted porous silicas are currently commercially available. Here we demonstrate the preparation of poly(4-vinyl pyridinium)-based zwitterionic polymer-grafted porous silica particles (Sil-$VP^+A^-_n$) and their application for HILIC mode stationary phase. Sil-$VP^+A^-_n$ was prepared the grafting of poly(4-vinylpyridine) with terminal reactive group (average polymerization degree = 33) onto porous spherical silica (average diameter = 5, average pore size = 12 nm), followed by the quaternization of pyridyl side chains using alkyl halides with different anions (A^-) such as sulfonate, phosphate and carboxylic acid. In this paper, we report the separation behaviors of geometric and positional isomers of substituted aromatics and compare the separation behaviours with commercially available monomeric zwitterionic stationary phase. Sil-$VP^+A^-_n$ showed highly selective separations for hydrophilic biomolecules such as nucleobases and nucleosides. The effect of anionic group will be also discussed in this presentation.

Keywords: zwitter-ion, hydrophilic interaction chromatography, biomolecules, quaternization, grafting-to

Ion Chromatography Analysis of Degradation Products in Lithium-Ion Battery Electrolytes

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A composition of the conducting salt lithium hexafluorophosphate in a mixture of cyclic ethylene carbonate with different short-chain linear organic carbonates represents the essential electrolyte formulation for modern lithium-ion batteries (LIB). The commercial success of the electrolyte was guaranteed by its well-balanced properties. However, its main disadvantage includes high chemical and thermal instability of $LiPF_6$ in the electrolytes leading to formation of i.a. hydrofluoric acid and inorganic as well as organic phosphates.

Ion chromatography (IC) is an important analytical method for analysis of ionic compounds and decomposition products in LIB to evaluate the electrolyte degradation. However, the analysis time of anions can be unacceptable long due to strong retention of highly polarizable PF_6^- requiring e.g., an additional gradient step with organic modifiers. Column-switching IC applied in this work is a solution for fast analysis of fluoride and related ionic degradation products in $LiPF_6$-based LIB electrolytes. The isocratic elution used in this method has an advantage to significantly shorten the total analysis time by avoiding the equilibration step normally necessary after the gradient step.

Keywords: lithium-ion battery, battery electrolyte, ion chromatography

Retention Depending on Ionic and Molecular Properties in Ion Exchange Chromatography

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Ion chromatography has an unquestionable significant part in the analysis of ionic and ionizable compounds, nevertheless, there are still unanswered questions about the retention-elution mechanisms of compounds.

In the classical modelling of ion exchange chromatography, the parameters of retention models are the mobile phase concentration, the formal ionic charges of analyte and eluent ions, the theoretical ion exchange capacity of resin, the ratio of stationary and mobile phases and an empirical constant called selectivity coefficient. The selectivity coefficient characterizes the equilibrium process based on the distribution coefficients of analyte and eluent ions, can be described using adsorption isotherms.

The conception of this research to discover the contacts between the physical and chemical characteristics of compounds and the retention of analytes applied to classical ion chromatographic models and isotherms. The aim of this work is to develop a multiple retention model system to predict the retention factors of analytes under different separation conditions and thereby give help for method optimization.

Acknowledgement: The research was supported by the Hungarian National Research, Development and Innovation Office (NKFIH FK128350).

Keywords: nature of retention, chemical structure, equilibrium isotherms, predictive modelling

$^{13}C_{11}$-Tryptophan Tracing Analysis in Hodgkin Lymphoma Cell Lines

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The tumor microenvironment plays an important role for tumor growth and development. In this context tryptophan catabolism is considered an important factor for the suppression of antitumoral immune defense.[1] The purpose of this work was the investigation of tryptophan metabolism in Hodgkin Lymphoma cell lines by $^{13}C_{11}$-tryptophan tracing. In cell culture experiments, cells were incubated in $^{13}C_{11}$-tryptophan-containing medium. The time to reach isotopic steady state was determined by time series experiments. Metabolites were extracted with 80% methanol and HPLC-MS/MS analysis was performed. Therefore, both LC- and MS-method were optimized for separation and detection of ^{13}C-labeled tryptophan metabolites. Interestingly, indole-3-lactic acid was found as one of the downstream metabolites of tryptophan in Hodgkin lymphoma cells.[2] Usually, this compound is considered as a microbial metabolite. Comparative experiments with Burkitt Lymphoma and Diffuse Large B Cell Lymphoma cell lines are ongoing.

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Keywords: metabolomics, tracing, tryptophan, ^{13}C stable isotope label, HPLC-MS/MS

Determination of Various PFAS in Drinking Water Using On-line SPE Coupled to LC-MS/MS

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Perfluoroalkyl and polyfluoroalkyl substances (PFAS) constitute a class of more than 4000 individual chemicals that have been widely used since the 1950s, e.g. as fire retardants, food packaging materials or non-stick coatings. These compounds offer heat-resistant, and oil- and water-repellant properties as well as chemical and thermal stability, resistance to UV light and weathering. Due to their anthropogenic origin, PFAS cannot be degraded, and hence they accumulate and can now be detected ubiquitously in the environment. Since drinking water is considered to be an important source of human PFAS intake, testing drinking water for PFAS levels has been essential for several years now. The aim of this work was to demonstrate the determination of all PFAS requested in the EU directive 2020/2184[1] on the quality of water intended for human consumption in an appropriate concentration range. Furthermore, the analysis includes 24 additional PFAS and 22 internal standards using the same method based on an on-line SPE approach which omits additional sample preparation steps.

The linearity of calibration curves ranges from 0.5 ng/L (resp. 1 or 2.5 ng/L) – 100 ng/L (50 ng/L for PFNS) with an R^2 of at least 0.99 for all PFAS. The lowest calibration point (0.5 ng/mL) can be determined in 77.3% of all PFAS included in this work. Control samples at 5 ng/L and 25 ng/L were analyzed in three-fold to measure analytical reproducibility. The RSD was typically lower than 20% (for >95% of the determined compounds resp. QCs) from these measurements.

Keywords: PFAS, LCMS, On-line SPE, Water analysis

Enantioseparation of Deschloroketamine and its Metabolites by Supercritical Fluid Chromatography Hyphenated to Mass Spectrometry

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Deschloroketamine (DXE) belongs to the family of dissociative anaesthetics, abused as new psychoactive substances. On the other hand, dissociative anaesthetics have currently been in the spotlight because of their potential for unipolar depression treatment demonstrated by a clinical application of ketamine. It is important to note that the plausible effect has been described for *R*-ketamine, which exerts rapid-acting antidepressant action probably via its conversion to a particular chiral metabolite.

Generally, information about metabolism, toxicity or the difference in the effects of DXE enantiomers is missing, although the chemical structure of ketamine and DXE differ only slightly. Hence, it is reasonable to expect a similar metabolic pathway and probably also effects for both the substances.

Therefore, as a part of a behavioural study on Vistar rats treated with DXE with the aim to deconvolute its antidepressant effect, we have developed a chiral separation method of the parent drug and its prominent metabolites using SFC-MS/MS. The first analysis of a brain tissue of rats indicates that there might be an active transport of the more potent enantiomer (*R*-enantiomer) over the blood-brain barrier.

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Keywords: enantioseparation, supercritical fluid chromatography, deschloroketamine, metabolites, mass spectrometry

Potential of ASAP-MS Technique and its Application for Metabolite Profiling in Plant Microsamples

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In this communication we introduce a new atmospheric solids analysis probe mass spectrometric (ASAP-MS) technique for direct analysis of solid plant microsamples. Lab modified glass capillaries were used as probe in connection with a high-resolution tandem mass spectrometer Cyclic IMS (Waters). The sampling was performed using a defined micromanipulation under microscopic control.

Long chain fatty acids (LFA), their hydroxy-derivates (HLFA) and alkyl esters of caffeic acid (AECA) were detected and identified by this technique in intact microsamples of pea seed coat parts. Effect of external degradation factors (UV light, ozonation or heat) on metabolite profile was studied. Significant degradation of (H)LFA and AECA was observed. On the other hand, sterols (e.g. β-sitosterol, stigmasterol and campesterol) and phenolic compounds (sinapic acid, catechin etc.) were identified in all microsamples without significant changes. Moreover, new degradation products (e.g. a signal at m/z 413.3811) appeared in exposed microsamples and their identification is an objective of present research.

Combination of micromanipulation with microscopic control for sampling and ASAP-MS analysis using modified capillaries appeared to be appropriate approach for metabolite profiling in plant microsamples.

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Keywords: ASAP-MS, microsamples, metabolites, degradation, direct analysis

How to Couple LC-IRMS with HRMS – A Proof-of-Concept Study

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Compound-specific stable isotope analysis (CSIA) is a unique analytical technique for determining small variations in isotope ratios of light isotopes in analytes from complex mixtures. A problem of CSIA using gas chromatography (GC) and liquid chromatography-isotope ratio mass spectrometry (LC-IRMS) is that any structural information of the analytes is lost due to the processes involved in determining the isotope ratio. To obtain the isotopic composition of, for example, carbon from organic compounds, all carbon in each analyte is quantitatively converted to carbon dioxide (CO_2). For GC-IRMS, open split GC-IRMS-MS couplings have been described that allow additional acquisition of structural information of analytes and interferences. Structural analysis using LC-IRMS is more difficult and requires additional technical and instrumental efforts. In this study, LC was combined for the first time with simultaneous analysis by IRMS and high-resolution mass spectrometry (HRMS), enabling the direct identification of unknown or coeluting species. We have thoroughly investigated and optimized the coupling and showed how technical problems, arising from instrumental conditions, can be overcome. To this end, it was successfully demonstrated that a consistent split ratio between IRMS and HRMS could be obtained using a variable post column flow splitter. This coupling provided reproducible results in terms of resulting peak areas, isotope values, and retention time differences for the two mass spectrometer systems. To demonstrate the applicability of the coupling, we chose to address an important question regarding the purity of international isotope standards. In this context, we were able to confirm that the USGS41 reference material indeed contains substantial amounts of pyroglutamic acid as suggested previously in the literature. Moreover, the replacement material, USGS41a, still has significant amounts of pyroglutamic acid as impurity, rendering some caution necessary when using this material for isotopic calibration.

Keywords: IRMS, HRMS, Isotope, Hyphenation, CSIA

Method Validation for the Analysis of Pesticides in Water Samples using Solid Phase Extraction and Liquid Chromatography coupled to Mass Spectrometry Tandem (SPE-LC-MS/MS) - Matrix effect assessing and monitoring results in water sources

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The Council Directive 2020/2184 of 16 December 2020 regarding the quality of water intended for human consumption states a parametric value of 0,10 ug/L for each individual pesticide and that the analytical methods should allow the quantification of pesticides at 30% of the parametric value. The aim of this work was the implementation and validation of an analytical method for the analysis of several pesticides in raw water and in water for human consumption, according to the documents referred above, namely: Carbofuran, MCPA, 2,4-D, Ipconazole, Metconazole, Penconazole, Prochloraz, Tebuconazole, Tetraconazole, Dimoxystrobin,and Terbuthylazine-desethyl. Some of these pesticides are included in the third watch list published by the Official Journal of the European Union 2020/1161 of August 2020.

Several mass spectrometer parameters were optimized to get the best formation conditions of the precursor and product ion for each pesticide. Two different MRM transitions (one for quantification and one for qualification) were selected for each pesticide. Some compounds showed significant ion suppression effects (-23% for MCPA). For those compounds surrogate labeled compounds were used for results correction. This method showed excellent linearity ranges for all pesticides (between 2,5 and 25 ug/L), with correlation coefficients greater than 0,9994. Recovery studies in several matrices with different fortification levels were performed using solid phase extraction with recoveries between 65% (Terbuthylazine-desethyl) and 101% (2,4-D) with RSD lower than 11,6% (Ipconazole). The Method Quantification Limits obtained for these compounds were between 0,0020 µg/L (Tetraconazole) and 0,0098 µg/L (Dimoxystrobin). The expanded uncertainty (k=2) of the analytical method was below 25,1% (Ipconazole). Interlaboratory studies showed good performance for this method with z-scores between -0,75 (2,4-D) and -0,26 (Carbofuran). Pesticide monitoring campaign around Tagus river and Alentejo regions during 2021, showed presence of Chlortoluron, Terbuthylazine-desethyl, MCPA, Tebuconazole and 2,4-D in some raw water samples, with a maximum concentration of 0,081 µg/L. None of these compounds were quantified in drinking water samples.

Keywords: Pesticides, Water, Solid Phase Extraction, Liquid Chromatography, Mass Spectrometry tandem; Matrix effects

Analysis of Naphthoyl Indole Derivative Synthetic Cannabinoid MAM-2201 in Blood by Liquid Chromatography Tandem Mass Spectrometry

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Abuse of fluorinated synthetic cannabinoid analogs to avoid existing legal regulations still maintains its popularity. At the same time the analysis of synthetic cannabinoids in human matrices is of particular importance in the fields of forensic and clinical toxicology, as cannabis users partly shift to the consumption of 'herbal mixtures' as a legal alternative to cannabis products in order to circumvent drug testing.

The aim of this research was to develop and validate target LC-MS/MS method for the detection and quantification of one of the most potent – Naphthoyl Indole derivative Synthetic Cannabinoid – MAM-2201 in the whole blood samples. The samples were prepared by liquid-liquid extraction. Chromatographic separation and detection were achieved using an Agilent Technologies 1290 liquid chromatograph coupled to a 6460-triple quadrupole mass spectrometer with an electrospray source. Separation was performed on Zorbax Eclipse plus C18 (100×3.0 mm, 1.8 µm) column. The mobile phases consisted of 0.1% water solution of formic acid: 0.1% acetonitrile solution of formic acid with gradient elation. Sample volume was 5 µl, flow rate - 0.4 mL/min. The MS was operated in positive ESI mode, and the analysis was operated in MRM acquisition: 373.6→231.4, m/z 373.6→140.9, m/z 373.6→168.6 m/z.

The method validation was carried out using drug free human plasma samples spiked with analytes. Both intraday and interday accuracy and precision data were all within acceptable limits ±15% RSD. Recovery ranged 86.4% and matrix effects were less than 15%. This method has shown to be selective and specific, providing no evidence of interference or carryover concerns.

Keywords: Cannabinoids, MAM-220, Chromatography, Mass Spectrometry

Glycomic Characterization of Liver Tissues with Cirrhosis and Hepatocarcinoma

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The aim of our work was to perform the glycomic (chondroitin sulfate, CS, and heparan sulfate, HS) analysis of samples from hepatocarcinoma (HCC), cirrhotic and cancer adjacent cirrhotic human liver parenchyma tissues with various etiologies, such as Hepatitis B and C infections (HBV and HCV), primary sclerotizing cholangitis (PSC), and alcoholic liver disease (ALD). During our study formalin-fixed paraffin-embedded human liver tissues were investigated. Glycosaminoglycan chains were degraded into disaccharides by on-tissue bacterial lyase digestion, followed by graphite+C_{18} TopTip purification. Samples were analyzed using nanoHPLC-MS/MS with self-packed HILIC-WAX capillary column. Statistical analysis of the results was performed with R Studio. Regarding total abundance of CS and HS chains, strong etiology dependence was observed between several sample groups. Examining the sulfation patterns of CS disaccharides, several significant differences were observed between cirrhotic tissues with different etiologies, and no etiology-dependence was shown for HCC. In the case of HS chains, PSC-associated cirrhosis showed decreased sulfation, but no other etiology-dependence was observed. However, a shift in *N*-sulfation and *O*-sulfation was observed between cirrhosis and HCC samples. During the glycomic research we observed differences in the total amount and sulfation pattern of CS and HS disaccharides in correlation with etiology of cirrhosis and HCC.

Keywords: heparan sulfate, chondroitin sulfate, mass spectrometry, high-performance liquid chromatography

The Effect of Make-Up Solvent Composition on Response in Supercritical Fluid Chromatography-Mass Spectrometry Using Single and Triple Quadrupole

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The make-up solvent is a crucial parameter of supercritical fluid chromatography-mass spectrometry (SFC-MS) method as its composition is strongly affecting MS response. However, the optimization of the make-up solvent composition is usually timeconsuming and includes testing of numerous solvents. Our study aimed at simplifying the optimization process by proposing equations that describe the correlations between MS responses obtained with different make-up solvents. Thus, 91 compounds, 3 stationary phases, 2 organic modifiers, and 24 make-up solvents including pure alcohols and methanol in combination with 6 commonly used additives with varying molarity, were used for the experiments, corresponding to over 10.000 data points. These analyses were carried out using both single and triple quadrupole MS. The obtained data were statistically evaluated using Pearson correlation test and regression analysis enabling us to describe the correlations between used chromatographic conditions, physicochemical properties of analytes, and MS response and to define the differences between optimal conditions on these two analyzers.

The study was supported by the STARSS project (Reg. No. CZ.02.1.01/0.0/0.0/15_003/0000465) co-funded by ERDF and the Czech Science Foundation (GAČR n. 21-27270S).

Keywords: supercritical fluid chromatography, mass spectrometry, SFC-MS interface, make-up solvent, quadrupole

Ultra-High Performance Liquid Chromatography in combination with High Resolution Mass Spectrometry for the analysis of Electrochemical Degradation Products of 17-alpha-ethinylestradiol (EE_2) and Biological Assessment of their Estrogenic Activity

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The well documented presence of 17-alpha-ethinylestradiol (EE2) in environmental waters is of growing concern due to its endocrine disruptive effects on aquatic organisms, even at very low concentrations. Conventional wastewater treatment plants (WWTPs) are ineffective in removing EE2 from wastewater, leading to its disposal in the environment. In this respect, electrochemical Advanced Oxidation Processes (eAOPs) are novel and promising chemical-free water treatment processes. eAOPs use diamond coated anodes to generate hydroxyl radicals, which serve as oxidants for the effective degradation of EE2. Nonetheless, the degradation process of EE2 can result in different transformation products (TPs) that can conserve the biological activity of the original molecule. Consequently, an extensive analysis of eAOP degraded water samples from a chemical and endocrine disruption point of view is required, before these techniques can be employed in real-life applications.

In this work, a boron-doped diamond anode was used to perform electrochemical degradation of EE2. Different parameters such as electrolyte type, electrolyte concentration and electric current were optimized to obtain the fastest and most efficient degradation of EE2. To separate, identify and quantify EE2 and its TPs, ultra-high performance liquid chromatography in combination with quadrupole time-of-flight mass spectrometry (QTOF-MS) was used. To evaluate the estrogenic activity of the degraded samples, pre-feeding transgenic chgh-gfp medaka fish fry were used. This animal model is suitable to monitor the net estrogen axis activity. Biological activity was determined by the quantification of green fluorescent protein (GFP) in response to the presence of estrogen active compounds.

The analysis of degraded samples, obtained at different time points, revealed a rapid decrease of the EE2 concentration. In concordance, the estrogenic activity decreased, although it remained relatively high, pointing at the generation of EE2 TPs that retain biological activity. Different TPs were identified in intermediate samples and were shown to also degrade during the degradation process. Finally, the detection of estrogenic

activity in the final degraded samples (obtained after 30 min) suggested that the removal of EE2 and its TPs was not complete, even though EE2 could no longer be detected by UHPLC-MS. This points at the high estrogenic activity of these compounds, even at very low concentrations.

Keywords: 17-alpha-ethinylestradiol, endocrine disruption, electrochemical degradation, liquid chromatography, mass spectrometry

Investigation of Mitochondrial Cardiolipins by Reversed Phase Liquid Chromatography-mass Spectrometry

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Cardiolipins (CL) are characterised as a phospholipid subclass located exclusively in the inner mitochondrial membrane of eucaryotic cells. Particular attention is paid to changes in the natural cardiolipin distribution. High contents of polyunsaturated, long-chain fatty acyl residues make these lipids sensitive to oxidative stress. Alterations of CLs potentially leading to mitochondrial dysfunction have been linked to cardiovascular diseases, diabetes or neurodegenerative diseases. The elucidation of the natural occurring CL distribution is therefore essential to understanding the pathogenesis on a molecular level.

Making complex biochemical processes accessible for analytical investigations, cell culture often becomes a suitable model. For this project, lipid extracts of isolated mitochondria from HeLa cells were used for analysis via reversed phase chromatography (RP-HPLC) hyphenated to a Q Exactive Plus Orbitrap mass spectrometer (HRMS). Chromatographic separation was optimised on a RP-C30 stationary phase with special focus on modified CL species such as monolyso-CL (MLCL), dilyso-CL (DLCL) and oxidation products. Efficient separation is achieved for homologous CL species based on the fatty acid residues, resulting in a suitable method for further analysis of mitochondrial lipid extracts.

A broad distribution of CL species were identified in the lipid extracts of HeLa cell mitochondria. Detection by high-resolution mass spectrometry (HRMS) allowed identification of the species by their accurate masses, confirmed by additionally performed data-dependent MS/MS experiments. Based on characteristic fragments, bound fatty acyl residues were identified for most CL species.

Keywords: Mitochondria, Cardiolipins, RP-HPLC, HRMS, MS/MS

De-Formulation of various (Bio)-Plastic Bags using Evolved Gas Analysis and Pyrolysis-GC/MS

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Environmental pollution by plastics has attracted much concern globally. As an alternative to petroleum-based plastics in the context of sustainability, “bioplastics” have recently been utilized as environmentally friendly plastics. Here, we define two types of bioplastics, one is bio-based (and not biodegradable) plastics and the other is biodegradable (and bio-based) plastics which are naturally degraded and completely broken down by micro-organisms at ideal conditions. Biodegradable plastics may contain additives which accelerate the degradation under light, oxygen, and heat. However, there could be the risk of the toxic residues and the production of small plastic fragments (micro-bioplastics) during the degradation. Thus, it is important to analyze both main constituents and additives of bioplastics. In this work, analysis of bioplastic bags was carried out by vertical micro-furnace pyrolyzer coupled to GC/MS using EGA-MS, single-shot, double-shot, and heart-cut measurement methods. 4 different plastics bags used as samples were commercially obtained: conventional plastic bag (STD), bio-based plastic bag (BP-A), polyethylene plastic bag with 30 % biomass resin (BP-B) and a biodegradable plastic bag (GP). From EGA thermograms, optimum pyrolysis and thermodesorption temperatures were defined for the different samples. The pyrograms of plastic bag STD and BP-A show a similar pattern characteristic to polyethylene (PE), showing that the main component is PE. The bio-based plastic BP-B shows characteristic peaks ascribed to the pyrolyzates of polypropylene and polysaccharides in addition to peaks ascribed to PE, suggesting the existence of the plant-derived components, maybe Rice Resin. GP shows quite different pyrogram patterns compared to other plastic bags and pyrolyzates of PBSA, PLA and PBAT could be identified. In all 4 plastics bags various additives could be identified and quantified. As described above, identification of main constituents and identification and quantification of additives of bioplastic bags could be easily done by Py-GC/MS.

Keywords: pyrolysis, GC/MS, polymers, biopolymers, additives

Development of mass spectrometry search algorithm for mixed microplastics by Py-GC/MS

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Ocean pollution by plastics has become one of the most serious environmental issues. Microplastics may affect marine life by accumulating in the food chain and have a possible impact on human health. Therefore, analysis of microplastics in the environment is of interest. In this study, a vertical furnace pyrolyzer coupled to GC/MS was applied to microplastics analysis, since it provides high-sensitivity analysis and quantitative data even when the sample is a mixture of multiple polymers. A dedicated mass spectral library was constructed from the data obtained by measurements of a 12-polymer reference mixture, and the usefulness of a developed software capable of rapid identification and quantitation of polymers was investigated and applied to the analysis of environmental microplastic samples. Mass spectra constructed by summing up all the intensities of characteristic ions of major pyrolyzates for each polymer were stored in a library. Using the library, the identification and quantitation of each polymer were performed. To isolate the major pyrolyzates of the polymers in the pyrogram, their characteristic ions were selected to obtain mass chromatograms (MC). Upon calculating the match quality between the summated mass spectrum of the characteristic pyrolyzates detected on the MCs of the test sample, and the summated mass spectrum of the pyrolyzates stored in the library, a high match quality was obtained. The mass of polymers calculated from the calibration curve was in a good agreement with the actual polymer amount in the test sample and could be also shown using a real environmental sample, demonstrating its applicability and effectiveness for both identification and quantitation of microplastics.

Keywords: Pyrolysis-GC/MS, microplastics, analytical pyrolysis, polymers, plastics

High Throughput CZE-MS Method for Determination of Triptorelin in Pharmaceutical and Biological Matrices

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Triptorelin is a synthetic decapeptide and a modulator of gonadotropin-releasing hormone, commonly used in the treatment of cancer, but can also be misused to improve athletic performance. In the present work a capillary zone electrophoresis-tandem mass spectrometry based on a triple quadrupole method with a multisegment injection and in-capillary field-enhanced sample injection stacking technique was developed for determination of triptorelin in pharmaceutical and biological matrices. Separation was performed in a background electrolyte consisting of 1000 mM formic acid at pH 1.88. The electrospray parameters were set as follows: sheath liquid composed of methanol and 5 mM ammonium acetate (50/50, v/v) with a flow rate of 8 µL/min was used. The nebulizing gas pressure was 10 psi, the drying gas temperature 300 °C, its flow rate 10 L/min, and the capillary voltage was set at 5000 V. Optimal values of the triple quadrupole parameters were obtained using individual operating modes of the tandem mass spectrometer. A doubly charged parent ion (m/z = 656.5) was identified, an optimal fragmentor voltage of 160 V, a collision energy of 20 eV and the two most intense daughter ions obtained by fragmentation, i.e. quantifier (m/z = 328.3) and qualifier (m/z = 249.0) were selected. The highly selective determination of triptorelin was carried out in multiple reaction monitoring mode, using two ion transitions: 656.5→328.3 and 656.5→249.0. The proposed method was characterized by favorable performance parameters, such as limit of detection (5 ng/mL in water matrix, 25 ng/mL in plasma matrix), precision (relative standard deviation, 1.5-9.4% for intraday and 2.3-11.9% for interday reproducibility), or accuracy (relative errors in the range of 80-109%). Satisfactory performance parameters predetermine the use of the developed method for its routine use in drug quality control laboratories and even in therapeutic drug monitoring.

This work was supported by the projects VEGA 1/0514/22, KEGA 027UK-4/2020, APVV-15-0585 and FaF/2/2022, and was carried out in the Toxicological and Antidoping Center at the Faculty of Pharmacy, Comenius University in Bratislava.

Keywords: triptorelin, capillary zone electrophoresis, tandem mass spectrometry, drug quality control, field-enhanced sample injection, multisegment injection

Separation and Identification of Four Regioisomers of a Sugammadex-related Impurity

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Cyclodextrins were used for many years as excipients; however, their therapeutic properties were discovered at the end of the 20th century only. Sugammadex (SGM) is one of the greatest success in the history of cyclodextrins, because of its selective encapsulation of steroidal neuromuscular blocking agents, such as rocuronium or vecuronium.

During the synthesis of SGM, the formation of process-related impurities, such as Mono-OH-SGM, Di-OH-SGM, Mono-halogen-SGM, etc., is inevitable. Therefore, powerful analytical methods (NMR, MS, HPLC, IR) are needed for the necessary characterization, identification and quantitation of these impurities.

This research focuses on the identification and quantitation of the four possible regioisomers (marked as AB, AC, AD, AE) of the Di-OH-SGM (Hexakis(6-deoxy-6-(2-carboxyethyl)thio)-gamma-cyclodextrin). The presence of the four regioisomers of a synthesized Di-OH-SGM reference material has already been proven by NMR. The four regioisomers were separated and their ratio was also given by an HPLC-UV/DAD method, and their molecular weight was confirmed by an HPLC-MS measurement. However, determination of the elution order, and assignation of each regioisomer to the four chromatographic peaks, requires another technique.

The aim of the project was the identification of the structure of the four isomers, based on their selective mass spectrometric fragmentation. It was supposed that certain fragments can be detected in the MS spectrum which are characteristic for each of the regioisomers, and the regioisomers can be assigned based on the presence or absence of these characteristic fragment ions.

Our results show that fragmentation of the sodium adducts of the separated regioisomers provides structure-selective fragment ions. Based on these, the assignation of the regioisomers were carried out by an appropriate HPLC-MS/MS method. The elution order of the four regioisomers was also determined successfully. Interestingly, fragmentation of protonated molecules resulted in highly similar MS/MS spectra, showing an unusual structure scrambling.

Keywords: cyclodextrin, Sugammadex, fragmentation, HPLC-MS/MS

Determination of Long-Chain Fatty Acids In Anaerobic Digester Supernatant And Olive Mill Wastewater Exploiting An In-Syringe Dispersive Liquid-Liquid Microextraction And Derivatization-Free GC-MS Method

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Gas chromatography with mass spectrometry (GC-MS) stands out among the analytical techniques for determination of long-chain fatty acids - LCFA (by-products of lipid hydrolysis of fats in wastewater and main inhibitor of anaerobic treatment of wastewater). However, reported preparation methods involve derivatization and a subsequent extraction process. These procedures are time and solvent consuming and increase waste generation. To overcome these problems, the automation of dispersive liquid-liquid microextraction (DLLME) technique is an interesting alternative to efficiently separate and preconcentrate the analytes. The aim of this work was to develop an In-Syringe-Magnetic Stirring Assisted (MSA) DLLME method, without derivatization of five LCFA (myristic, palmitic, stearic, oleic and linoleic acid), before analysis by GC-MS. Low LODs were achieved (0.01-0.5 mg L-1) and good precisions (RSDs≤7.9%) were obtained. The method was applied to quantify LCFA in supernatants of anaerobic biodigesters and olive mill wastewaters (recoveries: 81-113%).The developed method could be used as effective monitoring tool for routine analysis in complex environmental samples. This is the first automated method for extraction, preconcentration and individual quantification of LCFA with no need of derivatization.

Keywords: GC-MS, automation, Lab-In-Syringe, DLLME, wastewater

Ion Chromatography Mass Spectrometry Methods for Cationic Polar Pesticides Analysis

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The hyphenation of Ion Chromatography to Triple Quadrupole Mass Spectrometry (IC-MS/MS) is increasing in popularity for the analysis of Polar Pesticides. Advances in the Quick Polar Pesticides (QuPPe) method of sample preparation has made this analysis fast, accurate and routine for anionic polar pesticides such as glyphosate, but the development of methods for cationic polar pesticides has fallen behind. Nevertheless, cationic polar pesticides may occur as residues in food, but are often excluded from pesticide monitoring programs due to the difficulty in the determination of these target analytes. The EURL-SRM (European Union Reference Laboratory for pesticides requiring Single Residue Methods) lists 39 polar pesticides of interest, from which 23 fall into the category of cationic ones based on their ionic properties and molecular structures. This work is aimed to demonstrate capabilities of several cation-exchange columns in the analysis of the listed cationic polar pesticides via ion chromatography.

To achieve the stated goal, several columns with different cation-exchange functionalities have been evaluated. The key parameters influencing the choice of the columns were the functional site structure, surface hydrophobicity, column capacity, and retention of matrix components. Preliminary investigation was conducted in suppressed and non-suppressed IC modes with conductivity or UV detection. Retention of cationic polar pesticides was evaluated using acidic aqueous eluents, and separation was optimized using temperature variation and gradient elution mode.

The obtained data allowed one to select cation-exchange columns suitable for the separation of various types of cationic polar pesticides. The columns providing the best selectivity toward target analytes and their good separation from the matrix components were used for IC-MS/MS analysis of cationic polar pesticides in model solutions and in real world samples.

Keywords: ion chromatography, mass spectrometry, cationic polar pesticides, cation exchange column.

The Dual LC Concept for Productivity Increase in Various Applications and Industries

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In research and development or quality control laboratories of industries such as pharmaceutical, biotechnology or food & beverage, the number of (ultra) high performance liquid chromatography ((U)HPLC) analyses is tremendous. As a result, up to several dozen systems must be operated in one laboratory. The Thermo Scientific™ Vanquish™ Duo for Dual LC offers an ideal solution to significantly increase productivity. The system provides two separate flow paths with the footprint of a single (U)HPLC system, doubling throughput while efficiently utilizing laboratory bench space. Consequently, more applications can be performed with the same number of systems in one laboratory. In this study, four approaches are presented that benefit strongly from the unique Dual LC concept. One approach describes the simultaneous analysis of water-soluble and fat-soluble vitamins which, due to their very different hydrophobicities, require completely independent chromatographic methods, making analysis using the same method difficult. A second approach demonstrates how throughput can easily be doubled when identical methods are run on both flow path, as is often the case with isocratic stability-indicating methods for pharmaceuticals. In a third example, the parallel measurement of an assay and impurity determination of an active pharmaceutical ingredient (API) is shown. Here, the chromatographic methods and/or sample concentration are typically very different and can pose challenges to determine the content of API and related impurities in one run. Lastly, the fourth case describes accelerated method development to detect post-transcriptional impurities in mRNA using a time-effective scouting approach.

Keywords: productivity, throughput, food, beverage, pharmaceutical, biotechnology, vitamins, active pharmaceutical ingredient, drug, impurity, mRNA

Large Volume Electrophoretic Preconcentration as an Efficient Tool for Improvement of Two-Dimensional Liquid-Phase Separations

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Capillary electrophoresis is a widely used separation technique. Its advantages include high efficiency, short analysis time and low consumption of chemicals and samples. On the other hand, the sensitivity of the most commonly used UV/Vis detection is low due to the small volume of the injected sample and the short optical path length. A solution to increase the sensitivity of detection may be to use some of the sample preconcentration techniques. In the present work, different techniques based on various principles were tested for preconcentration of the samples of phenolic acids and flavonoids. The best results have been achieved using the large volume sample concentration method, where a capillary is filled from 75-90 % of the volume with the sample and a negative voltage is applied, which concentrate the sample into a narrow zone subsequently subjected to separation under typical electrophoretic conditions. Such preconcentration conditions are beneficial either i) in two-dimensional separations with the liquid chromatography in the first dimension followed by the capillary electrophoresis in the second dimension, or ii) within focusing modulation of capillary two-dimensional LC×LC, improving quantitative parameters of both setups.

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

Keywords: capillary electrophoresis, preconcentration, two-dimensional separation, antioxidants

Assessing the Orthogonality of Stationary Phases for Comprehensive GCxGC Measurements of Essential Oils

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To make the best use of a multidimensional separation system, the retention mechanisms of each dimension should be as different as possible. In gas chromatography, the retention is influenced decisively by the stationary phase. We present a procedure for the selection of stationary phases for GCxGC that aims at identifying the most orthogonal column pair to achieve the best available separation.

14 different essential oils were analyzed on more than 15 stationary phases under standardized conditions (flow rate, temperature ramp) with both MS and FID detection. All columns were capillary columns with mainly the same dimensions. Variations in length were accounted for by converting retention time to elution temperature, and variations in film thickness were investigated separately. For each essential oil, several target analytes were defined, and the elution temperature of each analyte was determined on each column. The elution temperatures on each column were evaluated statistically, and the column pairs with the least correlation were evaluated in practice on a system with flow modulation. The order of installation was also considered.

Since the set of screened columns comprised a medium and a highly modified version for most stationary phases, the influence of specific modifications was observed as well.

The use of authentic samples also permitted to use the number of separated peaks as an additional quality parameter, since a good separation in the first dimension reduces the demands on the second dimension, which typically has a low peak capacity.

This workflow is expected to be of general usefulness for assessing the orthogonality of GC columns for any kind of volatile sample.

Keywords: column, elution, interaction, screening, selectivity

2D chromatography based on Flow Programming

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Advanced Flow Programming means handling zones of liquids in bidirectional flow at variable speeds or a halt. It can perform the sampling, sample pre-treatment, online hyphenation of multiple analytical steps, and separation. Sequential Injection Chromatography (SIC), introduced in 2003 by Šatínský et al. [1], represents a quickly developing liquid chromatography method based on Flow Programming. A flexible manifold with a syringe pump and several selection/switching valves enables fast chromatography, reduces organic solvents consumption and waste production, and shows excellent analytical performance [2].

However, with a pressure limit of 10 MPa, the SIC manifold could utilize chromatographic columns up to 100 mm in length. Alternatively, the 2D separation methods in both comprehensive and heart-cutting modes can be programmed to a significant increase in the method selectivity desired for the real-life sample analysis.

Flow Programming helps to develop and tuition novel multistep procedures, when later transferable to the HPLC instruments. The development of 2D methods for analyzing active substances contents in food supplements of natural origin will demonstrate the key features of Flow Programming, SIC hardware, and software. The capability and contribution of SIC compared with HPLC will be discussed.

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Keywords: Flow programming, 2D chromatography, Sequential Injection Chromatography

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Combining Photodegradation in a Liquid-Core-Waveguide Cell with Two-Dimensional Liquid Chromatography

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Organic compounds can undergo photochemical conversion through exposure to (UV) light. Sometimes this is exploited, for example in water purification, but it is often undesirable. Examples of the latter include the fading of cultural-heritage objects, reducing their esthetical value, or the degradation of healthy food ingredients (*e.g.* vitamins). Studying such photochemical conversions is challenging and can be very time consuming. Often it is difficult to establish clear link between degradation products and the parent compounds, which results in poor degradation-prediction models.

To solve these issues and to perform photodegradation on small samples (60 μL), a new light cell based on the liquid-core-waveguide (LCW) principle was developed by our group. This cell was coupled to a liquid-chromatography separation to separate and detect the degradation products. To establish strong links between the parent compounds and its degradation products, the pure compound should be injected into the cell. While this may be possible for a small group of compounds, many are not available as single compound.

In this poster presentation we present a multiple-heart-cut two-dimensional liquid chromatography method with the LCW cell inserted between the two separations. The degradation of some target compounds will be shown, including *i)* fuchsin, a 19th-century dye and histological staining agent, *ii)* vitamin B complex pills, and *iii)* annatto extract, a common food dye for fatty products, such as butter and cheese. Furthermore, a simplified version of the setup will be presented, using fewer LC modules, but obtaining more analyte information.

Keywords: light stability, two-dimensional liquid chromatography, photodegradation, reaction modulation, transformative modulator

Off-line LC x SFC - HRMS development for the Characterization of the Wastewater from Algae Hydrothermal Liquefaction

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Biofuels based on cultivated algae are in development and in order to limit the environmental impact of this production, the aqueous waste resulting from the hydrothermal conversion process can be used as a culture medium for these algae. Some species of algae are even more productive when they grow in this recycled water. On the other hand, the number of recycling cycles carried out and the concentration of organic matter can inhibit the growth of algae. The hypothesis of an accumulation of toxins is considered. The analysis of these culture media involves the implementation of techniques with very high peak capacity and so GC x GC is traditionally used. However, it must be supplemented by an alternative allowing non-volatile molecules to be taken into account. Liquid chromatography coupled with supercritical fluid chromatography (LC x SFC) offers orthogonal mechanisms to separate these neutral molecules that elute over a very wide range of polarity. The development of the comprehensive off-line LCxSFC method is carried out on a watsewater resulting from the hydrothermal treatment of the algae Chlorella Sorokiniana sp. Particular attention is paid to the SFC injection process and the impact of the composition of the first-dimensional fractions. The two-dimensional maps show a very large occupation of space. The separation of isomers not detected in one-dimensional LC-HRMS is also demonstrated.

Keywords: two-dimensional chromatography, supercritical fluid chromatography, wastewater

Functionalization of Commercial Hydrophilic-lipophilic Balanced Copolymer for Automatic Magnetic Dispersive Micro-Solid Phase Extraction of Surface Water Contaminants.

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We present an easy approach to magnetic dispersive solid phase microextraction and its automation for the enrichment of water contaminants. Here, SupelTM-Select HLB (Hydrophilic modified styrene polymer) beads were made magnetically susceptible by introducing magnetite nanoparticles into the sorbent. The so-functionalized sorbent was used in a dispersive solid phase extraction methodology that was automated using the Lab-In-Syringe technique.

The methodology followed a previous study on using magnetic nanoparticles by enabling automated exchange of the sorbent, i.e., developing an in-syringe bead injection concept. In short, optimized volumes of sample and bead suspension were aspirated into the syringe void and dispersed by the aid of a magnetic stirring bar placed inside the syringe. By automatic activation and deactivation of magnetic stirring process, the analytes were retained on the dispersed sorbent, which was, when the stirring stopped, captured on the magnetic stirring bar. Afterwards, the sample solution was discarded, and beads were washed in-syringe with water. Thereafter, an optimized eluent was aspirated to strip the analytes from the anew dispersed sorbent.

The extraction system was coupled online to a liquid chromatograph for the determination of model analytes mebendazole, bisphenol A, benzyl 4-hydroxybenzoate, diclofenac, and irgasan. Essential parameters such as extraction time, elution time, sorbent suspension and eluent volume, as well as phase separation time were optimized. Limits of detection ranged from 1.2 µg/L to 6.5 µg/L with < 7 % RSD for all the analytes at 25 µg/L level. The method was linear over a tested range from 5 µg/L to 200 µg/L. The developed method was successfully implemented for the analysis of the chosen contaminants in surface water (lakes and rivers) with recoveries between 78.4 % and 105.6 %.

Keywords: Dispersive solid phase extraction, high performance liquid chromatography, lab-in-syringe automation, magnetic-functionalized sorbent, water contaminants.

Characterization of Humic Acids Isolated from Soil by Off-line Combination of Preparative Isotachophoresis and Size-Exclusion Chromatography

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The off-line combination of preparative capillary isotachophoresis (CITP), operating in a discontinuous fractionation mode, and size-exclusion chromatographic method was studied in this work for separation and characterization of humic acids (HAs). The CITP separations were performed using electrolyte system at pH 10. The use of three discrete spacers (DS), injected into the CITP column together with the HAs, allows the spatial separation of humic constituents and their reproducible fractionation using a micropreparative valve (with a volume of 22 mL). The presence of corresponding DS zones in collected fractions were controlled by analytical CITP in the same electrolyte system as used for preparative CITP. Individual fractions collected from the CITP were off-line analyzed by size-exclusion chromatographic method (SEC) using N,N-dimethylformamide (DMF)/aqueous phosphate buffer pH 3.0 (99/1, v/v) with Spheron HEMA 100 stationary phase filled in a 2.2 mm I.D. column using photometric (280 nm and 420 nm) and fluorimetric detection (ex. 470 nm/em 530 nm).

Keywords: CITP, SEC, Humic acids, Discrete spacers, Off-line combination

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Three-loop Modulator for Comprehensive Two-Dimensional Liquid Chromatography

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In the HILIC×RP comprehensive two-dimensional systems (2D-LC), different mobile phases used in the first and in the second dimension usually show limited compatibility of the elution strengths of mobile phases used in the HILIC and RP systems and may cause excessive band broadening or even band splitting in the second dimension. The band broadening can be suppressed collecting and transferring low fraction volumes (units of µl) to the second dimension. However, small transfer volumes and high flow rates in the second dimension lead to excessive dilution of the analytes and thus increase the detection and quantification limits.

We investigated suitability of a new developed modulator for on-line comprehensive two-dimensional liquid chromatography based on the electronically controlled 12-port/2-position switching valve equipped with three sampling loops. One additional loop compared to the conventional two-loop configuration gives us a possibility efficient electro-driven focusing of analytes into narrower zones, resulting in less dilution during fraction transfer and lower the detection limits. We compare three sampling loops modulator with commonly used interfaces for 2D-LC on a mixture of phenolic acid and flavone standards. The Agilent 2-position/4-port duo-valve and the active solvent modulation (ASM) were chosen as the reference modulator.

This work was supported by the Grant Agency of the Czech Republic under project 22-09556S.

Keywords: two-dimensional liquid chromatography, modulator, HILIC, RP

Expanding Detection Capabilities in SEC and APC: On-line SEC-ICP/MS and APC-ICP/MS Hyphenation

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The atomic spectrometric techniques, XRF and ICP/MS, are widely used for the determination of the elemental content in materials. ICP/MS and XRF are complementary techniques in terms of sensitivity and robustness, however, both approaches provide only the total amount of the element(s) of interest. When elemental distribution information is required, separation steps have to be included in the overall analysis.

Size Exclusion Chromatography (SEC) and the recently developed Advanced Polymer Chromatography (APC) are the preferred methods for the determination of the molecular weight (MW) and dispersity (Đ) of polymers. APC is superior to SEC in speed, resolution, and precision. Combining the resolving power and speed of these size-based separation techniques with the high selectivity and sensitivity of ICP/MS results in unique approaches to determining both the amount and the distribution of heteroatom-containing moieties. This presentation will cover both SEC-ICP/MS and APC-ICP/MS hyphenations to monitor heteroatom distribution within polymer MW.

Keywords: Size Exclusion Chromatography, Advanced Polymer Chromatography, Inductively Coupled Plasma-Mass Spectroscopy, SEC, APC, ICP-MS

Characterization of Automobile Material Emissions by TD-GCxGC-TOFMS and Correlation with Odor Hedonic Perception in Humans

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The study of pollutants and odorous molecules is of growing interest to automotive manufacturers within the frame of Vehicle Interior Air Quality (VIAQ) programs. The most common method to analyse material emissions is to heat them, collect their emissions on sorbent tubes, later thermally desorbed and analysed by gas chromatography coupled with mass spectrometry. This method is suitable for the target analysis of the dozen most intense molecules, but fails to uncover the true molecular complexity of the samples.

In the present work, we used a new instrumental configuration combining thermal desorption (TD), comprehensive bidimensional gas chromatography (GCxGC) and Time of Flight Mass Spectrometry (TOFMS) to elucidate the detailed composition of vehicle material emissions. This implied the testing of several column sets, including normal and reverse polarity configuration, and several second column dimensions, while managing constraints due to the TD coupling.

Over a hundred of potentially odorous polar compounds were observed for each sample. The material samples were also presented to an untrained panel of human noses while collecting their hedonic perception and semantic descriptions, in the aim of establishing links between the material emissions and perception.

Keywords: Thermodesorption, GCxGC, VIAQ, air quality

Unconventional Practice of SPE Extraction - the Simultaneous Application of Normal and Reversed Phases

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The macrofungi are a valuable source of secondary metabolites with different bioactivities. A whole extract generally contains major and minor bioactive components as well. When using the combination of high-performance thin-layer chromatography (HPTLC) with various bioactivity assays, the activity of major components may mask and conceal those of minor ones. The aim of our study was the development of an efficient sample preparation method for the HPTLC-effect-directed analysis of bioactive minor substances of an oyster mushroom species (*Pleurotus citrinopileatus*). The elimination of the inactive matrix compounds and the bioactive major components (mainly fatty acids) from the crude extract, and concentration of the minor components were achieved by a rapid and selective solid-phase extraction (SPE) process using a home-made dual-layer SPE that contains C18 (lower layer) and silica gel (upper layer). The obtained fractions were screened for bioactivity. Antioxidant compounds were monitored by HPTLC-DPPH and antibacterials by HPTLC-bioautography using *Aliivibrio fischeri* and *Bacillus subtilis*. Our results show that the novel SPE sample preparation method facilitated the investigation of the minor components as it reduced the amount of the interfering major components. The same method could also separate and collect the antioxidants and the antibacterial compounds into two fractions. Furthermore, the method could be utilized to fractionate other mushroom extracts. Its refinement will be the subject of our future research.

Keywords: SPE, normal and reversed phases, mushroom compounds, antibacterials, antimicrobials

Hyphenated Two-Dimensional Liquid Chromatography and Mass Spectrometry for Synthetic Oligonucleotides

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Nowadays, ion-pairing reversed-phase liquid chromatography (IP-RP-LC) is still the dominating state-of-art method for the analysis of nucleic acid related compounds, such as antisense-oligonucleotides (ASO), small-interfering ribonucleic acid (siRNA) or other DNA or RNA molecules. Despite of its reliable performance and robustness, the usage of a high number of ion-pairing reagents by IP-RP-LC complicates the hyphenation with mass spectrometry (MS) for an advanced characterization of the analytes. In this work, we tested an alternative stationary phase with polybutylene terephthalate-bonded silica for the separation of generically synthesized Patisiran as siRNA strands (antisense, sense and annealed) giving some unexpected selectivity without any presence of ion-pairing reagents. With that, a further MS and tandem MS (MS/MS) characterization was possible to be carried out. Additionally, hydrophilic interaction liquid chromatography (HILIC) was recombined in a further second-dimensional (^{2}D) LC setup to enhance the selectivity for more separation power.

Keywords: Oligonucleotide separation, Tandem MS, LC-MS Hyphenation, ^{2}D liquid chromatography, Principal Component Analysis

Hyphenated Techniques for Comprehensive Analysis of Glycolipidic Biosurfactants Based on Supercritical Fluid Chromatography

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Amphiphilic rhamnolipids, consisting of one or two L-rhamnose units and up to three β-hydroxy fatty acids, are among the most intensively researched glycolipids and best known for their use as a bio-based alternative to synthetic surfactants. Besides, they can disperse biofilms and have antimicrobial activity. The various applications show great potential, but the study of the biological diversity of rhamnolipid producers, i.e. diverse microorganisms, remains an analytical challenge, as previously established methods do not capture the full spectrum of rhamnolipids produced in a simple and straightforward way.

In this work, we developed analytical methods based on supercritical fluid chromatography (SFC) for the study of rhamnolipids covering the entire biosynthetic pathway from 3-(3-hydroxyalkanoyloxy)alkanoic acids (HAA) to di-rhamnolipids. Hyphenation with complementary detection methods enables their identification, structural characterization and quantification. In particular, SFC allows rapid separation of rhamnolipid subclasses differing in the number of L-rhamnose units and linked β-hydroxy fatty acids, as well as differentiation of rhamnolipids from their precursors and degradation products. By hyphenation with mass spectrometry (MS), identification on fatty acyl position level can be achieved based on accurate mass and data-dependent fragmentation, whereas coupling with charged aerosol detection (CAD) provides complementary quantification of each subclass. Data analysis and visualization were supported with Kendrick mass defect plots, allowing differences in composition and complexity of biotechnological samples within a screening of relevant bacterial strains to be identified at a glance.

Keywords: Supercritical Fluid Chromatography, Mass spectrometry, Charged Aerosol Detection, Glycolipids, Biosurfactants

Characterization of (co-)Polymer Mixtures by Employing Multiple Heart-Cutting Two-Dimensional Liquid Chromatography with Multi-detector Size Exclusion Chromatography

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Industrial synthetic (co-)polymer samples can often be a mixture of different polymeric components. These components may frequently have overlapping distributions in molar mass, chemical composition, and/or architecture, etc. resulting in co-elution in one-dimensional size exclusion chromatography (SEC) rendering analyses inaccurate even after employing absolute detectors. Employing two-dimensional liquid chromatography (2D-LC, e.g., LC x SEC), separations according to chemical composition and size can be obtained, simultaneously, without laborious sample collection. However, LC x SEC with the requirement of a fast 2nd dimension is challenging to implement with absolute detectors due to cycle time incompatibility. Here, we report the application of multiple heartcutting (MHC) 2D-LC for the accurate and detailed characterization of (co-)polymer mixtures with SEC in 2nd dimension coupled to multiple detection, i.e., ultraviolet (UV), refractive index (RI), multi-angle light scattering (MALS) and viscometry (IV). Normal phase LC with silica column in 1st dimension provided separation according to the chemical composition for a mixture of polystyrene, styrene-acrylonitrile copolymers and polyglycols. Utilizing MHC valves, chemically narrow fractions were injected to SEC in 2nd dimension with PL gel mixed bed columns, allowing for determination of chemical composition information via UV/RI ratios, and accurate absolute molar mass distributions, as well as conformations via MALS/IV detection. Additionally, in contrast to LC x SEC method, the MHC method allowed for a facile quantitation of sample constituents.

Keywords: Two-dimensional LC, molecular weight distributions, heart-cutting LC, multi-angle light scattering, copolymers

Ensuring Precise Isolation of Macromolecules by Hyphenating UHPLC Separation Power with Exact Fractionation for Analytical and Semi-preparative LC Purification

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The ultimate goal of fractionation is to isolate compounds of interest or impurities, all while conserving fraction integrity. Ensuring that the resolution achieved by the column is preserved throughout the process is essential for successful fractionation. A major influence is the delay volume (DV), which is defined by the volume between the detector used to trigger the fraction collector device and the tip of the dispensing needle. This work discusses the impact of the DV on analyte resolution, contributing factors, and how the DV can be optimized. Performance was evaluated experimentally using a mixture of oligonucleotides with incremental lengths (12mer to 40mer). Fractions containing the differing oligomer lengths were collected using either time-based or peak-based fractionation. The advantages and disadvantages of both approaches are discussed in detail. The recovery as the ratio between injected amount versus the resulting amount in the fraction of the optimized system was investigated. The results are correlated with the versatile options for fractionation parameters in modern instrumentation. Furthermore, due to the low dispersion characteristics of the instrument, impurities could be readily isolated for further analysis. Crucially, results also showed that pure fractions of the target analyte could be pooled without interference from surrounding impurities.

Keywords: Fractionation, oligonucleotide purification, fraction collector, analytical purification, fraction purity, HPLC, UHPLC, semi-prep purification

Functionalized Poly(ethylene glycol)s (PEGs) as a Reference Material for Detection of Polymeric end Groups via SEC Hyphenated with FTIR and ^{1}H-NMR (80 MHz)

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With the online coupling (or hyphenation) of chemically-resolved spectroscopy detection with classical size-exclusion chromatography (SEC) it is possible to correlate polymer molecular size with chemical structure in a single *in-situ* or on-flow measurement. Medium-resolution ^{1}H-NMR (60 MHz) spectroscopy (i.e., "desktop NMR") and Fourier-transform Infrared Spectroscopy (FTIR) have both been developed as coupled methods (SEC-MR-NMR, SEC-FTIR) for the detection of polymer analytes by monitoring e.g. alkene or carbonyl functionality.[1,2] Here, we have investigated the potential for the detection of polymer end groups, using a slightly higher field-strength spectrometer (80 MHz ^{1}H Larmor frequency) and taking advantage of a columnless injection method that provides bulk spectra for reference. End-functionalized polymers were synthesized by reaction of PEG with toluene isocyanate, yielding α,ω-functionalized polymers with exactly 2 IR- and NMR-detectable functional groups per chain, as proven by MALD-TOF mass spectrometry. We have investigated the sensitivity (limit of detection) and spectral resolution for these functional groups in chloroform, THF, and water. Results have demonstrated a good feasibility for the simultaneous online detection of PEG backbone (M~4000 g/mol) and these end groups at the level of ca. 2 mol%.

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Keywords: Size Exclusion Chromatography, Poly(ethylene glycol), Hyphenated SEC methods, FTIR- and ^{1}H-NMR- detection

Hyphenation of Temperature-responsive Chromatography and Ultrafast Chiral Chromatography as a Generic Comprehensive Two-dimensional Method for the Analysis of Chiral Pharmaceuticals

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Chirality is a prevalent property found in nature, caused by the presence of a (carbon) chiral center in a molecule. Many molecules with different chirality greatly vary in their chemical properties, therefore the ability for separation and detection of these molecules is of utmost importance in the biomedical and pharmaceutical industries. (1)

However, obtaining effective separation of chiral analytes in complex (biological or synthetic) samples requires high resolving power. Which can be theoretically achieved by Comprehensive two-dimensional liquid chromatography (LC x LC), as this technique would allow for a large increase in peak capacity per unit of time. However finding appropriate solvents for the second dimension might be difficult. The slow speed of chiral separations, on the other hand, has hampered the use of chiral stationary phases as the second dimension in 2D-LC. (2,3,4)

In this study, the enantioselective separation of a wide range of medicinal substances (log p: 0.9-4.1) is evaluated using a combination of temperature-responsive and reversed-phase chiral liquid chromatography. Temperature-responsive liquid chromatography (TRLC) in the first dimension enables analysis in purely aqueous conditions, allowing for complete (and more general) focusing of organic solutes prior to second-dimension separation. (2) By combining this with small particle (sub-2 micron) based chiral stationary phases as the second dimension in TRLCxChiral-RPLC, a chiral screening platform may be built, which in principle has a lot of potential for tackling chiral screening challenges. (4)

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Keywords: Chiral separation, two-dimensional liquid chromatography, Ultrafast chiral stationary phase, Temperature-responsive liquid chromatography (TRLC)

A 1D/2D-UHPLC-MS System and its use in the Field of Drug Metabolism and Pharmacokinetics

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Drug metabolism and pharmacokinetics (DMPK) plays a crucial role in the development of new active pharmaceutical ingredients. The elucidation of metabolites has a great importance for the identification of metabolic liabilities of pharmacologically active compounds and cross species comparison for validation of toxicological animal models. Even very low concentrations of metabolites may play a major role. Separation of the biodegradation products by Ultra-High Performance Liquid Chromatography (UHPLC) coupled with mass spectrometry (MS) detection is the technology of choice, with high importance of well synchronized hyphenation.

Thus, a very flexible system to separate a high diversity of molecules in a wide range of operation modes is required. The Vanquish UHPLC system consists of two binary pumps, DADs and column ovens equipped with four 2-position 6-port valves and one autosampler controlled by Chromeleon CDS. The arrangement of the individual modules allows analysis in 1D- as well as 2D-LC mode without hardware reassembling. The 2nd dimension column can be used directly. This allows 2D method development within one sequence. The MS (Thermo Fisher Scientific) is available in all operation modes, resulting in accelerated MS method development. The use of 2 pumps increases the variety of eluents. For complex matrix samples (plasma, urine) treated with high organic solvent proportions, online dilution capabilities enable the injection of up to 100 µL without further sample preparation (e.g. evaporation), which saves time and avoids analyte degradation during sample processing. Continuous flow to the MS during separation steps protects the ESI source, accomplished by a third pump cleaning the source while the analytical column is being equilibrated or washed. For the detection of radiolabeled metabolites an external radioactivity detector can be switched into the system. A flow splitter enables the simultaneous measurement of masses and radio signals. This new, utmost flexible approach enables a gentle measurement of metabolites in early DMPK investigations with high sensitivity and accelerated workflow providing chemists guidance for drug optimization.

Keywords: drug development, DMPK, 2D-LC, method development, pharmaceuticals

Non-Targeted Analysis of Depolymerized Lignin with Online 2D LCxLC-QTOF-MS Technique

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Lignin is an abundant renewable resource that is mainly obtained as a by-product from the wood industry. Most of the isolated lignin is considered a waste, and approximately only 2% is commercialized. However, because of the aromatic structure, it has high potential to be used even more as a natural resource for the isolation of valuable chemicals. The BIOPOLIOL (Bio-based Polymers from Lignin Oligomers) project aims to valorize lignin to manufacture polyhydroxyurethanes (PHU) that could replace polyurethane (whose synthesis requires toxic isocyanates) foams applied in construction. In this joint project, lignin is depolymerized using soft catalysis into monomers and oligomers, which in turn are demethylated to increase the number of hydroxyl groups beneficial to the PHU formation. The complex mixture has to be analyzed to monitor and improve the valorization of the processed lignin via a powerful chromatographic and mass-spectrometric technique.

In this study, online two-dimensional reversed-phase liquid chromatography combined with quadrupole time-of-flight mass spectrometry (LCxLC-QTOF-MS) was used. The utilization of this hyphenated technique benefits from both the increased peak capacity and high resolution of the mass detection. The two dimensions were separately optimized by analyzing 33 lignin monomers. The combined theoretical 2D plots demonstrated that the highest orthogonality and best separation of isomers could be achieved with a set of fluorophenyl and C18 columns. The analysis of processed lignin sample using optimized online LCxLC coupled with the high-resolution Q-TOF-MS enabled to determine the exact mass, formula, C/O ratio, and ring double-bond (RDB) equivalent for the separated compounds. Additionally, fragmentation was used to monitor the elimination of the benzene unit to obtain even more information about the oligomers. Based on the aforementioned data, the presence of a known monomer or the type of the oligomer was also tentatively determined.

Keywords: two-dimensional, liquid chromatography, lignin valorization, 2D plot

Determination of ^{13}C-palmitate using Thin-layer Chromatography Coupled with GC-MS to Determine de Novo Lipogenesis of Firsocostat

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Firsocostat is a highly sensitive drug targeting the liver to reduce hepatic de novo lipogenesis (DNL) by inhibiting acetyl-CoA-carboxylase. DNL inhibition by the drug was measured *in vivo* by a technique of analyzing ^{13}C-labeled plasma triglyceride after IV administration of stable isotope labeled ^{13}C-acetate. Human plasma samples were first extracted by the Folch technique. Plasma triglycerides were isolated and extracted via thin-layer chromatography. The isolated triglycerides were trans-esterified to fatty acid-methyl esters and then analyzed by gas chromatography-mass spectrometry (GC-MS). Fractional hepatic DNL was calculated using Mass Isotopomer Distribution Analyses (MIDA) and represents the fraction of palmitate in plasma triglycerides that was newly synthesized through the hepatic DNL pathway during the stable isotope labeling period. Quality control (QC) samples (human plasma with two different levels of ^{13}C-enriched palmitate) were included during all runs and prepared and extracted in the same manner as study samples. Low enrichment QC samples showed an interday precision (expressed as %CV) of 0.65 to 1.55%, and high enrichment QC samples showed an interday precision (expressed as %CV) of 0.9 to 2.03%. Thin-layer chromatography coupled with GC-MS technique was successfully used to determine DNL inhibition of Firsocostat in a randomized four-way crossover drug-drug interaction clinical study with twenty-four patients.

Keywords: de novo lipogenesis, GC-MS, thin-layer chromatography, triglycerides, bioanalysis, biomarkers

Quantitative Analysis of Nitrosamine Impurities using the LC-MS Methods from the United States Pharmacopeia General Chapter <1469>

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The United States Pharmacopeia (USP) published in December 2021 new procedures and four analytical methods as a response to unexpected detection of nitrosamines, classified as probable human carcinogens, such as N-nitrosodimethylamine (NDMA), in certain pharmaceutical active pharmaceutical ingredients (API), i.e., valsartan, ranitidine, etc. and corresponding final formulations. As a result, demand for nitrosamine analysis has rapidly increased worldwide. The list of concerned products manufactured from drug substances using specific synthetic routes has grown after extensive synthetic route assessments.

The general chapter <1469> provide recommendations regarding; a) the creation of controls of nitrosamine levels to ensure their elimination or reduction; and b) analytical procedure performance characteristics for procedures to monitor nitrosamine levels (two GC-MS and two LC-MS based procedures).

This poster discusses findings from work with establishing validated analytical protocols based on the new liquid chromatography high-resolution (HR) mass spectrometry (MS) method (procedure 1) and the new liquid chromatography MS/MS method (procedure 3) for the quantitative analysis of relevant nitrosamine impurities in pharmaceutical active pharmaceutical ingredients.

Keywords: Nitrosamine Impurities, LC-MS, United States Pharmacopeia, USP, General Chapter <1469>

Long-term Harmonized USP and EP Stability Studies of SBECD (Dexolve™) and CD-Screen-IEC HPLC Column

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The stability of the excipient widely used in the pharmaceutical industry, sulfobutyl ether beta-cyclodextrin (SBECD), was investigated. Analytical and microbiological samples from three batches were stored for 5 years at 25 °C and 60% relative humidity. The content of residual beta-cyclodextrin (BCD) and cyclodextrin and other impurities in the samples were tested quarterly in the first year, semi-annually in the second year and annually monitored by HPLC according to the valid American Pharmacopoeia monograph method. In addition to HPLC assays, the water content, purity and pH of the 30% solution, and microbiological status were monitored. During the five years, the examined properties did not change detectably or only within the limits of the measurement error. In the third year of the stability study, the SBECD monograph was published in the European Pharmacopoeia, according to which the analysis of residual BCD, cyclodextrin and other impurities contents should be measured by another HPLC method. The pharmacopoeia prescribes the use of a new type of HPLC column, CD-Screen-IEC, has been manufactured by Bio-Sol-Dex Ltd. The stability of the IEC column following the change in resolution values between the impurity and the main peak were investigated. Columns were stored in various solvents for 2 years at room temperature or refrigerated.

Keywords: cyclodextin, Dexolve™, stability studies

Greening Reversed-phase Liquid Preparative Chromatography using Alternative Solvents to Acetonitrile for the Purification of a Therapeutic Peptide

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In recent years, research in the field of analytical chemistry has been focusing on the use of methods and technologies in line with the 12 principles of green chemistry. This applies also to purification techniques for biopharmaceuticals, especially peptides. Indeed, the most widely used method for their isolation is reversed phase preparative liquid chromatography even if large volumes of an organic modifier are required. Acetonitrile (ACN) has always been the preferred organic solvent due to its excellent characteristics (e.g., UV transparency and good elution properties). However, this solvent is toxic to the environment and human health, and the aim is to reduce its consumption by replacing it with more environmental friendly solvents. The aim of this work is to demonstrate the possibility of purifying biomolecules while respecting the principles of green chemistry. To this end, a peptide of therapeutic interest, Icatibant, was purified using several alternative organic modifiers to ACN. Results showed that some of the tested solvents can be promising alternative to achieve good separation and purification performance.

Keywords: Green solvents, Green analytical chemistry, Preparative Liquid chromatography, Peptide purification

Method for the Analysis and Quantitation of Pharmaceutical Counterions Utilizing Hydrophilic Interaction Liquid Chromatography

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In this study, Hydrophilic Interaction Liquid Chromatography (HILIC) using a new zwitterionic stationary phase is combined with Evaporative Light Scattering Detector (ELSD) to separate and detect both anionic and cationic pharmaceutical counterions commonly found in drug substance. This single method was capable of quantifying pharmaceutical counterions over a linear range of 2mM to 60mM with excellent retention, resolution and reproducibility. Further, this method was developed to be compatible with mass spectrometry as a means to provide identification for several counterions from the active pharmaceutical ingredient (API) free bases in multicomponent drugs.

Keywords: HPLC, ELSD, HILIC, Counterions, Pharmaceuticals

Performance of Different Models to Estimate Skin Permeability

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Skin permeation (K_p) is a rate constant that defines the transport of drugs from the external layer of the skin, the *stratum corneum*, into the inner layers and the systemic circulation. The knowledge of K_p values is of utmost importance in many fields such as dermal toxicology, exposure to environmental pollutants, and dermal studies of pharmaceutical and cosmetic interest. However, the experimental difficulty and ethical implications in the determination K_p values through *in vivo* and *in vitro* experiments has given rise to the development of alternative methodologies to estimate skin permeation.

This work compares different models to estimate K_p of neutral compounds: a QSAR model based on molecular volume and the octanol-water partition coefficient, a chromatographic model based on the retention factor in a C18 column and volume of the solutes, and finally a model based on skin-PAMPA (parallel artificial membrane permeability assay).

The three models perform similarly in the estimation of K_p values, although the one based on chromatographic measurements presents slightly improved statistics. Moreover, the high-throughput of chromatographic systems makes this model very suitable for K_p estimation.

Keywords: Skin permeation, permeability, liquid chromatography, skin-PAMPA, estimation

Investigation of Potential Mutagenic N-Nitrosamines in Active Substances by LC-MS/MS

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In 2018 N-nitrosamines came under the spotlight, when large number of batches of medical products for the treatment of hypertension containing Valsartan was recalled by EMEA and by FDA, because *N*-nitrosodimethylamine (NDMA) was detected in drug substances.

Investigation of circumstances of formation of different nitrosamines and also development of different analytical methods were started over the world.

In *Pharmeuropa 32.2 (2020)* a draft monograph was published *(2.4.36. N-Nitrosamines in active substances)* where three analytical procedures were detailed for determination of the content of N-nitrosamines in sartans, including an LC-MS/MS method. The limit of the method is 30 ppb, which can be achieved only with the preparation of high-concentration sample solutions, with specific sample preparation and with state-of-the-art instruments.

The aim of this presentation is to compare the optimization of the component dependent and the ion source parameters and to demonstrate the achievable sensitivity of the instruments and to summarize the observations related to the method.

Keywords: N-Nitrosamines, LC-MS/MS, pharmaceuticals

Using Novel Stationary Phase Selectivity to Address Potential NDMA Over-Quantification due to Isobaric Interference in the LC-MS/MS Analysis of Nitrosamines

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Detection of mutagenic nitrosamines in drug substance and product is an area of global concern. The polar nature and low molecular weight of some nitrosamines makes achieving chromatographic retention by LC challenging and presents possibilities for interference from other low molecular weight impurities. During development of an LC-MS/MS method for eight target nitrosamines in API, significant over-quantification of N-nitrosodimethylamine (NDMA) was observed. Spiking experiments confirmed this was due to isobaric interference from co-eluting DMF. At a spiking level of 100 ppm, 307% over-quantification of 1.0 ng/mL NDMA was recorded, highlighting the implications for quantification in pharmaceutical samples. Alternative MRM transitions were assessed, although accuracy improved, DMF co-elution remained problematic.

Several stationary phase chemistries were found to provide enhanced retention and separation of NDMA and DMF. Additionally, fully porous particles, as opposed to solid core, were found to enhance hydrophobic retention of NDMA, sufficient to resolve it from DMF. Two alternative methods were developed and both demonstrated excellent linearity ($R^2 \geq 0.99$), accuracy (93.3 to 109.1%) and precision (%RSD 0.3 to 9.5%). Limits of detection ranged from 0.02 to 0.55 ppb with respect to drug substance (0.04 to 1.5 pg on column), significantly lower than limits specified by regulatory authorities. The improved chromatographic selectivity successfully improved NDMA quantification accuracy in the presence of DMF (accuracy = 104.0% and 102.7% at the 100 ppm spiking level). Highly selective MRM transitions for DMF were established to further safeguard against potential for NDMA over-quantification.

Keywords: Isobaric interference, nitrosamine, NDMA, mutagenic impurities

Simultaneous Determination of Small Molecular Weight Nitrosamines by Fast Gas Chromatography Mass Spectrometry (Fast GC-MS)

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Detection of N-nitrosamines has been an increasing interest in recent years because many of them are probable human carcinogens. In July of 2018, the U.S. Food and Drug Administration (FDA) announced a recall for valsartan-containing medicines due to contamination with the possible carcinogen *N*-nitrosodimethylamine. It has become clear that the problem can not only exist in the case of sartans, but in any API/drug product manufacturing of which secondary or tertiary amines are used or can be produced and a nitrosating agent is available. The decision was made by regulators, according to which all pharmaceutical manufacturers are obliged to perform a risk assessment for the potential presence of nitrosamines in active ingredients and drug products.

The aim of our work was to develop and validate a fast GC-MS method suitable for the quantitative determination of nitrosamines in pharmaceutical products, which include *N*-nitrosodibutylamine, *N*-nitrosodiethylamine, *N*-nitrosodimethylamine, *N*-*N*-nitrosodiphenylamine, *N*-nitrosodipropylamine, *N*-nitrosomethylethylamine, N-nitrosomorpholine, *N*-nitrosopiperidine, *N*-nitrosoethylisopropylamine, *N*-nitroso-diisopropylamine, *N*-nitrosomethylphenylamine, *N*-nitroso-*N'*-methylpiperazine, *N*-nitrosopyrrolidine. The advantage of the method is that it is possible to screen small molecular weight nitrosamines in extremely low concentrations with a short analysis time.

Keywords: nitrosamines, genotoxic/mutagenic impurities, gas chromatography, fast gas chromatography, mass spectrometry

Development of a Fast and Robust UHPLC Method for Apixaban In-Process Control Analysis

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In-process control (IPC) is an important task during chemical syntheses in pharmaceutical industry. Despite the fact that each chemical reaction is unique, the most common analytical technique used for IPC analysis is high performance liquid chromatography (HPLC). Today, the so-called "Quality by Design" (QbD) principle is often being applied rather than "Trial and Error" approach for HPLC method development. The QbD approach requires only for a very few experimental measurements to find the appropriate stationary phase and optimal chromatographic conditions such as the composition of mobile phase, gradient steepness or time (tG), temperature (T), and mobile phase pH. In this study, the applicability of a multifactorial liquid chromatographic optimization software was studied in an extended knowledge space. Using state-of-the-art ultra-high performance liquid chromatography (UHPLC), the analysis time can significantly be shortened. By using UHPLC, it is possible to analyse the composition of the reaction mixture within few minutes. In this work, a mixture of route of synthesis of apixaban was analysed on short narrow bore column (50×2.1 mm, packed with sub-2 µm particles) resulting in short analysis time. The aim of the study was to cover a relatively narrow range of method parameters (tG, T, pH) in order to find a robust working point (zone). The results of the virtual (modeled) robustness testing were systematically compared to experimental measurements and Design of Experiments (DoE) based predictions.

Keywords: apixaban, design of experiments, liquid chromatography, method development, quality by design, robustness

Comparative 90-day Study of Toxicokinetic Profile of Fixed Combination of Antihypertensive Drugs in Rats

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The purpose of this study was to compare toxicity (primary objective) and toxicokinetic profile (secondary objective) of two antihypertensive drugs when together administered to rats orally for 90 consecutive days. The concentrations of both drugs were determined by LC-MS/MS methods in the prepared formulation and in the rat plasma samples. The development of the methods and full validations in both matrices (formulation and rat plasma) in the terms of linearity, accuracy, precision (within-run and between-run), limit of detection, limit of quantification, selectivity, robustness, practicability, stability (in matrix, processed sample stability, stability of standard solutions), and system suitability according to EMA guidelines were performed.

This study deals with concentration data of both drugs in rat plasma obtained in the 90-day oral toxicity study with toxicokinetics performed after multiple oral administration. The administrated doses were 4 mg/kg bw of the 1st drug and 2 mg/kg bw of the 2nd drug (Group D1-FC, low dose), 9 mg/kg bw of the 1st drug and 4.5 mg/kg bw of the 2nd drug (Group D2-FC, middle dose) and 20 mg/kg bw of the 1st drug and 10 mg/kg bw of the 2nd drug (Group D3-FC, high dose). The toxicokinetics parameters (Cmax, Tmax, kel, t1/2, AUCt, AUCi, Residual area, AUMC, MRT and R) were calculated on day 1 and on day 90 separately for each dose and then for all doses together as well as separately or female and male rats.

The accumulation factors were calculated for both drugs separately for each dose. Accumulation factors for the 1st drug was in most cases higher than 1, therefore accumulation was approved for middle and the high dose. No accumulation was proven for the 2nd drug, due to its faster elimination.

Keywords: LC-MS/MS method, toxicokinetic profile, rat plasma

Study of the Degradation of Human Insulin

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Insulin is an important peptide hormone regulating glucose metabolism. Currently, the majority of insulins used for medicinal purposes is produced by recombinant DNA technology. These products can undergo several post-translational modifications (PTM). Deamidation is one of the most prominent PTMs, which occurs as a result of the removal of amide groups in asparagine (N) and glutamine (Q) residues by hydrolysis resulting in free carboxylate groups. Asparagine is converted to aspartic acid and iso-aspartic acid through the formation of a succinimide intermediate. PTMs cause alterations in biological activity, immune response and stability, therefore their characterization during manufacture and storage is essential.

Capillary zone electrophoresis (CZE) with UV and MS detection was tested to determine the deamidation isoforms of human insulin (Humulin R). For the degradation studies insulin pharmaceutical formulations were subjected to acidic condition (pH=1) at room temperature. Sample analysis took place on multiple occasion at different incubation times. At least five isoforms can be seen with UV detection in the acidified sample stored at room temperature for 12 hours. Additional ten components appeared in the sample stored for one month. CE coupled with mass spectrometry was used to determine the molecular mass of each deamidated isoform. The deamidation of one amino acid leads to a 1 Da mass increase. CZE was suitable for the separation of isoforms having different charge-to-mass ratios and MS detection enabled the identification of the degradation products.

Keywords: capillary electrophoresis, mass spectrometry, deamidation, insulin

Development of an Analytical Method for the Determination of N-bromosuccinimide in Two Different Active Pharmaceutical Ingredients by High-performance Ion Chromatography with Suppressed Conductivity Detection

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N-bromosuccinimide is used as a bromination agent in the synthesis route of certain active pharmaceutical ingredients. The determination of N-bromosuccinimide is challenging because of its high reactivity. This poster presents the method development for the high-performance ion chromatographic determination of N-bromosuccinimide. The ion chromatography measurement is the analysis of generated bromide ion by an anion exchange column. The determination is a gradient elution with potassium hydroxide eluent and the detection is performed by suppressed conductivity detector in both cases. Two different types of active pharmaceutical ingredients (prasugrel, favipiravir) were chosen for testing the developed method. For both active pharmaceutical ingredients the sample preparation was performed in a single vial, and it consisted of liquid-liquid extraction with an alkaline reagent. The developed anion exchange chromatography method was validated at limit level, harmonized with guidelines (specificity, accuracy at limit level, precision, and limit of detection and quantification) for prasugrel.

Keywords: N-bromosuccinimide, prasugrel, favipiravir, LLE, ion chromatography

The Bioequivalence Study in Dogs

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The purpose of this study was to investigate the bioequivalence between a new generic formulation (Test Item) and approved veterinary medicinal product (Reference Item). An in vivo bioequivalence study was conducted in dogs in order to compare pharmacokinetic parameters of the approved veterinary medicinal product and the newly developed generic product after administration in dogs.

Concentrations of test and reference item in canine plasma samples were determined by an LC-MS/MS method which was developed and validated before the study samples analysis. The analysis was performed on Quattro Micro API LC/MS-MS System (Waters). Waters XTerra MS C18 (3.5 µm, 150 x 3.0 mm) column was used. The column temperature was maintained at 35 ◦C. The gradient of mobiles phases A (10 mM ammonium formate in water) and B (methanol) was applied. Injection volume was 10 µL and total run time was 8 min. Concentrations were evaluated using an internal standard (IS). Samples were prepared by pipetting 200 µL of canine plasma into a 2mL Eppendorf vial and then it was spiked with 100 µL of working solution of IS (except blank samples). 400 µL of acetonitrile (MeCN) were added to all samples. Samples were shaken and centrifuged at 15,000 rpm for 5 min. Dried samples were dissolved in 200 µL of water:methanol (50:50 (v/v)) solution, transferred into the HPLC vial and injected to the LC-MS/MS instrument.

Pharmacokinetic analysis was performed for individual plasma concentrations by standard non-compartmental methods and the bioequivalence between new generic formulation and approved veterinary medicinal product was confirmed.

Keywords: Test Item, Reference Item, LC-MS/MS, canine plasma

Predicting Pharmacokinetic Properties of Potential Anticancer Agents via Their Chromatographic Behavior on Different Reversed Phase Materials

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Three classes of fused azaisocytosine-containing congeners synthesized in our laboratory were considered and tested as promising drug candidates. For this purpose, different biological parameters describing their bioactivity in human body were established. Quantitative Structure-Activity Relationships (QSARs) equations show the relationship between substance's bioactivity and its lipophilic, electronic and steric descriptors. These independent variables were measured during the experiments (lipophilic parameters) or calculated with a specialist programs (electronic and steric properties). In this work, during *in vitro* chromatographic experiments, the special columns (IAM and ODS) or methods (biopartitioning micellar chromatography) imitating biological systems were applied. The statistically significant QSARs equations were derived using multiple linear regression, then validated and found to be very good. This way of conducting research allows to avoid expensive and questionable animal testing, yet the results obtained are still claimed to be reliable.

Keywords: micellar liquid chromatography, immobilized artificial membrane chromatography, QSARs, fused azaisocytosine-containing congeners

Direct Determination of Chloride Ion by HPLC-DAD? Yes, it Works

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The usual chromatographic method of choice for the direct analysis of amino acids (including L-lysine) is ion chromatography with conductivity detection (CD) or hydrophilic interaction chromatography (HILIC) with charged aerosol detection (CAD). However, due to presence of a weak chromophore some HILIC-UV methods were also suggested (J. Pharm. Biomed. Anal. 145 (2017) 751–757) in the case of L-lysine.

Recently we ran into a problem requiring a rapid, robust, and routinely applicable HPLC-UV method for the determination of L-lysine. Besides the "everyday" method development struggles, this project presented a, at a first sight quite surprising result. It appeared, that, after HILIC separation, Cl^- ions could be directly detected at 200 nm.

As it is often the case, in retrospect, this finding was not surprising anymore. Cl^- has a UV spectrum with a max. at 197 nm. There are a few literature examples highlighting the possibility of direct Cl^- determination by UV detection (J. Chrom. A. (1984), 284, 510). It is simply not in our everyday thinking. Firstly, because short wavelength UV detections are usually avoided (due to UV cutoff, interference, stability…). Secondly, because of the usual textbook recommendations to use CD or CAD when dealing with chloride. Being "trained" by these "experiences" one can easily fall (as we did!) into the mental trap (Cs. Szántay: Anthropic Awareness, (2015)) namely, into "thinking in a paradigm nest" that *chloride ions cannot be directly detected by UV*.

Some aspects of this journey are presented here, while highlighting the key points of the development and the applicability of the method.

Keywords: L-lysine, chloride ion, HPLC-DAD, pharmaceuticals

Co-polymer Characterization by Combination use of HPLC and Raman Microscopy

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One of the important properties of polymer compounds is that they have a molecular weight (MW) distribution, and the average MW value and the degree of polydispersity are usually used for the polymer characterization.

It is common to design polymers consisting of multiple monomers and multi-component polymers for the purpose of developing more highly functional products, called co-polymers. The composition of the co-polymer may not be uniform and may vary, resulting in a distribution of monomer composition as well as molecular weight distribution.

The MW distribution and the composition distribution are parameters that directly affect the thermal and mechanical properties of synthetic polymers. Therefore, a detailed understanding of the molecular structure is essential for controlling the functionality of the polymer materials. Based on this background, the importance of structural analysis of co-polymers is increasing.

Fourier transform infrared spectroscopy (FT - IR), nuclear magnetic resonance spectroscopy (NMR), and reversed-phase chromatography in combination with gel permeation chromatography (GPC) have been reported as methods for the characterization of co-polymers, but few measurements have been performed in combination with Raman microscopy.

In this study, we report a case study for the application of HPLC (GPC) combined with Raman microscopy to the quality analysis of co-polymers and obtained the following conclusion.

A combination use of HPLC and Raman detection afforded quick and easy polymer characterization, which was previously difficult by HPLC alone or required complicated complex methods. In addition, the use of commercial spectral libraries is expected to lead to a wide range of applications.

Keywords: Co-polymer, Raman spectroscopy, SEC, GPC, Characterization, monomer composition

Optimization of the 3D-Printing Fabrication Processes for Prototyping Microfluidic Devices for Spatial Multi-dimensional Liquid Chromatography

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3D-printing is a novel method for the fabrication of layer-by-layer three-dimensional features with high precision. Digital Light Processing (DLP) 3D printing is the most suited technique for the fabrication of complex microfluidic devices with a large number of interconnected microchannels, as encountered in spatial multi-dimensional liquid chromatography (MD-LC). This novel separation concept requires the construction of a device in which analytes are separated by their position in a three-dimensional separation space.

In this research, several aspects of DLP 3D-printing were investigated and optimized, including the optimization of the printing parameters, microchannel design, and post-fabrication process. A comprehensive-experimental study about effects of exposure time and layer thickness on microchannel geometry and surface waviness was conducted. A spatial 2D-LC chip was designed and prototyped targeting isoelectric focusing followed by size-exclusion chromatography (IEF x SEC). A novel methodology for localized synthesizing UV-photoinitiated polymer monolithic frits in-situ in microdevices created from a UV absorber 3D-printing resin was developed. Furthermore, the parallel channel structure was packed with 5 µm SEC particles and employed for the separation of intact proteins. The pressure resistance of the 3D-printed chips was determined to be around 230 bar in presence of organic solvents.

Keywords: 3D printing, multi-dimensional LC

Towards a 3D Printed Highly Customizable Lab-on-Chip-Platform

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Miniaturization of separation systems is the goal of many developments in science and technology. The miniaturization of structures leads to an increase in performance and speed as well as a reduction in the consumption of resources and energy. However, the implementation of such systems has not yet become established in the field of instrumental analysis and the chemical-pharmaceutical industry. Among other things this is due to the complex manufacturing steps and non-standardized microfluidic layouts. Therefore, the aim of this work is to develop a modular 3D-printed lab-on-chip (LoC) system. This system consists of freely configurable modules for mixing, separation, and detection, as well as a base station. The technical innovation is that the individual LoC elements can be easily arranged according to the respective analytical workflow. This eliminates the need for transfer capillaries, which otherwise contribute to a significant reduction in separation efficiency. Within the frame of the work, the self-developed 3D printer capable of processing PEEK and the first additively manufactured chip modules as well as the corresponding base station are presented.

Keywords: Miniaturization; additive manufacturing; Lab-on-Chip

Preparation of Nickel ion substituted hydroxyapatite (NiSHAp) matrix for L-asparaginase purification

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Hydroxyapatite (HAp, $Ca_{10}(PO_4)_6(OH)_2$) is a naturally occurring calcium-phosphate ceramic that has long been utilized as a packing material in the separation of proteins and enzymes. The HAp crystal structure is hospitable to a wide range of substitutions, which allows altering the properties of the materials. In this study, we prepared nickel ion substituted HAp through the spray-drying method and characterized it with a Scanning electron microscope (SEM), X-ray diffraction (XRD), and Fourier transform infrared spectroscopy (FTIR). The results confirm the material is phase-pure NiSHAp without other impurity phases. The particle size analysis revealed that the particles are micron in size with a narrow distribution. The chromatographic experiments showed that the nickel ion substitution has the most significant effect on L-asparaginase purification since the normal HAp failed to separate the protein from impurity. These encouraging results demonstrate that the spray-dried nickel ion substituted hydroxyapatite is a promising matrix for L-asparaginase protein separation.

Keywords: spray drying, nickel ion, hydroxyapatite, L-asparaginase, matrix

Applicability of Centrifugal Partition Chromatography in Downstream Bioprocessing

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Chromatography is dominant and inevitable among separation technologies utilized in the complex and multistep purification of therapeutic macromolecules: a myriad of methodologies, such as reversed-phase column chromatography (RP-LC), affinity chromatography (AC), ion exchange (IEX) or size exclusion (SEC) techniques have been widely implemented in the industry. However, it has also become obvious that the limitations of these approaches in terms of efficiency, capacity, compatibility, or sustainability manifest in enormous costs. To provide alternative solutions, significant research and progress have been made in the field of liquid-liquid chromatography (LLC) in the last decade: viability of this approach has been demonstrated on the purification (in capturing and polishing as well) of synthetic oligopeptides, oligonucleotides, enzymes, transport proteins, monoclonal antibodies (mAb), and virus-like particles (VLP).

Centrifugal partition chromatography (CPC) is one realization of LLC, where both the stationary and the mobile phases are liquids and resolution is simply governed by the partitioning of solutes between these two immiscible liquid phases (i.e., biphasic liquid system or BLS). In practice, one of the two phases is immobilized by a strong centrifugal force inside a rotor, while the other one containing the sample to be purified is pumped through the rotor. Thanks to the liquid nature of the chromatographic system, CPC possesses numerous unique features: (i) flexible and versatile since the chemistry and polarity range of the BLS can be freely varied and adjusted; (ii) total sample recovery can be realized since no irreversible adsorption can occur; (iii) robust and highly loadable due to high stationary phase ratio; (iv) green by appropriate solvent recycling; (v) and can be easily scaled up.

In this presentation, based on an extensive literature survey and unpublished in-house data, CPC-based solutions to the purification requests emerging in the biotech industry will be reviewed through case studies focusing on aqueous two-phase systems (ATPS), operating modes (pH-zone), instrumentation, and solvent recycling possibilities.

Various Applications of Centrifugal Partition Chromatography as a Means of Liquid-Liquid Chromatography for Purification of Small Molecule Compounds and Proteins

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Chromatography is one of the most used technologies for purification in the pharmaceutical industry. Many alternative techniques have been implemented over the past few decades but none of them could operate with comparable efficiency and sustainability while maintaining large capacity and compatibility for a wide variety of compounds. The realizable selectivity in these systems is quite constrained due to the limited number of commercially available stationary phases and their compatible mobile phases. Substantial effort and research have been made in the area of liquid-liquid chromatography (LLC) in the last couple of years to overcome these limitations and find an alternative – not to mention cost-effective – technology for large-scale separations.

Centrifugal partition chromatography (CPC) is a liquid-liquid chromatographic technique that operates under similar principles as traditional solid-liquid chromatography but with one major distinction: CPC does not utilize a solid stationary phase, both the mobile and stationary phases are liquids. Separation occurs between the two immiscible liquid phases, solely based on the partitioning properties of the solutes in the system. In practice, one of the two phases is immobilized by a strong centrifugal force inside a rotor, while the other one containing the sample to be purified is pumped through the rotor. CPC has many advantageous features that make this technology unique:

a) no sample loss due to irreversible adsorption
b) versatility thanks to the large polarity range of applicable solvent systems
c) simple scalability from pilot-scale to industrial-scale
d) high loadability and excellent repeatability due to the robustness of CPC

RotaChrom is currently working with a broad array of industries including pharmaceutical, botanical, food and beverages, nutraceutical industry, and cosmetics at the pilot- and industrial-scale level. Six in-house applications are presented here as examples: polishing of an anticancer API, isolation of the indole alkaloid, Mitragynine, isolation of plasma proteins, polishing of a steroid API, isolation of chemotherapeutic drug precursor, and preparative separation of epimer diterpenes in an achiral environment.

The significance, flexibility, and versatility of centrifugal partition chromatography will be pointed out in this poster through these six applications from various fields of the industry. The diversity of the purified compounds proves the brilliance and untapped potential of this technology.

High Throughput Continuous Refolding of L-Asparaginase Inclusion Bodies

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Chromatography-based refolding is emerging as a promising alternative to dilution-refolding of solubilized inclusion bodies (IBs). The advantages of this matrix-assisted refolding (MAR) lie in its ability to reduce aggregate formation, leading to better recovery of active protein, and enabling refolding at higher protein concentration. However, batch chromatography has the disadvantage of ineffective solvent utilization, under-utilization of resin, and low throughput. In this work, we overcome these challenges by using a 3-column Periodic Counter-current Chromatographic (PCC) system for continuous refolding of IBs, formed during the production of L-asparaginase by recombinant *E. coli* cultures. Initial experiments were conducted in batch processes using single-column immobilized metal-affinity chromatography (IMAC) and Size exclusion chromatography (SEC). Different gradient operations with varying buffer compositions were designed to improve the protein loading for the single-column, batch-MAR processes. Optimized conditions, based on the batch-MAR experiments, were used for designing the continuous-MAR processes using the PCC system. The continuous-MAR was demonstrated using both SEC and IMAC in a 3-column PCC system. A detailed quantitative comparison based on recovery, throughput, buffer consumption, and resin utilization was made for the three modes of operation: pulse-dilution, single-column batch-MAR, and 3-Column PCC-based continuous-MAR processes. These quantitative comparisons clearly establish the advantages of the continuous-MAR process over the batch-MAR and other conventional refolding techniques.

Keywords: Inclusion bodies (IBs); Matrix-assisted refolding (MAR); Periodic Counter-current Chromatography (PCC); Continuous refolding; L-Asparaginase

Simultaneous Online LC Reaction Monitoring of Small Molecules and Trace Impurities using UV Detection

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In the production process of small molecule active pharmaceuticals, a regular monitoring of the reactor is crucial for reaction control. During the reaction, educts and main products are prominent in high concentrations, while impurities are present in low concentrations. Although UV detection is a quick and easy approach, the concentration differences during reaction monitoring are challenging due to limitations by dynamic range and linearity of the detector.

Manual sampling and operation of the analytical system are time consuming and need frequent interactions by the analyst. At the same time, optimization and simplification are required to increase lab efficiency. Automation approaches these needs by merging the various tasks in one setup. Facing the challenges of detection, sampling, and analysis, we present the simultaneous online LC reaction monitoring of small molecules and trace impurities with a two-detector setup.

During the small molecule reaction, samples were drawn regularly and automated. The analysis was performed on a 2.1 x 30 mm, 1.9 µm, RP-C18 column with two UV detectors. Data was analyzed with an integrated software-based combination of both chromatograms. The reaction monitoring showed the product formation resp. educt decrease and, in the same chromatogram, the formation of impurities.

Online LC sampling and combined analysis enabled automated reaction monitoring without interaction of the analyst. Software integrated combination of two detectors allowed quick and easy compound analysis and, therefore, helped speeding up reaction monitoring.

Keywords: Online LC, reactor monitoring, small molecules, UV detection

Systematic Study of Long-Term Retention Time Stability in SFC

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The current supercritical fluid chromatography (SFC) uses a broad spectrum of stationary phases (SP) from pure silica to C_{18} and mobile phase consisting of a carbon dioxide, organic modifier, and an additive in some cases. The use of additives is crucial to enable elution of acidic/basic analytes and to obtain symmetric peak shapes. However, additives in SFC mobile phase bring several negatives features, including (i) long column equilibration time to achieve the retention time repeatability, (ii) difficult additive removing from SP, and (iii) change of SP surface and separation selectivity over time. Moreover, the free SP silanols can interact with organic modifier, forming silylether structures on SP surface. As a consequence, a decrease in analyte retention time can be observed supported by water as a side reaction product acting as a polar additive. This phenomenon designated as column aging effect is pronounced in SFC where the non-aqueous mobile phase is used.

An extensive experimental study was carried out to evaluate the effect of column aging and the effect of additive in SFC using a set of 10 stationary phases and 112 analytes. The retention behavior was studied over the period of one year. Then, the column regeneration procedure was applied and the retention was re-evaluated.

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Keywords: SFC, stationary phase, column aging, effect of additive, retention behavior

Exploring the Difficulties of Mass Flow Measurements in Supercritical Fluid Chromatography

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The variation of pressure, temperature and volumetric flow-rate is most noticeable in supercritical fluid chromatography (SFC), when the mobile phase contains only neat carbon dioxide. This can be attributed to the compressibility of CO_2, that introduces several difficulties to the work of chromatographers. The only flow parameter considered to be constant across the SFC system is the mass flow-rate. It has been shown that the Coriolis flow meter (CFM) provides different types of information depending on its placement in the instrument.

The goal of this work is to investigate several factors affecting the variation of mass flow-rate in SFC, including four different configurations around the column, four sets of experimental conditions along with two columns and a zero-volume union. The effect of disturbances introduced by injections is studied as well. The results show different mass flow-rates when taken at the inlet or the outlet of the column. In addition, different columns produced different tendencies of variations. Study of the effect of injections showed that the initial drop of mass flow is reduced when the averages are taken until the elution times of the chosen compounds. Additional testing related to possible leaks and CFM calibration showed that even if all standard operating procedures are strictly followed, reproducibility of the mass-flow rate can still be problematic.

Keywords: supercritical fluid chromatography, mass flow-rate, Coriolis flow meter

Evaluation of Supercritical Fluid Chromatography and Ultra-high Performance Liquid Chromatography for the Analysis of Polar Analytes

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Polar analytes are often used as building blocks for Active Pharmaceutical Ingredients (APIs). Determining the Chiral and Achiral composition of these molecules is often challenging due to their polarity and often due to the lack of chromophores. Since synthesis is often performed using High-Throughput Experimentation (HTE), analytical methods with short turnaround times are required.

To achieve this, we have developed generic Supercritical Fluid Chromatography (SFC) and Ultra-high Performance Liquid Chromatography (UHPLC) screening setups with different separation mechanisms and using different detectors to rapidly develop analytical methods for this category of compounds.

Keywords: SFC, UHPLC, Polar analytes, Chiral compounds, High-Throughput

Competitive Adsorption of the Mobile Phase Components in Supercritical Fluid Chromatography

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The use of water as an additive in the modified mobile phase in supercritical fluid chromatography is one of the topics which is under spotlight.

The use of combination of water and methanol in the mobile phase will give rise a competition between methanol and water for the adsorption sites on the stationary phase. For investigating the competition between water and methanol, the competitive equilibrium isotherm for methanol and water mixture was determined using the inverse method (IM) which requires a priori selection of an isotherm model. The competitive bi-Langmuir model was selected to fit the equilibrium isotherm of the mixture.

By comparing the results of the single-component adsorption isotherm parameters for the methanol and water with those of the competitive isotherm for the mixture methanol/water we found out that there is a decrease in the saturation capacity values in case of the competitive isotherm, confirming a competition between methanol and water to adsorb on the stationary phase.

The obtained information on the adsorption behavior of the components of a mobile phase could help to get insight about the influence on separation of polar compounds in SFC, which is one of the current challenges in SFC.

Supercritical Fluid Chromatography as a Green Alternative to Normal Phase Liquid Chromatography for the Separations of Phyto Cannabinoids

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One of the chromatographic techniques that can be used for the resolution of mixtures of enantiomers is high performance liquid chromatography under normal phase conditions (NP-HPLC). The mobile phase is made up of a mixture of a non-polar solvent (e.g. hexane) and an alcohol. The solvents used are volatile and dangerous, and often capable of causing damage to the neuro-cerebral system. Supercritical Fluid Chromatography (SFC) could be the alternative candidate technique to the use of NP-HPLC. The use of SFC has grown in recent years due to its green characteristics due to its great versatility in many fields (e.g., medical one). SFC is environmentally friendly as it reduces the use of toxic and dangerous solvents. The mobile phase is usually a mixture of CO_2 together with a certain percentage of organic modifiers that allows to increase the solubility of analytes but also to induce selectivity variations due to the formation of weak interactions (e.g., hydrogen bonds). In this work, SFC and NP-HPLC have been compared for the separation and isolation of complex natural mixtures such as the phytocannabinoid class, obtaining very promising results also in SFC. To the best of our knowledge, this is the first work in which fundamentals of cannabinoid retention using these two techniques have been compared.

Keywords: NP-HPLC, SFC, cannabinoids, green, fundamentals

Evaluation of a Novel Hybrid Silica Solid Phase Extraction plate by HPLC-MS

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Aim: Demonstrate a new technology that will be used to allow for more reproducible sample preparation when performing solid phase extraction (SPE) in a high throughput 96-well plate format. This work will look at a variety of pharmaceutical drug compounds to demonstrate advantages over the current loose filled products used for SPE.

The work: Traditional solid phase extraction (SPE) products packed with loose resin have been used for decades for sample preparation in chromatographic analysis. However, these can suffer from issues which could reduce recovery and reproducibility - channeling, packing mass and height consistency and voiding. A novel hybrid technology using a co-sintered mix of porous plastic and chromatographic SPE resin has been used to help eliminate the issues of loose fill products. Early stage work has been performed using this new technology in a 10 mg format. This test demonstrated for a selection of neutral pharmaceutical compounds that it was possible to get high recovery in a reproducible format. Work between now and the final submitted poster will look to collect data on how this format compares to a loose fill format as well as expanding the compounds looked at.

Results: Current data has looked at two pharmaceutical drugs – carbamazepine and hydrocortisone-21-acetate. Solid phase extraction was performed using a standard reversed phase protocol for silica C18 products with 32 replicates. Recovery and reproducibility values can be seen in Table 1. Recovery was excellent for both compounds tested showing all sample was recovered. The reproducibility of recovery showed there was very little variation sample-to-sample.

	Carbamazepine	Hydrocortisone-21-Acetate
Mean recovery (%)	103.8	105.1
Std Deviation	1.2	1.3
% RSD	1.2	1.3

Table 1. Recovery and reproducibility data for the hybrid C18 silica product (n=32)

Keywords: SPE, HPLC-MS, Pharmaceutical, Reverse phase SPE

Simultaneous Determination of Three Mycotoxins – Ochratoxin A, Zearalenone, and Citrinin in Plant-based Liquids and Comparison Three Various Sorbents using On-line SPE-HPLC with Fluorescence Detection

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Various advanced sorbents for on-line extraction and determination of citrinin, zearalenone and ochratoxin A in plant-based liquid samples have been compared. The plant-based milk is a complex matrix containing high contents of proteins, carbohydrates, lipids, and fiber. Our approach to sample preparation presents a new on-line method including fast extraction using precolumn coupled to liquid chromatography with fluorescence detection. Twelve types of fibrous sorbents including polyethylene (μPE) and polypropylene microfibers (μPP), polycaprolactone microfibers/polyvinylidene difluoride nanofibers composite (μPCL/nPVDF), poly(3-hydroxy butyrate) microfibers (μPHB), poly(3-hydroxy butyrate) microfiber/polypropylene microfibers composite (μPHB/μPP), polycaprolactone nanofibers (nPCL), polycaprolactone nanofibers with 1%, 3% and 5% graphene, polyacrylonitrile nanofibers (nPAN), polyurethane nanofibers (nPUR) and polyamide 6 nanofibers (nPA6) were compared in term of extraction and clean-up efficiency with manually prepared molecularly imprinted polymers for citrinin, zearalenone and ochratoxin A in ration 1:1:1 and restricted access media sorbent RP-18 ADS. The polymer fibers filled in a cartridge and also commercial sorbents were directly connected to HPLC system and the clean-up step and the subsequent chromatography separation optimized. The separation was carried out using core-shell particle analytical column Kinetex Biphenyl (150 × 4.6 mm, particle size 5 μm) followed by fluorescence detection. Excitation wavelength was set to 335 nm and the emission wavelength was set to 497 nm for CIT and OTA and 270 and 458 nm for ZEA. Solvents suitable for separation were acetonitrile with 0.5% anhydrous formic acid under gradient elution, for extraction 5% methanol or 5% acetonitrile was used. The sorbents with the best results were used for control of mycotoxins contamination in plant-based milk. The measured results were compared with tolerable limit established by the European Union.

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Biopolymers as Sustainable Microextraction Materials for the Environmental Monitoring of Polycyclic Aromatic Hydrocarbons and Personal Care Products

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Synthetic polymers offer numerous advantages in analytical sample preparation, likely due to their unique two- and three-dimensional structures, possessing numerous functional groups capable of establishing a variety of interactions with analytes of different nature. However, their degradation capacity is usually limited to their fragmentation, thus causing a high environmental impact if not properly disposed. Besides, there is a current trend of utilization of less harmful materials in analytical sample preparation. Most biopolymers are biodegradable materials obtained from natural sources, and thus included within the group of renewable materials. Various polysaccharides, proteins, and lipids derived from animal and plant sources, can be cited as biopolymers of interest. Therefore, this study focuses on the utilization of various biopolymers from the polysaccharide (chitosan, agarose, and cellulose) and protein (keratin) families as neat sorbents, and not as support for other extraction materials. These biopolymers were studied as novel sorbents in a miniaturized dispersive solid phase extraction method for the analytical determination of environmental contaminants such as polycyclic aromatic hydrocarbons, using the method in combination with HPLC-FD, and other emerging contaminants such as personal care products, using the method in combination with HPLC-DAD.

Keywords: Analytical Microextraction, Biopolymers, HPLC, Personal care products, Polycyclic aromatic hydrocarbons

Occurrence and Bioconcentration of Organic UV Filters in Primary Marine Consumers from Gran Canaria (Spain)

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Organic ultraviolet (UV) filters are added in different products to absorb UV radiation. Due to their extensively production, these compounds are continuously released into the aquatic environment. Their presence in the marine environment poses a hazard to living organisms subjected to their exposure.

The occurrence of eight widely used organic UV filters was analysed in five different primary marine species from Gran Canaria Island (Spain). Analytes were extracted using microwave assisted extraction and determined by ultra-high performance liquid chromatography coupled to mass spectrometry in tandem. The method was applied to 20 samples.

All analysed samples reported at least one target compound. The highest detection frequency corresponded to butyl methoxydibenzoylmethane (BMDBM) (55%), and the second correspond to octocrylene (OC) which was found at the highest concentration (1735 ng·g-1 dry weight, dw) in the sea hare. From the preliminary bioconcentration and biomagnification assess for the found UV filters, bioconcentration factors (Log values) over 3.7 and a biomagnification factor over 1 were obtained in some cases, which suggest a possible bioaccumulation and a potential biomagnification.

Keywords: organic UV filters, marine organisms, microwave assisted extraction

Occurrence and Environmental Hazard of Organic UV Filters in Seawater and Wastewater from Gran Canaria (Spain)

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Organic ultraviolet (UV) filters are extensively used in different products, thus they are constantly released to the environment. The occurrence of eight widely used organic UV filters in seawater and wastewater from Gran Canaria Island (Spain) were analysed. Target compounds' post-treatment removal efficiencies were also reported. Sampling was carried out for 6 months. Extraction was carried out by solid phase extraction with Sep-pak C18 cartridges and determined by ultra-high performance liquid chromatography coupled to mass spectrometry in tandem. The potential environmental hazard was also assessed for several marine organisms.

Benzophenone-3 (BP3) was the most recurrent compound in the seawater (83%) and wastewater (100%). The highest concentrations for seawater (172 µg·L-1) and influent wastewater (208 µg·L-1) was for octocrylene (OC), while to methylene bis-benzotriazolyltetramethylbutylphenol in the secondary treatment effluent (34.0 µg·L-1) and BP3 for the tertiary treatment effluent (8.07 µg·L-1). All the analysed samples showed that at least one target compound. The removal efficiencies reported an average of higher than 50 % for most of the compounds. Primary, secondary and tertiary treatments are unable to completely remove many studied compounds. An environmental hazard quotient above 1 was found for some compounds, which indicates a potential high hazard for living species.

Keywords: organic UV filters, solid-phase extraction, seawater, wastewater

Preconcentration of OTCs (OrganoTin Compounds) in Water Matrices, Speciation Investigation by HPLC-ICP-MS

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OTCs (*OrganoTin Compounds*) are well known global pollutants. They are considered endocrine disruptors, responsible for genetic, reproductive, and metabolic disorders [1]. Due to their persistence, OTCs presence and bioaccumulation in living organisms is still a current issue [2]. The Legislative Decree *D.Lgs 172/2015* set the AA-EQS (*Annual Average value – Environmental Quality Standard*) for TBT (*Tributyltin*) compounds in surface water at 0.2 μg L^{-1}. Therefore, efficient and sensitive analytical methods in compliance with the *D.Lgs 172/2015* are needed. Since these pollutants are present at low concentrations in natural waters, it is necessary to pre-concentrate the sample in order to obtain a detectable concentration. The purpose of this study was to develop a method for the preconcentration and determination of organotin compounds (tributyltin TBT, dibutyltin DBT, monobutyltin MTB), applicable to seawater samples, characterized by a complex matrix. In this work, we used Beta zeolite with Silica/Alumina Ratio 25 (β25) to adsorb OTCs, and then a solution of methanol and tropolone to extract the analytes. The determination of the concentration of OTCs in the solutions was carried out by high performance liquid chromatography hyphenated to inductively coupled plasma mass spectrometer (HPLC-ICP-MS) which can satisfy the low LOQ required, does not involve derivatization steps and allow the speciation of organotin compounds. Satisfying results were obtained for the preconcentration method developed, with extraction recoveries up to 90%. In addition, HPLC-ICP-MS allowed to achieve a good separation of the OTCs investigated.

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Keywords: OTCs, Sample preparation, Adsorbent material, Environmental sample

Automated Sample Preparation for Bioanalytical SPE Method Development & Optimization using Andrew+ Pipetting Robot

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Bioanalysis, or the analysis of drugs and their metabolites from biological fluids, are key assays used in support of drug discovery, development, clinical and forensic toxicology research. Development of quantitative bioanalytical methods focus on the detection, separation and extraction of pharmaceutical drugs from biological matrices. Solid phase extraction (SPE) is often the preferred technique affording high recovery, selectivity, and the ability to concentrate the sample for improved analytical assay sensitivity. SPE optimization, however, is a lengthy and often laborious process requiring extensive manual labor.

This work aims to demonstrate a fully automated reversed-phase SPE method development strategy using a novel sequential elution SPE scouting method, optimizing pH solvent polarity, and various aqueous/organic compositions to determine optimum analyte SPE elution. This SPE scouting method enabled rapid screening of 90 different SPE elution conditions within one experiment with high efficiency. This strategy incorporates the use of the compact Andrew+ Pipetting Robot, an automated liquid handling device, controlled by its intuitive, cloud-based OneLab Software to autonomously executing this multi-step SPE scouting method. The top 3 SPE elution profiles, determined from the initial SPE scouting method, were used to assess final SPE protocol performance (recovery and matrix effects) in neat and extracted plasma with fully automated assessment using the Andrew+ Pipetting Robot. Using the target analyte, carbamazepine, all 3 methods achieved SPE recovery between 67-93% in neat solution, with best performance of one method yielding 84 % recovery and only 3% matrix effects in extracted plasma. The Andrew+ Pipetting Robot controlled with OneLab Software greatly simplifies protocol creation and execution for simple to complex sample preparation and extraction protocols, thus providing a fast, standardized, and fully automated approach to SPE method development.

Keywords: Automation, SPE, Optimization, LC-MS/MS, bioanalysis

Critical Re-Investigations of the Detection of Per- and Polysulfide Species in Cells for Metabolomic Measurements

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Reactive Sulfur Species (RSS), especially per- and polysulfides, are becoming increasingly important in metabolomic studies, but their detection is challenging because these molecules are quite reactive and labile and present in relatively low concentrations in living organisms. Due to their labile nature, most methods employ an alkylation step to conserve these species before separation and detection. In our work, we have focused on this alkylation step and tried to carefully and systematically revise critical parameters affecting the reaction. We used β-hydroxy-4-phenylethyl-iodoacetamide, an alkylation agent that protects against polysulfide chain cleavage and stabilizes the product.

First, an LC-MS/MS method was established and optimized to detect biologically important persulfide species (Hamid et al. Red.Biol. 2019). After that, we have systematically examined how key parameters of the alkylation step, like temperature and duration influence the ratios of certain RSS species and we also investigated the important aspects of alkylation before or after lysis. Our results highlight the importance of a well validated and carefully executed protocol and the possible problems associated with the alkylation reaction of persulfides.

This work was supported by the Hungarian National Laboratory (under the National Tumorbiology Laboratory project (NLP-17)) and the Hungarian Thematic Excellence Programme (TKP2021-EGA-44).

Keywords: alkylation, HPLC-MS/MS, reactive sulfur species, metabolomics

Microwave-Assisted Extraction and Ultra-High-Performance Liquid Chromatography tandem Mass Spectrometry (MAE-UHPLC-MS/MS) Methodology for the Determination of Steroid Hormones in Solid Samples from Natural Purification Systems

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Steroid hormones are a group of emerging pollutants that are having a growing interest in the scientific community and, in recent years, also among legislators with competences in the environmental field. This type of compounds can reach the environment through treated water, since the treatments of wastewater treatment plants are not always effective in their elimination. An interesting area of study is the capacity of natural purification systems (NPS) to achieve the elimination of this type of pollutants. These treatment systems use the purification capacity of the soil, plants, and microorganisms to purify wastewater and therefore, it could be possible that micropollutants such as steroid hormones accumulate in them. In this work, an analytical methodology has been developed for the extraction of different steroid hormones that may be present in solid samples from NPSs. The extraction technique used has been the microwave assisted extraction due to multiple advantages such as the use of small volumes of organic solvents or the easy handling of the technique. The optimized extraction method, coupled to ultra-high-resolution liquid chromatography tandem mass spectrometry (MAE-UHPLC-MS/MS) presents very good detection limits, in the $ng \cdot g^{-1}$ range, as well as extraction efficiencies higher than 75% and relative standard deviations (RSDs) lower than 15%. After optimization and validation of the extraction method, it was applied to solid samples from different natural purification systems such as facultative ponds and constructed wetlands.

Keywords: steroid hormones, microwave-assisted extraction, ultra-high performance liquid chromatography, wastewater

Cotton-HILIC and Graphite Based Solid Phase Extraction Purification Methods for Chondoritin Sulfate Disaccharides

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Chondoritin sulfates (CS) are long-chained macromolecules built up of repeating disaccharide units. Disaccharide building blocks are frequently analyzed using HPLC-MS following enzymatic digestion of the polysaccharides. Between the enzymatic digestion and the instrumental analysis, a purification step is also necessary to remove contaminants and salts from the sample. We aimed to improve our currently used tissue surface enzymatic digestion method to increase sample extraction of CS disaccharides by testing the digestion efficiency as a function of enzyme amount and solution composition. Another goal was to develop a solid phase extraction (SPE) method, that could be used for purification of CS disaccharides extracted from small size biological samples. We compared different graphite-based commercial SPE systems and tested self-packed cotton SPE spin tips (HILIC-type resin) as well. Using double amount of enzyme, and an extraction solution containing 1% ammonia, successfully improved extraction efficiency. Furthermore, we developed an SPE purification method combining the cotton-HILIC and graphite based methods, and maximized the recovery of CS disaccharides. As the last step we tested the applicability of the methods by investigating prostate cancer tissues.

Keywords: chondroitin sulfate, glycosaminoglycan, purification, solid-phase extraction, HILIC

A Sustainable and Innovative Analytical Approach Based on µSPEed/UHPLC-PDA for the Determination of Pesticides in Food and Environmental Samples

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In this work, a fast and innovative procedure, µSPEed, operated by a semiautomatic electronic syringe, digiVol®, was optimized for the simultaneous extraction of eight pesticides (paraquat, thiabendazole, asulam, picloran, ametryn, atracine, linuron, cymoxalil) from food matrices and residual waters. Upon the selection of C18 as the best of the eight sorbents assayed, the optimized extraction involving minor solvent and sample volumes (2×250µL methanol activation, water equilibration and sample loading and 2×50µL methanol elution) and less than 5 min to complete, was coupled to a 7.5 min chromatographic separation using a 1.8 µm ACQUITY UPLC HSS column, at 40ºC, an acidified acetonitrile gradient and PDA detection. The good analytical performance achieved for the target analytes, combined with the simple, semiautomatic and fast extraction and analysis, confers to the µSPEed/UPLC-PDA method here proposed a great potential for pesticides analysis in different food matrices and residual waters.

Keywords: pesticides, µSPEed, UPLC-PDA, residual water, food matrices

Evaluation of Molecularly Imprinted Solid Phase Extraction Cartridges based on Estrogens to Analyze the Presence of Different Steroid Hormones in Wastewaters

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Steroid hormones are an important group of emerging pollutants that may affect aquatic ecosystems even at trace concentrations. The main source of this type of compounds into the environment are the wastewaters and their extraction is very challenging due to the complexity of this type of matrices. For this reason, it is necessary the development of selective analytical methodologies that permit the extraction of these compounds with the least possible amount of interferences.

Molecularly Imprinted Polymers (MIPs) have revealed as selective materials as solid phase extraction (SPE) and an alternative to traditional sorbents. Considering that all steroid hormones share a basic molecular structure and that they can be grouped in C_{18}, C_{19} and C_{21} steroid hormones, in this work we evaluate the extraction efficiency of C_{18}-based MIP to extract and preconcentrate different steroid hormones present in wastewater samples. The developed extraction methodology coupled to ultra-high performance liquid chromatography tandem mass spectrometry (MISPE-UHPLC-MS/MS) shows very good recoveries and reproducibilities as well appropriate detection limits, in the range of $ng \cdot L^{-1}$.

Keywords: steroid hormones, molecularly imprinted polymers, solid phase extraction, ultra-high performance liquid chromatography, wastewater.

Monitoring of Organic ultraviolet Filters and Stabilizers Adsorb in Microplastics from Beaches of the Macaronesia

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Organic ultraviolet filters (UVFs) and stabilizers (UVSs) are a group of emerging pollutants present in marine and coastal waters. These contaminants have been described as bioaccumulative, pseudo-persistent and toxic; therefore, it is of great importance to investigate them and determine their presence and distribution in the environment. They can be adsorbed to the microplastics present in the marine environment, being able to travel great distances in the ocean. In this way, the harmful effect of microplastic is combined with that of these micropollutants, being able to affect areas in which there are no direct sources of contamination of said contaminants. In this study, the occurrence of UVFs and UVSs was monitored during two years in microplastics samples recollected from beaches of the Macaronesia. An ultrasound-assisted extraction (UAE) followed by ultra-high-performance liquid chromatography with tandem mass detection (UHPLC-MS/MS) was applied. Several of these pollutants were identified and quantified with concentrations in the range of $ng \cdot g^{-1}$. Furthermore, in specific samples high concentrations were obtained even up to 5176.45 $ng \cdot g^{-1}$.

Keywords: UV filters, UV stabilizers, microplastics, UAE, UHPLC-MS/MS

The Role of Sample Preparation Techniques in Liquid Chromatography Analysis of Pollutants of Active Pharmaceutical Ingredients

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One of the fundamental tasks of pharmaceutical analysis is to determine potential contaminants (genotoxic) in different active pharmaceutical ingredients (APIs). Sometimes this is a real challenge for analysts. The identification of low concentrations of genotoxic contaminants should be carried out in the presence of the high concentration of the active substance. This high API concentration can cause several problems in liquid chromatography measurements. One of the most important keys to the successful identification of difficult-to-test substances is the development and successful application of an appropriate sample preparation procedure. This poster presents four sample preparation solutions for four genotoxic contaminants, such as sodium-azide, acetaldehyde, N-bromosuccinimide and hydrazine analyzed by liquid chromatography. These sample preparation techniques are liquid-liquid extraction, solid-phase extraction and their combination with derivatisation.

Keywords: sample preparation, pharmaceutical APIs, genotoxic components

Aerial Drone Furnished with Miniaturized Versatile Air Sampling Systems for Selective Collection of Nitrogen Containing Compounds from Air Samples

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A wide variety of nitrogen-containing compounds, present in the environment contributes to air pollution and affects human health and the climate. Their determination and especially the determination of amines in the atmosphere is quite challenging because of their high volatility and polarity. Moreover, due to complex mixtures of compounds present in low concentrations in the air require the development of specific methods and techniques that are selective, efficient, and reliable for nitrogen-containing compounds in the atmosphere.

In this study, aerial drone with miniaturized air sampling system containing both passive and active miniaturized sampling techniques, such as solid-phase microextraction Arrow (SPME Arrow) and in-tube extraction (ITEX) with selective mesoporous silica-based sorbent materials, was employed for the reliable collection of nitrogen-containing compounds in both gas phase and aerosol particles. The nitrogen-containing compounds collected by SPME Arrows and ITEX were after collection desorbed from the samplers, separated, and detected by thermal desorption gas chromatography-mass spectrometry. Sampling accessories, located prior to ITEX, based on adsorbent coatings and polytetrafluoroethylene filters, improved the selectivity of the sampling system further and eliminated particles from collected gas phases. The ITEX sampling system with the drone as carrier was successfully exploited for the evaluation of diurnal patterns and spatial distribution of the nitrogen-containing compounds in boreal forest SMEAR II station, Finland.

Nitrogen containing compounds in the gas phase had the highest concentrations usually in the warmer afternoons, while in the particle phase the highest concentrations were obtained in the mornings and evenings. In the case of the vertical profile, the concentrations of those nitrogen-containing compounds that were mostly emitted by biogenic sources were considerably decreased at the higher altitudes, while those, produced by the anthropogenic sources had highest concentrations at the highest altitude studied. This study forms an excellent platform for future research to evaluate the impact of nitrogen-containing compounds on human health and the environment. Furthermore, due to the flexibility of the sampling systems developed they have great potential in several other environmental application studies (the volatile nitrogen-containing compounds in indoor, soil, water, and food samples).

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Keywords: Nitrogen containing compounds, miniaturized air sampling, ITEX, SPME Arrow, sorbent selectivity, aerial drone, mesoporous silica, atmospheric air, aerosols

Disposable Rubber Gloves as Evidence Samples After Chemical Attack with Nerve Agents

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Videos made after the chemical attacks in Syria show that medical teams did not wear any protective gear, except for sporadic use of single-use gloves and surgical masks. Single-use gloves do not provide sufficient protection for the wearer, however, it has not yet been studied whether they can be used as samples to confirm an attack by chemical weapons.

The military mobile laboratory is a preforensic team that can be deployed quickly to identify toxic substances in various samples. In the study, 3 types of disposable gloves were studied as samples - nitrile, latex and vinyl. The contaminants were the nerve agent sarin (GB) and soman (GD). Six standard solvents used in deployable laboratories were used as extractants. The optimal extraction method and extraction time were monitored. Furthermore, the extraction efficiency of individual solvents was studied, as well as the extraction of other substances from the matrix, which would interfere in the chromatogram. The time after contamination, for which the analyte can still be found in the sample, was observed.

The outputs show different results for each material, as well as for different extractants and contaminants. In latex gloves, it was possible to detect GD using 5 solvents up to 480 min after contamination (GB 30 min). The best extractants for nitrile gloves was acetone, which extracted GD up to 11 days after contamination (GB 480 min). For vinyl gloves, GD could be traced in the sample 3 days after contamination using acetone (GB 180 min, hexane and acetone). The optimal extractants were selected after considering several parameters affecting the analysis. Finally, a sampling scenario was simulated. The sampling team collected the sample according to their SOP 2 days after the incident and the effect of handling and packaging on the subsequent laboratory analysis was observed.

Keywords: military, deployable laboratory, gas chromatography-mass spectrometry;

Comparison of Different Chromatography and Mass Spectrometry Methods for the Analysis of Selected Estrogens at Ultra-Trace Level

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Estrogens are important endocrine disrupting compounds that are released into the environment from various sources and have been shown to be detrimental toaquatic ecosystems[1]. The EU water framework directive (WFD) sets environmental quality standards (EQS) for the environmental concentrations of several endocrine disrupting compounds (EDCs), including the estrogens estrone (E1), 17β-estradiol (E2) and 17α-ethinyl estradiol (EE2) at sub-ng/L concentrations. Preliminary surveys indicated than more than 12% of European rivers by length do not meet these standards[2]. However, robust and traceable measurements at EQS-concentrations are still challenging.

As part of the project EDC-WFD to deliver reliable measurements of estrogens for better monitoring and risk assessment of EDCs, we evaluated the suitability of gas chromatography (GC) and high-performance liquid chromatography (HPLC) separation, each coupled to triple quadrupole and time-of flight mass spectrometers with electron ionization and electrospray ionization, respectively. Detection and determination limit under repeatability conditions are determined according to DIN 32645 for all methods.

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Keywords: Estrogens, HPLC, GC, MS/MS, HRMS, Comparison, Ultra-trace level

Simultaneous Determination of Fatty Acids and Fatty Acid Methyl Esters by HS-SPME arrow GC-MS/MS

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Fatty acids (FAs) and fatty acid methyl esters (FAMEs) are relevant substances in the food industry, microbiology, water analysis and biodiesel production and are analyzed for quality or process control. FAs and FAMEs are related and often appear together, as they can easily be transferred into each other. The fully automated method enables simultaneous determination of 48 FAs and FAMEs in aqueous samples by in-situ FA esterification followed by headspace solid-phase microextraction (SPME) arrow extraction. FAs are esterified before analysis with deuterated methanol to achieve a mass shift of +3 m/z compared to natural FAMEs. The deuterated methyl group results in a slightly shorter retention time (delta-RT = 0.03 min) and leads to specific transitions by using GC-MS/MS operating in multiple reaction monitoring mode. By utilizing these features of the developed method, the distinction of FAs and FAMEs is straightforward, whereas it is not possible by conventional methyl-esterification processes using non-deuterated esterification agents. Esterification parameters (pH, time, temperature, content of deuterated methanol) were optimized by design of experiment. In-situ esterification and headspace extraction make the method not only fast but also applicable to many different matrices.

Keywords: fatty acids, fatty acid methyl esters, derivatization, GC-MS/MS, multiple reaction monitoring

LC-MS Bioanalytical Quantification of a GalNAc-siRNA Conjugate Oligonucleotide Using Semi-Automated Solid Phase Extraction

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Development of robust, sensitive, and selective sample preparation and LC-MS methods for oligonucleotide therapeutics (ONTs) can be challenging due to the complex and diverse characteristics of ONTs. Compounding these challenges, mistakes during sample preparation or user introduced variability can lead to additional uncertainty and poor extraction performance. The use of automation in the preparation of calibrators and QC samples and for solid phase extraction (SPE) can minimize variability in extraction results, especially for compounds that can be technique sensitive or that may have complex SPE protocols.

This work describes a semi-automated sample preparation and extraction method of a GalNAc-siRNA conjugate oligonucleotide (GalNAc) from urine and plasma. Andrew Alliance Pipette+ and the Otto SPEcialist were used to carry out sample preparation and solid phase extraction (SPE) providing a semi-automated approach that can mitigate manual errors and improve analytical recoveries. This was combined with the ACQUITY Premier system and Oligonucleotide column for chromatographic separation, which improves analytical results by reducing metal adsorption, ultimately improving recovery of GalNAc conjugated ONTs.

Mean SPE recoveries ranged from 89-110% with %RSD values <10%. Both intra- and inter-day QC accuracies in plasma and urine met the bioanalytical regulatory requirements of accuracy and precision. Limits of detection were 1 ng/mL for both urine and plasma and linear dynamic ranges were 2.0-1000 ng/mL for urine and 4.0-1000 ng/mL for plasma. This method achieves high oligonucleotide SPE recovery and achieved low (ng/mL) levels of detection from neat and extracted plasma and urine samples. The use of Pipette+ and Otto SPEcialist simplified and streamlined the sample preparation and extraction, maximizing productivity, reducing errors, and ensured overall analytical method performance.

Keywords: Automation, SPE, Oligonucleotide, LC-MS/MS, bioanalysis

Software-supported HPLC Method Development to Baseline Separate Four Regioisomers of a Sugammadex-related Impurity

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Cyclodextrins were used for many years as excipients but their therapeutic potential only discovered at the end of the 20th century. From this point of view, Sugammadex (SGM) is one of the greatest success in the history of cyclodextrins, because of its unique ability to selectively encapsulate steroidal neuromuscular blocking agents, such as rocuronium or vecuronium. However, during the synthesis of SGM, the formation of process-related impurities, such as Mono-OH-SGM, Di-OH-SGM, Mono-halogenSGM, etc., is inevitable. In order to support the upstream synthetical process, powerful – often cost- and resource-intensive – analytical methods (NMR, MS, IR) are being employed for establishing a comprehensive characterization, identification and quantitation of these impurities.

This research focuses on a simple, relatively inexpensive reversed-phase high-pressure liquid chromatography (RP-HPLC) application in combination with UV-detection to separate the four relevant regioisomers (marked as AB, AC, AD, AE) of the Di-OHSGM (Hexakis(6-deoxy-6-(2-carboxyethyl)thio)-gamma-cyclodextrin). The presence of these regioisomers of the synthesized Di-OH-SGM reference material has already been proven by MS and NMR spectroscopy. Thus, the main goal of our project was to baseline resolve these regioisomers – as this is required for the qualification of the synthetized Di-OH-SGM material.

Although our preliminary results on the selected C18 stationary phase already provided with promising initial separation, baseline separation could not be realized. Hence, we decided to apply a systematic modeling approach (DryLab) to optimize critical method parameters. We found that by fine-tuning column temperature and eluent composition (%B), not only baseline separation of peaks but also higher method throughput and robustness could be achieved.

The poster will present this software-supported development process, underlining the key benefits of modeling approaches in establishing the economic separation of regioisomers of the Di-OH-SGM.

Keywords: cyclodextrin, DryLab, HPLC, software method development