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# Plenary Lectures Abstracts

# Future Trends in HPLC Column Technology

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Column technology remains at the forefront of chromatographic advancements, enabling greater resolution, speed, and efficiency in separations. This lecture will explore the state-of-the-art innovations shaping the field, focusing on three transformative developments: micro-pillar array columns, 3D-printed chromatographic supports, and multi-capillary channel systems.

Micro-pillar array columns represent a paradigm shift in stationary phase design. By employing precisely engineered microstructures, these columns deliver superior separation efficiency, reduced backpressure, and enhanced reproducibility. Their potential applications in high-throughput and miniaturized analytical workflows will be discussed, highlighting their compatibility with modern liquid chromatography systems.

Additive manufacturing has revolutionized chromatographic support fabrication. The advent of 3D printing enables the creation of intricate and customizable stationary phases with unprecedented control over geometry and porosity. This lecture will examine how these supports enhance mass transfer, reduce eddy diffusion, and expand the horizons for novel stationary phase chemistries.

Finally, multi-capillary channel columns introduce a multi-dimensional approach to chromatographic separation. By leveraging parallel capillary pathways, these systems achieve faster analyses without compromising resolution. Key insights into their design, integration with existing chromatography platforms, and potential for ultra-fast separations will be presented.

Together, these innovations underscore the rapid evolution of column technology, addressing the growing demands for efficiency, sensitivity, and sustainability in analytical separations. Attendees will gain an understanding of these cutting-edge developments and their implications for future research and industrial applications.

*Keywords*: 3D printing, stationary phase, kinetic performance, band broadening

# In vivo cross-linking mass spectrometry to decipher large scale protein conformations and interactions

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In vivo cross-linking mass spectrometry (CX-MS) enables the large-scale analysis of protein conformations and interactions in living cells, but challenges remain in biocompatibility, cross-linking coverage and identification sensitivity. To address these issues, we designed various biocompatible, multifunctional, and membrane-permeability adjustable cross-linkers, and established the methods for enrichment, identification and data-mining of crosslinked peptides.

A glycosidic bond-based MS-cleavable cross-linker of trehalose disuccinimidyl ester was designed and synthesized, which was not only of excellent biocompatibility for living cells, but also could be fragmented in MS under CID/HCD, simplifying the cross-linked peptides to conventional single peptides via selective cleavage between glycosidic and peptide bonds. Furthermore, the crosslinked peptides could be enriched by HILIC materials, enabling the identification of over 6000 and 1000 cross-linked peptides from 1E7 and 1E3 cells respectively by one LC-MS run, making the applications in primary cells and tissues possible.

By integrating targeted proximity labeling and in vivo cross-linking, we identified 327 PPIs in stress granule, with 78 upregulated upon $NaAsO_2$ stimulation and 30 showing high confidence ($p < 0.05$) among which 63% matched known interactions, while 11 were novel, involving functions such as DNA/RNA processing and protein translation. Enhanced interactions under stress were validated by fluorescence colocalization and Western blot, and further confirmed for both high- and low-confidence novel PPIs by co-immunoprecipitation, highlighting new PPIs related to translation and mRNA regulation. These results demonstrate that in vivo CX-MS enables the comprehensive analysis of dynamic protein assemblies in living cells, offering a powerful tool to elucidate the mechanisms underlying cellular compartmentalization and rapid responses.

Furthermore, protein dynamics play a crucial role in executing diverse functions. The intracellular environment significantly influences protein dynamics, particularly for intrinsically disordered proteins. Recently, we developed a hierarchical decoding strategy enabling comprehensively capture structural information from various proteins within cells and characterize protein dynamics. Computational analysis based on distance restraints derived from cross-links was used to infer protein dynamics, facilitated by the prior structure obtained from AlphaFold2. Furthermore, we can provide a comprehensive description of the intrinsic motion of IDPs, demonstrating the potential in understanding the protein functions in cells.

*Keywords*: CX-MS, *in-vivo* analysis, protein conformation, protein-protein interaction

## From Sample to Data: Enhancing Analytical Workflows with Automation

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Abstract body (up to 350 words)

Chromatography and mass spectrometry are indispensable tools across diverse research fields, from pharmaceutical development to environmental monitoring. Improving chromatographic and mass separation performance, as well as detection selectivity, not only enhances data quality but also drives innovations toward more efficient and reproducible analytical workflows.

In parallel, laboratory automation has advanced rapidly. The integration of artificial intelligence (AI) and machine learning (ML) in research supports experimental design, parameter optimization, and data processing. While these technologies reduce human error and improve analytical consistency, they can also create new challenges: automated workflows often generate large numbers of samples that require preparation, instrumental analysis, and post-processing, potentially extending the time needed to obtain final results unless the entire workflow is optimized. Advances in separation science have improved the efficiency of data acquisition; however, bottlenecks remain, particularly in sample preparation and analytical method development, which often involve labor-intensive operations and repeated trial-and-error cycles.

To address these challenges, Shimadzu has developed solutions that support lab automation with a focus on sample preparation and method development. Examples include automated platforms for sample clean-up, dilution etc., as well as AI-assisted workflows for rapid chromatographic method optimization. These technologies aim to reduce manual workload, shorten turnaround time, and improve reproducibility.

By integrating automation across the entire analytical workflow—from sample preparation to data interpretation—laboratories can achieve higher throughput and better resource utilization. Moreover, these approaches contribute to more sustainable operations by reducing waste, energy consumption, and overall environmental impact. The examples which will be discussed illustrate how combining separation technologies with intelligent automation can help laboratories meet the growing demands of modern research efficiently and sustainably.

*Keywords*: HPLC, LC/MS, Laboratory Automation

# Emerging Trends in AI for Chemistry and Its Applications to Analytical Chemistry

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Artificial intelligence (AI), as a next-generation deep technology, holds the potential to transform a wide range of scientific and societal domains fundamentally. In this lecture, we explore the emerging potential of AI in chemistry and materials science, with particular emphasis on its applications for analytical chemistry.

A critical prerequisite for enabling transformative advances through AI is the development of large-scale, high-quality foundational training datasets. However, in many areas of chemical research, obtaining sufficient data to train AI models remains a significant challenge.

To address this issue in the field of polymer science, we have constructed the world's largest computational property database for polymers, encompassing diverse physical properties for over 100,000 unique polymer structures. This omics-scale database was generated using RadonPy, a Python-based, fully automated pipeline for all-atom molecular dynamics simulations of polymeric materials.

Machine learning models pretrained on the RadonPy database can be fine-tuned with limited experimental data to perform a variety of real-world downstream tasks, achieving superior generalization performance compared to models trained from scratch. Notably, the generalization capability of these pretrained models increases significantly with the size of the RadonPy database, following a power-law scaling behavior across multiple prediction tasks—underscoring the importance of large, high-fidelity simulation datasets.

Building on this case study, we explore the potential applicability of AI in analytical chemistry. In particular, we present an application example focused on retention time prediction in liquid chromatography.

# Surrogate Optimization using Multivariate Adaptive Regression Splines for On-Line Supercritical Fluid Extraction – Chromatography Method Development

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Abstract

It is valuable to have multivariate optimization strategies for analytical instruments, such as supercritical fluid extraction – supercritical fluid chromatography – mass spectrometry (SFE-SFC-MS), a sophisticated on-line sample preparation and analysis system. SFE-SFC-MS has a very wide application base, but its optimization involves the consideration of many variables and variable interactions. Attempts using factorial design and response surface methodology (RSM) was generally successful [1], but clearly revealed the potential for complex response surfaces, which were highly variable with sample type and analyte type. To advance this methodology, we are developing a surrogate optimization (SO) approach. Rather than collecting a full data set of responses at different variable settings, as with RSM, SO uses an iterative process. The SO model is built in sequence with smaller sets of runs, so that an explore-and-exploit strategy for studying variable space can make more efficient use of experimental runs. SO can also accommodate more complex modeling algorithms. Multivariate adaptive regression splines provide the potential for multi-linear modeling of complex response surfaces. A series of pharmaceutical compounds were considered. From the set, a representative subset was chosen using molecular encoding techniques. The molecular encoding of chemical structures also enables a quantitative assessment of analyte similarity, so that the performance of similar and dissimilar chemical compounds can be assessed. Different response output functions were evaluated, including a composite response function that includes evaluation of extraction efficiency, chromatographic efficiency, peak symmetry, and the reproducibility of each. SO is an effective optimization process can be completed in fewer runs than RSM. Current efforts are aimed toward a) multi-analyte optimization and understanding the overlap of response surfaces based on molecular similarity, and b) the comparison of optimized extraction and analysis conditions for different analytes from different sample matrices. This work benefits from the integration of a variety of data science tools with analytical method optimization; we are just scratching the surface of possibilities.

*Keywords*: Pharmaceutical; multivariate optimization; response modeling; data science; molecular encoding

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# Keynote Lectures Abstracts

# Non-destructive forensic document examination of ballpoint cationic inks by blotting-capillary electrophoresis

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We present a nondestructive chemical analysis method for examining documents written with black ballpoint pens. A simple and convenient ink-blotting technique was developed to extract trace amounts of ink from original document surfaces onto a nylon membrane filter (NMF) using isopropyl alcohol, effectively avoiding smudging, blurring, or physical damage. Small circular sections of the blotted NMF were punched, extracted with methanol, and analyzed by nonaqueous capillary electrophoresis (NACE) using a methanol-based run buffer optimized for water-insoluble cationic dyes commonly found in ballpoint inks. To enhance detection sensitivity, large-volume sample stacking with an electroosmotic flow pump (LVSEP) was employed as an online preconcentration technique. LVSEP was performed under normal polarity using a polybrene-coated capillary. A one-time rinsing with water for 6 h following the coating process was essential for achieving stable electroosmotic flow, enabling about 100 runs without requiring polybrene supplementation to the run buffer. The limits of detection were below 1 ng/mL for standard dyes, corresponding to a ~200-fold sensitivity enhancement. The integrated blotting–LVSEP–NACE method enabled high-resolution analysis of fine ink features, including individual strokes and dots as small as ~0.5 mm, and was successfully applied to the forensic examination of real-world documents. This approach offers a robust, nondestructive analytical tool for chemical analysis of original writings, with significant implications for forensic science, cultural heritage preservation, and archival research.

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**Epitachophoresis for purification and concentration of biopolymers from large sample volumes**

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Most bioanalytical applications require sample pre-separation. Non-affinity-based separation methods such as electrophoresis take advantage of differences in electro-migration to separate and concentrate selected analytes from crude samples. Recently, we have introduced epitachophoresis as a new electrophoretic technique for the concentration and separation of nucleic acids from milliliter sample volumes.

This communication reports on new instrumental systems for processing the crude samples by discontinuous electrophoresis in a circular arrangement – epitachophoresis (ETP) with almost unlimited concentration factor. Several experimental parameters have been studied, including the size, shape, and size of the zone stabilizing media and devices for large samples. Polyacrylamide or agarose gels are the most frequently used sieving and stabilizing media in slab gel electrophoresis; however, such sieving materials limit the size of the concentrated nucleic acids. In part of this work, we have also explored large pore materials and 3D printing to form rigid stabilizing manifolds to minimize liquid flow during the epitachophoresis. The device was printed using the stereolithography technique from a low water-absorbing resin. Different geometries of the 3D printed stabilizing manifolds were tested to concentrate ionic sample components in the anionic or cationic mode. Depending on its geometry, the devices can focus analytes from 1 to 50 ml of the sample into the collection cup with a size of 150 µL or less. Depending on the stabilization media and power used, the concentration time ranges from minutes to one hour. We have used the ETP to isolate DNA and RNA from biologically relevant samples in a single run, including formalin-fixed paraffin-embedded tissue. While the system was initially designed for extraction and focusing of nucleic acids, this presentation will also discuss the potential of the ETP for the separation and concentration of other analytes, including peptides and proteins.

This research was co-funded by the European Union under the ATEBIO project (Advanced Techniques for Biomedical Diagnostics, Project ID CZ.02.01.01/00/23_020/0008535

# Portable Chip Electrophoresis Sensing for Biomedical Assay Based Moving Reaction Boundary


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**Abstract:** Recently, the concepts of electrophoresis titration (ET) chips were developed based on moving reaction boundary (MRB), including the ET of protein in milk sample forprotein contentassay,[1-3] the ET of enzyme catalysis for assays of enzymatic dynamics[4,5] and the ET of photocatalysis for melamineassay.[6] Herein, the two ET models of ELISA and uric acid were advanced based on the previous work.[1-6] In the first model,the ET was proposed for constructing ELISA chip of POCT just with a sextuplet electrode pairs and laminated cells. The chip had an anodic well, bridge channel, middle well, titration channel and cathode well in order. ELISA process was conducted in the bottom of middle well, where HRP catalyzed TMB as blue dimer of $TMB^+$. Under an electrical field of 29 V, the TMB dimer migrated into the titration channel and reacted with the ascorbic acid, creating a MRB. The MRB motion was a function of antigen content, indicating a visual ELISA. As a proof of concept, C-reactive protein was chosen as a model antigen. The experiments validated the ET ELISA model and method. Particularly, the chip was detector-free, power supply-free and sulfuric acid-free, making the method extremely simple, portable and safe. The method has potential to visual & portable ELISA in clinic, environmental and food safety immunoassay.

In the second model, uricase catalyzes UA in the anode well to allantoin, producing $H_2O_2$, which oxidizes colorless luceocrystal violet dye without charge as crystal violet (CV+) with one positive charge. Under an electric field, a MRB was created between the violet CV+ moving from the anode well into the channel and the alkaline sodium acetate in channel, resulting in an ET sensing. The model indicated that the distance of MRB under given conditions was as a function of UA content, implying an extraordinary simple sensing for UA. Based on the model, a series of experiments were conducted. The results evidently validated the model and method of ET-MRB. The experiments not only demonstrated the high facility and portability of MRB-ET model, but also showed the visuality, selectivity and rapidity. In addition, the experiments showed the sensitivity (< 0.1 mM), linearity (0.1-4.0 mM, $r^2$ = 0.9948), recovery (85~106%) and stability (RSD 3.8-7.1%). Finally, the developed method was successfully used for the determination of UA in urine and blood samples. All these results manifested the simple, portable and visual sensing of UA, and implied the potential of MRB-ET method to real POCT assay of UA in urine and blood samples.


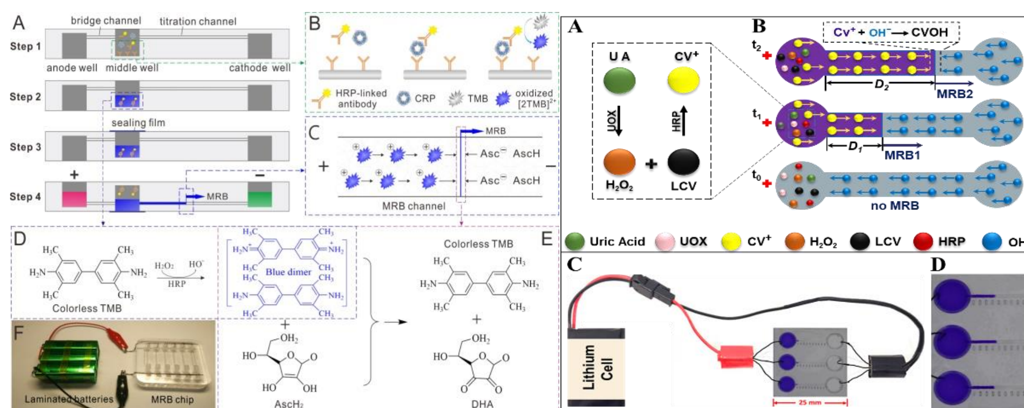


**Figure 1. Left:** ET model for ELISA of POCT. (**A**) ET ELISA assay in ET chip; (**B**) ELISA catalysisreaction in the middle well without use of $H_2SO_4$; (**C**) MRB formation between blue TMB dimer and ascorbic acid; (**D**) TMB substrates catalyzed by HRPenzyme as blue TMB dimer; (**E**) blue TMB dimer reduced by ascorbic acid as colorless TMB; (**F**) photograph of ET ELISA chip. **Right:** ET chip model for UA assay. (**A**)Uricase inducedcatalysis reaction of UA to $H_2O_2$ which further reacts with LCV in the presence of HRP forming $CV^+$; (**B**) MRB created with basic buffer $OH^-$ and alkali $CV^+$; (**C**) Lithium cell providing electric field for ET; (**D**) Photo of a single MRB-ET run via an iPhone.

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# Microfluidic Chip Combined with Mass Spectrometer for Single Cell Analysis

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**Abstract**
The micron-scale space of the microfluidic chips can be well matched to the cell size, which can easily simulate and precisely manipulate the cell growth microenvironment, and is an ideal platform for studying cell-cell interactions, signal transduction and communication, and cellular drug metabolism and delivery. Our research group has carried out research related to the development and application of microfluidic chip and mass spectrometry [1-3], successfully developed the world's first microfluidic chip mass spectrometry analysis device, and industrialized the technology transfer enterprises. This report mainly introduces the development of microfluidic chip combined with mass spectrometry for single cell analysis [4-6].

***Keywords**:* Microfluidics, Mass Spectrometry, Single cell analysis

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## Biography

Dr. Jin-Ming Lin is a professor of Tsinghua University. From 1992 to 2002, He had studied and worked at Showa University and Tokyo Metropolitan University since 1992-2002. In 2004, he was appointed as a professor at the Department of Chemistry, Tsinghua University. In 2008, he was selected as ChangJiang Scholar Professor of the Ministry of Education, China. His main research interesting: 1) Microfluidic chip combined with mass spectrometry for cell analysis; 2) Research on rapid detection methods of foodborne pathogenic bacteria; 3) Research on the preparation method of air negative ions and its mechanism on promoting health. He is currently the deputy director of the Committee of Analytical Chemistry of the Chinese Chemical Society, the president of the Microfluidic System Branch of the Chinese Biophysical Society, the executive director of the Chinese Association of Analysis and Testing, the vice chairman of the Micro-nano Fluidics Technology Branch in Chinese Micro and Nanotechnology Society, the contributing editor of Trends in Analytical Chemistry, Associate Editors of J. Pharm. Anal., Chinese Chemical Letters.

## Microfluidics for Extracellular Vesicles: from diagnosis to therapy

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Cancer is a leading cause of global mortality. Extracellular vesicles (EVs) play a crucial role in the occurrence and progression of cancer. In this presentation, we introduced our recent advancements on microfluidics-based EVs, new tools for EVs isolation, detection, and clinic diagnosis, prognosis, therapy as well. At first, we would discuss some our developed microfluidic-base methods for EVs analysis, and their applications for clinics, particularly with help of artificial intelligence. Then, clinical utilization of EVs as drug carriers for tumor therapy were investigated. We modified EVs by chemical modification and designed microfluidic chips and biomimetic particles for rapid and efficient isolation of EVs for tumor target and drugs delivery, which provides a new idea for the precise treatment of cancers. Finally, future prospective would be discussed.

*Keywords*: Extracellular Vesicles, microfluidics, diagnosis, therapy

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# Lipid-Binding Peptides for Size-Selective Exosome Isolation and Molecular Profiling in Neurodegenerative Disease Liquid Biopsy

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Exosomes, characterized by high membrane curvature, lipid-packing defects, and carrying a diverse array of biomolecules, have garnered significant attention. High-performance isolation of exosomes as a promising liquid biopsy target is of great importance for both fundamental research and clinical applications. However, this is challenged by the prevalent heterogeneity of exosomes and the highly complex nature of biosamples. Here, we introduce a dual-targeting strategy based on the identification of a cationic peptide that can specifically recognize and bind phosphatidylserine (PS) exposed on the outer leaflet of exosomal membranes for the efficient isolation of exosomes from diverse biofluids. Apart from lipid recognition, we further demonstrated that the peptide can sensitively respond to membrane curvature, thus vesicle size in a PS-dependent manner. Leveraging synergistic effect of PS targeting and curvature sensitivity, polymer-based peptide-functionalized affinity separation materials were constructed with tailored molecular interactions toward lipid membrane of exosomes. High yield and purity for targeted fishing of exosomes in complex samples including cell culture medium, urine, and serum were achieved. The rapid and efficient isolation of exosomes from patient biofluids enabled downstream analysis of misfolded proteins as neurodegenerative markers, thereby differentiating healthy individuals, patients with mild cognitive impairment, and those with Alzheimer’s disease. Moreover, integration of peptide-affinity capture with mass spectrometry–based proteomic profiling revealed differentially expressed exosomal proteins, highlighting their potential as theranostic targets for neurodegenerative disorders.

*Keywords*: affinity separation, peptides, lipid membrane, exosome, biofluids

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# Advanced Molecularly Imprinted Polymers for Sample Pretreatment and Disease Diagnosis

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**Abstract**
Molecularly Imprinted Polymers (MIPs), as mimics of antibodies, are fabricated via template-directed polymerization, creating three-dimensional polymeric matrices that form tailored molecular recognition cavities after template removal. These imprints enable specific rebinding to original templates, generating antibody-like affinity and specificity. Particularly, MIPs can provide specificity that antibodies often fail to provide, such as the recognition toward glycans. Moreover, MIPs can be fabricated into nanoscale or incorporated with nanocores of diverse properties and functions, endowing nanoscale MIPs (nanoMIPs) with unique potential in many challenging applications such as separation, sensing, imaging, disease diagnosis, cancer therapy and viral inhibition. In this talk, the speaker will present the recent progresses of his team in the development of advanced MIPs and their applications in sample pretreatment and disease diagnosis.

## New Methods Contributing to Metabolomics Analyses of Single Cells

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Metabolomics analyses of tissues, body fluids and cells are playing an increasingly important role in various fields of life science. For traditional cell metabolomics, millions of cells are generally required. However, in some special cases, such as stem cells, circulating tumor cells, primary cells and so on, the number of cells is very limited, and conventional metabolomics methods run into problems. It is necessary to develop novel methods for a small number of cells, even for single cell analysis. Although single cell is analyzed, to achieve statistically significant results, it is necessary to analyze a large number of cells under physiological conditions. Therefore, high-throughput single-cell analysis methods are required.

Over the past years, we have concentrated on the development of mass spectrometry (MS)-based single cells analysis techniques including capillary microsampling combined with high-resolution spectral stitching nanoelectrospray ionization direct-infusion MS, laser capture microdissection (LCM)-sample micromanipulation-MS, inertial microfluidics and pulsed electric field-induced ESI-MS, and concentric nanoESI-APCI hybrid source.

In this lecture our recent advancements on the single cells-related analysis methods for metabolomics and heterogeneity studies will be reported. The related work can be used not only for tens of cells or single cell metabolomics, but also for spatially resolved metabolomics study coupled with LCM.

*Keywords*: Single cell, Metabolomics, Mass spectrometry

# Dynamic single-cell metabolomics platform and its application in cell-cell interaction

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Cellular heterogeneity plays an important role in many key biological processes such as tumor, aging, immunity and development, etc. From the perspective of metabolites, single-cell metabolomics helps to reveal the precise life activities and physiological states of individual cells, and achieve the analysis of cell heterogeneity and interactions in complex microenvironments of tissues [1]. Based on the single cell work in our group, for example, single cell organic mass spectrometry [2] and in-depth organic mass cytometry [3], a dynamic single-cell metabolomics platform and an automated single-cell dynamic metabolomic data analysis platform were constructed. A total of 40 isotopic labeled metabolites were traced in single cells, disclosing the heterogeneity of metabolic activity among single cells. A linear neural network machine learning model based on metabolic features was successfully established for binary classification of tumor cells and macrophages. The analysis results not only disclosed the metabolic alterations of the two interactions but also unveiled the heterogeneity of macrophage differentiation in the tumor microenvironment [4].

*Keywords*: Single cell, Metabolomics, Mass cytometry, cell-cell interaction

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## Preparation of cation-exchange stationary phases for rare earth ion separation

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### Abstract

Rare earth elements have unique optical, magnetic and electrochemical properties, and have been widely used. However, it is complicated to be separated and to get a single high-purity rare earth element [1]. Ion-exchange chromatography is a useful technology that can be used for rare earth separation.

In this presentation, several cation-exchange chromatographic stationary phases were designed and synthesized, and a variety of rare earth elements were completely baseline separated [2-4]. Further experiments show that these new kinds of facile chromatographic stationary phases have good repeatability and stability, and is expected to be used for separation and analysis of real rare earth samples.

*Keywords*: HPLC; Stationary phase; Chromatographic separation; Rare earth ions.

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### Biography

Hongdeng Qiu received his PhD from Lanzhou Institute of Chemical Physics (2008). He did his postdoctoral research in Kumamoto University from 2009-2012 (JSPS Fellow). He worked in Lanzhou Institute of Chemical Physics in 2012-2024. Currently he is a professor in Ganjiang Innovation Academy, Chinese Academy of Sciences. His research interests are the applications of new materials in chromatography and sample preparation, especially for the separation and analysis of rare earth ions. He has published more than 260 papers and 50 patents. He is one of the chief-editor of Chinese Chemical Letters, and he is the Editorial Board Member of Chromatographia, Separation Science Plus, Chinese Journal of Chromatography and Progress in Chemistry.

# Multi-Dimensional Characterization of Environmental Nanoparticles by Mass Spectrometry Techniques

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Characterization at nanoscale plays a crucial role in in-depth understanding the nature and processes of environmental nanoparticles (NPs). However, currently available characterization techniques suffer from many limitations, like lack of accurate molecular information, inability to real-time monitor intermediates, or vulnerability to sample matrix interference. To probe NPs in the environment, we developed a series of mass spectrometry-based techniques to analyze and characterize NPs. Specifically, we developed a chemical multi-fingerprinting platform (integrating elemental fingerprinting, high-resolution structural fingerprinting, and natural isotopic fingerprinting) for particle characterization and source tracing in environmental and biological samples. We found that exogenous NPs are widely present in the blood samples with extreme diversity in chemical species, concentration, and morphology. Furthermore, we have also developed a detection platform for airborne magnetic NPs and soot particles in complex media based on different MS techniques. These methods will greatly rich the toolbox of nanotoxicological research and nanomaterial risk assessment.

*Keywords*: mass spectrometry, nanoparticle, environmental analysis, stable isotope, source tracing

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# Taylor Dispersion Analysis for Size Characterization of Charged Polymers and Silica Nanoparticles

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Abstract

Taylor dispersion analysis (TDA) is an absolute method that allows the determination of the hydrodynamic radius (RH) and molecular diffusion coefficient (D) of a solute by analyzing peak broadening in a laminar Poiseuille flow. TDA is normally used for size analysis of UV-absorbing or derivatized compounds using a commercial capillary electrophoresis (CE)-UV instrumentation. In this work, we investigate the capability of TDA for non-UV absorbing charged polymers and nanoparticles. A capacitively coupled contactless conductivity detector (C4D) was employed as a detector inline equipped with CE-UV instrumentation. Another application is to size the silica nanoparticles using the concept of light scattering using the equipped UV detector in CE instrumentation. Detection sensitivity is carried out using a continuous flow as 'frontal mode' and the sizing is achieved by using a hydrodynamic injection as in the 'plug injection mode' under the suitable eluent and TDA regime. Comparison of sizes obtained from the proposed alternative detection to the nominal size will be discussed.

*Keywords*: Taylor dispersion analysis; taylorgrams; light scattering; silica nanoparticle

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**Chemical proteomic exploration of intercellular signaling**

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Abstract:
Cell-cell interactions in tumor microenvironment are often mediated by ligand proteins secreted by one cell that are recognized by specific receptors presenting on neighboring cells. This intercellular communication activates signaling pathways through the induction of dynamic posttranslational modifications such as phosphorylation and protein-protein interactions. Mass spectrometry-based proteomics have been proven to be a robust approach for characterizing these signaling events on a global scale. However, proteomic studies of intercellular signaling directly from living systems have been challenging. In this talk, I will present our recent development of a series of chemical proteomic approaches toward this end, especially for new proximity labeling approaches. Beside our recent discovery of LIF as the new drug target and potential biomarker for pancreatic cancer, our work explored many new secreted ligand-membrane receptor pairs and provided valuable resources for systematically understanding dysfunctional signaling networks in pancreatic tumor microenvironment in a context closer to physiological condition than was previously possible.

## Superior Selectivity of Copper Single-Atom Nanozyme Mimicking Galactose Oxidase

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Since some natural enzymes are expensive and unstable, nanozymes—nanomaterials that mimic enzymes—have been created. Notably metal-centered nanozymes known as single-atom nanozymes resemble the structure of naturally occurring enzymes based on metals. In order to replicate the activity of natural galactose oxidase, Cu-N-C single-atom nanozyme (SAN) is created here with superior peroxidase- and increased oxidase-like capabilities. By oxidizing D-galactose and primary alcohol but not L-galactose or other carbohydrates, Cu-SAN exhibits stereospecific activity similar to that of natural galactose oxidase. When galactose is exposed to oxygen, the SAN can catalyze its oxidation, resulting in the production of hydrogen peroxide as a byproduct. After being catalyzed by the SAN, the generated hydrogen peroxide oxidizes 3,3′,5,5′-tetramethylbenzidine to form the characteristic blue product. With a detection limit as low as 0.23 μM, the absorbance and galactose concentration have a linear relationship in the 1–60 μM range. This method can be used to identify galactose in some dairy products and other commercial products, as well as to diagnose galactosemia condition.

# From Bottles to Bodies: High-Resolution Microplastics Detection Using Laser Direct Infrared in Malaysia

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Abstract

Microplastics (MPs) have become a growing environmental and health concern due to their persistence, ubiquity, and potential biological effects. Leveraging the state-of-the-art Laser Direct Infrared (LDIR) chemical imaging spectroscopy, this study provides the first integrated assessment of MPs contamination in Malaysia across multiple exposure pathways [1]. Analysis of bottled water revealed the widespread presence of MPs, predominantly smaller than 50 µm, with a diverse range of polymer types detected, including natural polyamide, cellulose, chitin, rubber, polyamide (PA), and polyethylene terephthalate (PET) (Fig. 1). Almost all of the EU's priority MPs were identified [2], with PA, PET, polyethylene (PE), polypropylene (PP), and polyurethane (PU) being among the most common. Extending the scope, carbonated and isotonic beverages packaged in PET bottles, aluminium cans, and laminated cartons were examined, showing marked differences in MPs abundance and composition depending on the packaging material, highlighting packaging as a key factor influencing contamination. Finally, MPs in human fecal samples were analysed from healthy individuals and patients with colorectal disease, revealing distinct differences in polymer types, size distributions, and patterns between the groups, suggesting possible links between MPs exposure and gastrointestinal health. These findings demonstrate the versatility and precision of LDIR in detecting and characterising MPs across diverse matrices, underscore the impact of beverage packaging on contamination levels, and point to the urgent need for further research into the health implications of chronic MPs exposure.

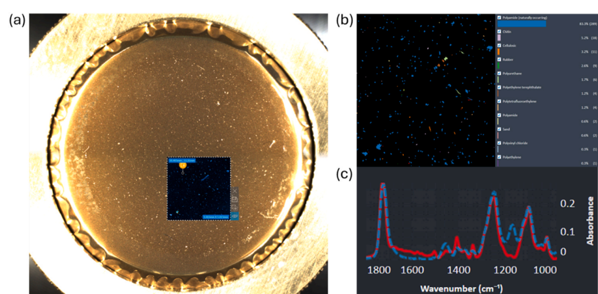


**Fig. 1.** (a) Gold-coated membrane with particles from a bottled water sample (F-U), showing a 5 mm × 5 mm scanned area (black square) on the 15 mm membrane, (b) IR image of detected particles coloured by polymer type, and (c) LDIR spectra of identified MPs (blue: reference; red: measured).

*Keywords*: Microplastics, Laser Direct Infrared, drinking water, colorectal disease

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# Multi-organoid Microphysiological Systems and Their Applications in Pharmacology and Toxicology Research

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Abstract body (up to 350 words)

Multi-organ-on-a-chip technology demonstrates significant potential in disease modeling, drug screening, and precision medicine due to its ability to simulate multi-organ interactions. Theoretically, simulated blood flow within microfluidic chips should ensure adequate interactions among organoids. However, in practical organ-on-a-chip applications, inter-organoid interactions are not always observable. Furthermore, variations in interaction intensity between identical organoid combinations on chips are often attributed to functional differences in organoids, though whether this is the sole cause remains unclear.

To address this issue, the presenter's team developed a microphysiological system incorporating nine interconnected organoids. Through a series of experiments, they comprehensively investigated how microfluidic chip design—including spatial arrangement, chip parameters, functional detection, and communication media—affects organoid interactions and their underlying patterns.

our study revealed that the spatial sequencing of organoids on the chip significantly alters their functionality and interaction patterns. Specifically, functional changes in target organoids are primarily influenced by the type of their upstream adjacent organoids, with this effect being independent of physical distance. Other chip parameters — such as flow rate, organoid chamber design, and simulated blood volume — non-linearly impact organoid interactions. Additionally, intact vascular barrier structures substantially influence inter-organ communication.

These findings establish new research directions for precisely simulating dynamic interactions at the human organ level. They will enhance the capability of multi-organ-on-a-chip systems to fulfill their potential in medical research and novel drug discovery.nd.

*Keywords*: organ-on-a-chip, multi-organ-on-chip, organ-interaction

# Development of isotope dilution-liquid chromatography/tandem mass spectrometry (ID-LC/MS/MS) for the accurate determination of aflatoxins and vitamin $B_{12}$ in food

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Certified reference materials (CRM) are crucial for disseminating national measurement standards in chemical metrology, ensuring the reliability of testing laboratories. As the National Metrology Institute of Korea, KRISS has been developing CRMs for food analysis. As a part of this effort, we need to develop higher-order reference methods using isotope dilution-liquid chromatography/tandem mass spectrometry (ID-LC/MS). In this talk, I will present two recent projects focused on analytical method development. Aflatoxins are highly toxic mycotoxins produced as secondary metabolites by various fungi. They frequently occur in a wide variety of foodstuffs, including cereals, coffee, and nuts. We have developed an accurate ID-LC/MS/MS method for simultaneous analysis of four aflatoxins in grains and coffee samples [1,2]. We optimized the sample preparation procedures for each food matrix and evaluated the matrix effects to eliminate the measurement bias. Next, I will discuss the analysis of Vitamin $B_{12}$ analysis in infant formula. Vitamin $B_{12}$ is an essential nutrient used for formation of red blood cells and DNA synthesis and cyanocobalamin has been employed for fortification of vitamin $B_{12}$. Due to the hygroscopic property of cyanocobalamin, we used quantitative NMR to determine the concentrations of standard solutions. The sample preparation procedure, including cyanidation and extraction conditions, was optimized. The developed methods were validated by evaluating their performance and subsequently applied to samples obtained from local markets.

*Keywords*: Isotope dilution-mass spectrometry, aflatoxins, vitamin $B_{12}$, validation

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# Portable Sensors Utilizing Small Sample Volumes for Forensic and Security Applications

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This presentation highlights recent research on the development of portable sensors for on-site chemical analysis using small sample volumes. Emphasis is placed on low-cost, disposable platforms fabricated from accessible materials such as paper, pencil leads, and gold leaf, enabling both electrochemical and colorimetric detection. The target applications focus on forensic and security-related scenarios, particularly in field-based or resource-limited settings. Demonstrated devices include: (i) a microfluidic paper-based analytical device (µPAD) [1,2] and a gold leaf-based electrochemical sensor [3] for gunshot residue (GSR) analysis, including the estimation of shooting distance, and (ii) a pencil-based voltammetric sensor for the detection of 2,4,6-trinitrotoluene (TNT), integrated with drone-assisted sampling [4]. These examples illustrate practical strategies for deploying portable, low-volume chemical sensing platforms to support rapid forensic and security screening beyond conventional laboratory environments.

***Keywords***: Paper-based, electrochemical sensor, gunshot residue, pencil, drone, explosives

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# Portable Capillary Electrophoresis Instrument for On-Site Forensic Analysis

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The field portable instrument was designed in our laboratory for ease of construction and can be assembled with limited mechanical and electronic manufacturing required. It is contained in a robust Peli protector case with the approximate dimensions of 40 x 33 x 17 cm and a weight of 8 kg. Polarity switching allows the determination of either cations or anions by capillary zone electrophoresis. For ease of use and high reproducibility, the injection of the sample was automated. The electropherograms are obtained with a universal contactless conductivity detector. This is of a differential design, which features automatic background correction to facilitate its use and suppression of drifts. Control of the instrument and data acquisition is achieved by attaching a notebook computer. The use of the instrument was successfully demonstrated for two forensic applications: the determination of the date rape drug gamma-hydroxybutyrate (GHB) in beverages and in urine, and for the estimation of the post-mortem interval (PMI) by determining the potassium concentration in the vitreous humour.

*Keywords*: capillary zone electrophoresis, contactless conductivity detection, portable instrument, forensic analysis

## Ion-pairing in peptide RPLC separations: unexpected features and their consequences

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Reversed-phase HPLC is the most popular method for peptide separations applied in various formats from preparative scale down to nano-flow applications in proteomics. While the dominant role of ion-pairing RPLC separation mechanism is widely acknowledged, its consequences for peptides separation selectivity are still poorly understood except for apparent differences in hydrophobicity of different ion-pairing additives (e.g. formic acid vs. trifluoroacetic acid).

Proteomic LC-MS technology provided separation scientists with virtually unlimited datasets to explore these selectivity features. First, we established a significant effect of ion-pairing environment on hydrophobicity of the residues adjacent to positively charged peptides' functional groups. This led to the development of the first position-dependent peptide retention time prediction models such as Sequence-Specific Retention Calculator [1]. Recently we explored peptide retentivity and selectivity features for the sorbents of different pore sizes and found a significant reduction of available surface area due to ion-pairing induced size exclusion for the sorbents in 60-200 Å pore size range [2]. Apparently, the size of hydrated counterions (acetate, formate, trifluoroacetate) is the major driver behind selectivity changes observed between RPLC packings of different pore sizes. It also determines variation in retention time shifts following peptides' chemical or post-translational modifications. This presentation reviews the ion-pairing driven selectivity changes in peptide RP HPLC, our 20-years progress in better understanding of the separation mechanism and outline future developments in designing optimal stationary phases for peptide separation.

*Keywords:* peptide RP HPLC, peptide retention time prediction, ion-pairing mechanism

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**Noncovalent Fluorophore Labeling of Biotherapeutics in Sodium Dodecyl Sulfate Capillary Gel Electrophoresis**

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Sodium dodecyl sulfate capillary gel electrophoresis is one of the frequently used methods for size-based protein separation in the biopharmaceutical industry. To increase throughput quite a few multicapillary electrophoresis systems have been recently developed but most of them only support fluorescent detection, requiring fluorophore labeling of the sample proteins. To avoid the time-consuming derivatization reaction, we developed an on-column labeling approach utilizing propidium iodide in SDS-CGE of proteins, a dye only used before for nucleic acid analysis. As a key ingredient of the gel-buffer system, the oppositely migrating positively charged propidium ligand *in migratio* complexes with the SDS-proteins, i.e., supports *in situ* labeling during the electrophoretic separation process, not requiring any extra pre- or post-column derivatization step. A theoretical treatment is given to shed light on the basic principles of this novel online labeling process. Considering the increasing number of protein therapeutics on the market next, we focused on the labeling optimization of a therapeutic monoclonal antibody and its subunits, including the addition of the non-glycosylated heavy chain. Peak efficiency and resolution were compared between non-covalent and covalent labeling. The effect of ligand concentration on the effective and apparent electrophoretic mobility, the resulting peak area, and resolution were all evaluated in view of the theoretical considerations. In addition, the effects of the three most important user-adjustable operational parameters (temperature, gel concentration, and electric field strength) were also investigated on the electrophoretic mobility and resolution of SDS-protein complexes in the presence of propidium iodide in the gel-buffer system. Our results underline the importance of optimizing these key parameters in SDS-CGE with propidium-mediated LIF detection to obtain rapid and high-resolution separation of complex protein samples such as biopharmaceuticals.

# Novel Approaches in Surface-Enhanced Raman Spectrometry for Analysis in Complex Matrices

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Surface-enhanced Raman spectrometry (SERS) is a powerful analytical technique that overcomes the low sensitivity of Raman scattering by amplifying the signal of analytes near metal nanostructures. This phenomenon opens new possibilities for applications, especially for tracing low-abundance compounds.

We developed a nanospray-assisted deposition method for the precise and homogeneous application of silver nanoparticles onto hydrophilic nanofibrous layers. This controlled deposition process enabled the detection of a biologically important peptide otherwise undetectable by conventional Raman spectrometry.[1] However, a significant challenge in analyzing real samples is matrix interference, which complicates SERS spectra interpretation.

To address this, we initiated the development of a hyphenated system coupling SERS with capillary electrophoresis (CE).[2] Our early-stage work involves designing etched-glass microfluidic chips that allow introducing colloidal silver nanoparticles downstream from the separation capillary. This configuration is intended to prevent nanoparticle interference with the separation process while enabling real-time SERS detection. To further enhance SERS performance and stability, we are also developing advanced nanomaterials to maintain colloidal stability and SERS sensitivity in high-ionic-strength solutions.[3] Furthermore, we are exploring composite nanostructures for the targeted analysis of specific compound classes.[4]

*Keywords*: Raman spectrometry, nanostructures, separation, matrix, deposition

Acknowledgement

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# Developing Ambient Ionization Mass Spectrometry Strategies and Their Applications in Comprehensive Natural Medicine Analysis

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Ambient ionization mass spectrometry (AIMS) enables direct, open-air ionization with virtually no sample preparation, offering a rapid and versatile analytical window for complex matrices. Leveraging representative modalities such as direct analysis in real time (DART), desorption electrospray ionization (DESI) and liquid-extraction surface analysis (LESA), our group has established a suite of workflows targeting the characteristic active or toxic constituents of traditional Chinese medicine (TCM). DART-MS supports straightforward screening of hepatotoxic pyrrolizidine alkaloids in herbal decoctions, finished preparations and related food products; Laser-assisted thin-layer chromatography (LA-TLC) coupled to DART and TLC spray-ionization platforms extends coverage to alkaloids, flavonoids, anthraquinones, volatile oils and organic acids and enables in-situ distribution mapping of triterpenic acids in *Poria cocos*. LESA-MS provides non-destructive surface fingerprints for high-value materials such as *Panax notoginseng* and agarwood, while a chip-based nano-ESI interface facilitates high-throughput authentication of bear-bile preparations and screening of natural enzyme inhibitors. To translate these laboratory capabilities to point-of-need contexts, we have integrated paper capillary spray with a handheld mini-MS. This portable platform delivers rapid chemical fingerprints that distinguish and grade agarwood, fragrant rosewood and aged Citrus peels, and simultaneously monitors pyrrolizidine alkaloids, thereby supporting authenticity verification, safety assessment and quality benchmarking outside the laboratory. Together, these complementary AIMS strategies create a coherent laboratory-to-field continuum that aligns with green-chemistry principles and modern regulatory expectations, positioning AIMS as a practical, sustainable solution for comprehensive quality evaluation and mechanistic exploration of natural medicines.

*Keywords*: Ambient Ionization, mass Spectrometry, natural Medicine, traditional Chinese medicine, quality evaluation

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# Isomer-Specific Separation and Glycomic Insights into Spatiotemporal Dynamics of the Mammalian Brain

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Liquid chromatography–mass spectrometry (LC–MS) provides powerful capabilities for resolving glycan and glycolipid heterogeneity, yet isomer separation has remained a major challenge in brain glycosylation research. Here, we present separation-based workflows enabling isomer-specific characterization to uncover spatial and temporal dynamics of the mammalian brain glycome. Using ultra-high-performance LC coupled with tandem MS, we achieved baseline separation of intact ganglioside isomers, including GD1a and GD1b, and profiled their region-specific distribution across nine mouse brain areas. This isomer-specific approach revealed conserved core structures, differential abundance of a- and b-series gangliosides, and distinct O-acetylation features, emphasizing the functional relevance of isomer separation in molecular neuroscience. Complementary glycomic profiling was performed using porous graphitized carbon nano-LC/MS for N-glycans in mouse and human brains. Separation-based analysis demonstrated that while a conserved glycan core is maintained, extensive developmental remodeling occurs, particularly in the prefrontal cortex. Sialylated and fucosylated glycans emerged as age-dependent markers, and a synthetic glycosylation map delineated pathways underlying maturation processes. Together, these studies establish chromatographic isomer separation as a critical foundation for decoding brain glycosylation. By integrating isomer-specific separation with LC–MS, we provide a systems-level view of glycan and glycolipid organization that captures both spatial heterogeneity and developmental plasticity. These findings underscore the central role of separation science in advancing glycomics and highlight its potential for identifying therapeutic targets in neurodevelopmental and neurodegenerative disorders.

*Keywords*: Isomer separation, LC–MS, glycome, glycolipids, brain

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# Advancing Food Safety from Bench to Field: Portable Electrochemical Devices with In-Situ Sample Pretreatment for Sulfite Detection

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Ensuring food safety remains a critical global challenge, particularly in products prone to chemical preservative abuse, such as frozen shrimp. Although sulfite preservatives are effective in preventing spoilage, their use must be strictly controlled due to potential health hazards. We will present the translational journey from laboratory-based chemical analysis to the development of a compact, user-friendly bench-top instrument that incorporates a unique electrochemical sensor for quantifying sulfite residue in frozen shrimp and other types of seafood. The electrochemical sensor operates in the gas phase above the closed vessel containing the sample. The sensor measures the sulfur dioxide gas generated from the sulfite in an aqueous suspension within a sealed extraction chamber. This modular device integrates an extraction unit with a reusable electrochemical sensor mounted in the lid, capable of providing consistent readings for at least 60 consecutive measurements. The system is electronically controlled and integrated with a software platform that enables fine-tuning of electrochemical parameters, signal monitoring, and real-time reporting sulfite concentration on a graphical display. Our instrument not only delivers a useful quality control tool for the frozen food industry but also serves as a product for a local start-up entrepreneur to explore the commercialization of this sensor innovation.

*Keywords*: sulfite, electrochemistry, sensor, gas phase detection, innovation, food safety

# Oral Lectures Abstracts

# Programmable Flow Injection for online solid phase extraction and more

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Programmable Flow Injection (pFI) was developed from the older FIA and SIA concepts [1]. It is based on a precise computer-controlled combination of flow reversals, flow accelerations, and stop flow periods of the µL zones of liquids mainly for volume metering, the sample collection, injection, confluence or sequence zone mixing for sample dilution, reaction or interaction with a solid phase, followed by detection of a result of the underwent processes in a flow cell or further transport to the next step of the analysis. pFI extends the capabilities of both a nonequilibrium flow-based and an equilibrium batch-flow based tasks that cannot be accomplished in a legacy continuous flow format, while maintaining the highly flexible software control of a clearly arranged manifold, high precision online detection, easy data evaluation, and automated feedback based on results.

The presentation provides examples and innovations in wet chemistry-based analysis of nutrients in seawater, automated sample preparation based on solid phase extraction, and hyphenation with separation techniques such as HPLC or sequential injection chromatography. News about the pFI concept is continuously published in the online tutorial "Programmable Flow Injection Analysis" [2] and relevant literature [3].

This work was supported by Advanced Techniques for Biomedical Diagnostics (ATEBIO), Project ID CZ.02.01.01/00/23_020/0008535 – co-funded by the European Union.

*Keywords*: programmable Flow Injection, tutorial, automation, online solid phase extraction, sequential injection analysis

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# Nano-liter Sample Pretreatment of Glycans for Capillary Electrophoresis Analysis

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Capillary electrophoresis (CE) is a powerful analytical technique that utilizes micro/nano-meter-scale inner diameter separation channels, facilitating efficient heat dissipation and minimizing analyte diffusion, thus significantly enhancing separation performance. However, the narrow channels also limit the sampling volume, posing a challenge to detection sensitivity. Typically, samples are handled in micro-liter scales, whereas CE sample injection volumes are only in the nano-liter range, resulting in substantial sample loss, often exceeding three orders of magnitude. To address this issue, large-volume sample stacking methods have been developed, which can increase the injected volume to a few micro-liters but still leave a significant portion of the sample unused [1].

This study introduces a novel approach by challenging sample pretreatment workflow using a nano-liter scale container. An open capillary tube, with dimensions of 5 mm length, 100 µm inner diameter, and a volume of 39 nL, was employed as a reaction container. The workflow for 8-aminopyrene-1,3,6-trisulfonic acid (APTS) derivatization of maltoligosaccharide standards was successfully realized within the capillary tube [2]. The molecular derivatization solution consisted of 100 mM glycan standards, 100 mM APTS, 7.5% acetic acid, 500 mM $NaBNCH_3$, and 50% tetrahydrofuran, and the derivatization process was conducted at 37°C overnight. The nano-liter reaction products were analyzed using CE coupled with laser-induced fluorescent detection (Fig. 1). The intensities of each oligosaccharide were found to be comparable with those from bulk-scale reaction. Future studies will focus on applying this nano-liter reactor to natural-source glycan sample pretreatment, aiming for whole sample injection. The proposed approach is expected to find applications in trace sample analysis, such as single-cell, tissue micro-section, minimally invasive clinical sample, and so on.

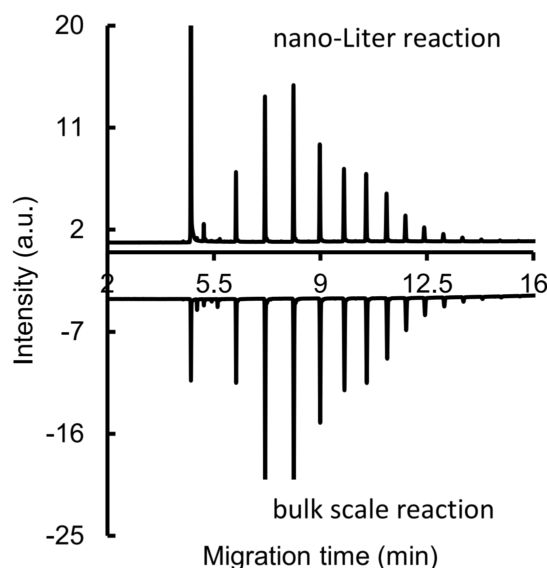


Figure 1. Electropherograms of malto-ligosaccharide standards in (a) nano-liter reactor and (b) bulk scale tube.

*Keywords*: capillary electrophoresis, glycans, sample preparation, nano-liter sampling.

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# ELECTRODRIVEN ION FOCUSING AND STACKING ON A POLYMER INCLUSION MEMBRANE

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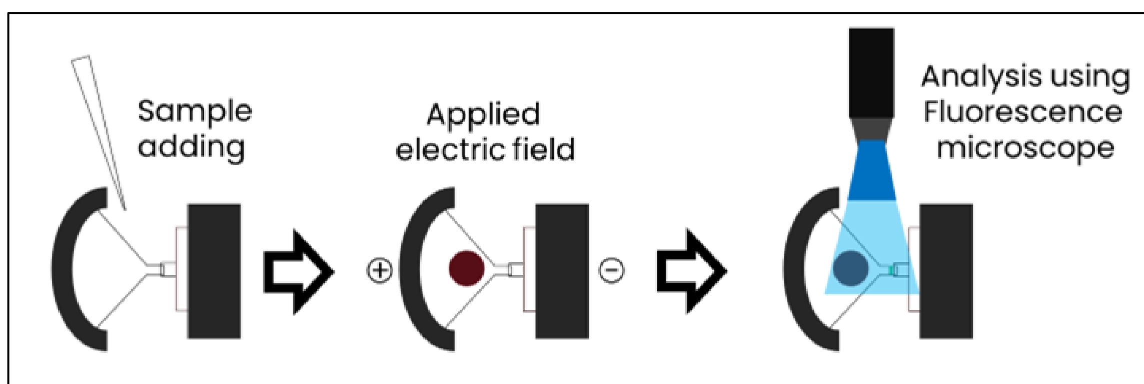
The detection of analytes from dried blood spots (DBS) is often hindered by low analyte concentrations and limited sample volumes, leading to weak signal responses. To address this challenge, a novel electrokinetic method has been developed to enable the focusing of cationic analytes at the interface of cationic and anionic polymer inclusion membranes (PIMs), as well as their separation across membranes with differing conductivities under an applied electric field. Two distinct PIMs were fabricated using cellulose triacetate (CTA) as the polymer matrix, 2-nitrophenyl octyl ether (2-NPOE) as plasticizer, and Di-(2-ethylhexyl) phosphoric acid (D2EHPA) and Aliquat 336 serving as functional carriers. The effects of CTA, carrier type, and plasticizer composition on ion mobility and stacking efficiency were systematically examined. Under optimized conditions (250 V/cm for 30 minutes), efficient focusing of Sanguinarine was achieved using PIM A, composed of CTA (100 mg), 2-NPOE (250 mg), and D2EHPA (40 mg), and PIM B, composed of CTA (100 mg) and Aliquat 336 (150 mg). A 20 µL aliquot of 1.0 ppm Sanguinarine was successfully stacked at the PIM interface in a fan-shaped configuration and visualized using fluorescence microscopy. Further enhancement of ion focusing and analyte separation was achieved by integrating Field-Amplified Sample Stacking (FASS) into the PIM platform. This enabled the electrophoretic separation of both cationic dyes and bioactive Sanguinarine. The results highlight the potential of this electrokinetic PIM-based method for miniaturized sample preparation and sensitive analysis, particularly for applications involving low-volume biological matrices such as DBS.

*Keywords*: Ion Focusing, PIM, Electrophoresis, FASS

Graphic:



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# Aptamer selection based on microscale electrophoretic filtration

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Abstract

DNA/RNA aptamers are versatile recognition molecules with high specificity for their targets, making them attractive for applications in biosensing, diagnostics, and pharmaceuticals. However, conventional selection methods suffer from limitations such as complicated procedures, low separation efficiency, and the need for expensive equipment, which hinder their practical use. To address these issues, we developed a new selection method using a microscale electrophoretic filtration device [1].

In this method, a capillary device partially filled with hydrogel enables a sequence of aptamer selection steps: electrophoretic filtration of target proteins via molecular sieving, electrokinetic injection of a random DNA library to interact with the trapped proteins, washing to remove weakly or non-binding DNAs, and elution of strongly binding aptamer candidates.

To demonstrate the concept, immunoglobulin E (IgE) was chosen as a model target. Following the electrokinetic injection of fluorescently labeled IgE, strong fluorescence was observed near the upstream hydrogel interface, confirming successful filtration. When unlabeled IgE was filtered, subsequent introduction of a fluorescently labeled random DNA library produced fluorescence from DNAs bound to IgE. During the washing step, the fluorescence gradually decreased but remained detectable, and it disappeared after the elution step, indicating successful recovery of strongly binding DNAs. The recovered DNAs were then amplified by PCR to generate the next-generation library.

After three selection cycles, two aptamer candidates for IgE were obtained. Affinity capillary electrophoresis revealed strong binding, with dissociation constants ($K_D$) in the nanomolar range. These aptamers showed no binding to non-target molecules such as bovine serum albumin or immunoglobulin G. This method enables aptamer selection through simple operations, high-efficiency separation via electrophoresis and filtration, and low-cost instrumentation using disposable devices, offering strong potential for practical applications.

*Keywords*: Aptamer, aptamer selection, microscale electrophoretic filtering

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## Polyacrylamide-modified Monolithic Silica Capillary Columns for the Separation of Polar Analytes

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Monolithic silica capillary column has been used for fine separation of challenging targets, such as peptides [1], isomers, and isotopologues in revered-phase mode [2]. It can be modified by polar functional group to be hydrophilic interaction chromatography (HILIC) column, too [3]. For better retentivity and selectivity, functionalization of the silica monolith by on-column polymerization by acrylamide [4], acrylic acid [5] and so on. To impart higher retentivity and better selectivity to the monolithic silica capillary column, higher concentration of monomers in the feed composition, however, such a polymerization conditions often resulted in column clogging. Capillary columns of 100 to 200 micron I.D. contain too small amount of silica to take samples of the polymer on the silica for molecular weight determination. For further improvement of the functionalization of monolithic silica capillary column, the nature of polyacrylamide bound onto silica surface was examined using polymer samples taken from silica particles modified by the same polymer, and the same polymerization conditions. Both of a monolithic silica capillary column and a particle-packed column modified by polyacrylamide showed similar separation characteristics, i.e., selectivity for OH group and $CH_2$ group, selectivity between vidarabine and adenosine, and theobromine and theophylline [6], were comparable that showed similar polymerization and similar binding of them to silica have undergone. The retention of the capillary column was one third of that by the particle-packed column, due to the lower silica content per one column. Molecular weight determination by size exclusion chromatographic analysis of the polymer on silica, and in solution resulted in 70,000 to 140,000 ($M_W$ on silica), and 570,000 to 740,000 ($M_W$ in solution), and the high molecular weight polymer in the solution part can be a reason of column clogging. High molecular weight, above 120,000 ($M_W$) was shown to be essential to impart good retentivity and selectivity for structural differences.

*Keywords*: HPLC, monolithic silica columns, polymerization, polyacrylamide

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# Gold nanostructures as a tool for biothiols preconcentration from non-invasive samples

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Endogenous low-molecular-mass biothiols, such as homocysteine, cysteine and glutathione, are a promising group of potential biomarkers for oxidative stress and various pathological states [1]. Their quantification in non-invasive biological samples presents a significant analytical challenge, especially due to their extremely low concentrations, demanding highly sensitive analytical methods for their detection. In this work, we present a procedure that utilizes gold nanostructures in various arrangements for biothiol preconcentration.

We developed and compared three distinct approaches based on gold nanostructures for the selective capture and enrichment of biothiols. These include: (i) colloidal gold nanoparticles (AuNPs), (ii) composite gold magnetic nanoparticles (Au-MNPs) enabling simple magnetic separation, and (iii) AuNPs grafted on stationary phase particles for use in a micro-column solid-phase extraction (SPE) format.

The developed procedures were applied to preconcentrate endogenous biothiols directly from EBC samples. Following the selective capture, the adsorbed biothiols were efficiently desorbed by dithiothreitol (DTT), achieving significant enrichment (preconcentration factors ranging from 8 to 34). For subsequent analysis, capillary electrophoresis with laser-induced fluorescence detection (CE-LIF) was employed. Prior to analysis, the thiol moiety was capped with N-ethylmaleimide (NEM), followed by a fluorescent derivatization via the amine group with naphthalene-2,3-dicarboxaldehyde (NDA) and potassium cyanide.

Gold nanoparticle-aided preconcentration procedure enhanced the overall sensitivity of the method, enabling the detection of biothiols at the nanomolar range (0.24-0.71 nM) [2]. This approach is a powerful tool for analysis of biothiols in non-invasive samples, especially with automation of the procedure by either magnetic manipulation or a flow-based sample treatment using grafted columns.

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*Keywords*: gold nanostructures, biothiols, capillary electrophoresis, exhaled breath condensate

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# Bone Marrow Derived Mesenchymal Stem Cell Purification Using Thermoresponsive-Cationic Copolymer Brush Modified Beads Packed Column

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Cell therapy using mesenchymal stem cells (MSCs) has received attention as an effective regenerative therapy for treating intractable diseases [1]. To increase the therapeutic efficacy of cell therapy, an effective cell separation method is required that does not modify cells and maintains cell activity. In the present study, we developed a temperature-modulated mesenchymal stem cell separation column using thermoresponsive and cationic block copolymer brush-modified silica beads as packing materials (Fig.1). Temperature-responsive cationic block copolymer, poly(*N,N*-dimethylaminopropyl acrylamide)-*b*-poly(*N*-isopropylacrylamide) (PDMAPAAm-*b*-PNIPAAm) brush were grafted on silica beads through two-steps of atom transfer radical polymerization (ATRP). The prepared copolymer brush-modified silica beads were characterized by CHN elemental analysis and FTIR. Using the prepared copolymer brush-modified silica beads-packed column, the elution behavior of MSCs and BM-CD34+ cells was observed at 37°C and 4°C. CHN elemental analysis and FTIR indicated that thermoresponsive cationic copolymer was successfully modified on silica beads through the ATRPs. The prepared silica beads were packed into a column, and the elution behavior of the cells from the column was observed. At 37° C, MSCs were adsorbed on the column through both hydrophobic and electrostatic interactions with the PNIPAAm and PDMAPAAm segments of the copolymer brush, respectively. By reducing the temperature to 4°C, adsorbed MSCs were eluted from the column by reducing hydrophobic and electrostatic interactions attributed to the hydration and extension of the PNIPAAm segment of the block copolymer brush. The suitable DMAPAAm composition in the block copolymer brush was determined from the temperature-modulated adsorption and elution behavior of MSCs. Using the column, a mixture of MSC and BM-CD34+ cells was separated by simply changing the column temperature while maintaining the cellular activity of MSCs. The separated cells exhibited viability and differentiation potency. The results indicated that the prepared bead-packed column can separate MSCs from contaminant cells by simply changing the temperature and without modifying the cells.

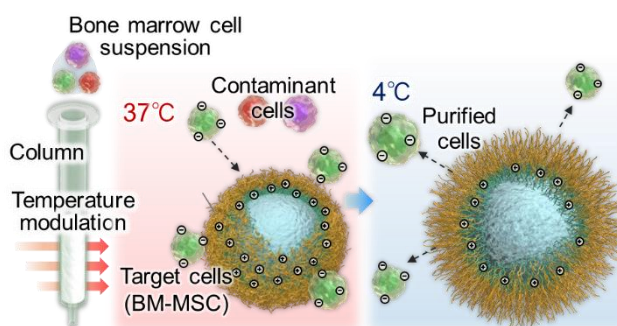

Fig.1 Temperature-responsive stem cell separation column for human bone marrow separation

*Keywords*: Stem cell, Cell separation, Cell therapy, Column separation, Bioseparation

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# Salivary Lysozyme Determination by Simple CZE-UV: Possibilities and Challenges

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Saliva presents an attractive, noninvasive biological fluid for disease monitoring due to its ease of collection and its content of diagnostically relevant biomarkers. Lysozyme, an antimicrobial enzyme naturally present in saliva, has shown significant potential as a biomarker for various pathological and inflammatory conditions. In this study, we present the development and validation of a novel, rapid, and simple capillary electrophoresis (CE) method with UV detection for the quantitative analysis of human salivary lysozyme.

The method employs capillary zone electrophoresis with transient isotachophoresis as an in-capillary preconcentration technique to enhance sensitivity. Key method optimizations included the use of 1 M formic acid with 10% (v/v) isopropyl alcohol as the background electrolyte, and suppression of non-specific adsorption via surface deactivation using carbonic anhydrase. Sample pretreatment was minimized to simple dilution, supporting the method's applicability in clinical settings.

The method demonstrated very good analytical performance, with precision (RSD < 5%), accuracy (−2.03% to +5.36%), and a limit of detection of 0.5 µg/mL. Lysozyme stability in saliva stored at −80 °C for up to 7 days with protease inhibitors was confirmed, and short-term biological variability was evaluated over multiple time points and days.

The validated method was applied to saliva samples from 25 healthy volunteers, with results showing a mean lysozyme concentration of 14.3 ± 10.5 µg/mL. To our knowledge, this is the first CE-UV method developed for salivary lysozyme determination in its intact form. The method is characterized by its simplicity, speed (total time under 45 minutes), robustness, and suitability for real-world sample analysis, making it a promising tool for future clinical applications in disease screening and monitoring.

*Acknowledgements:*

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*Keywords*: capillary zone electrophoresis, lysozyme, proteins, saliva, transient isotachophoresis

# *Pros* and *Cons* of the Schlieren Effect in Flow-based Analysis and Its Application for Quality Control in Food and Pharmaceutical Formulations

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In flow analysis, schlieren effect is the phenomenon of light refraction at the boundary of two liquids with different refractive indices. The signal profile arising from this phenomenon often perturbs the measurement of light absorption in spectrometry. Albeit the '*Cons*' of the schlieren effect in flow-based analysis, there is a '*Pros*' side to this effect, since the schlieren signal correlates with the concentration of a solute in the solution. In this work, utilization of the schlieren effect is demonstrated for the quantitative analysis of alcohols, acetic acid and dissolved sodium chloride in binary-mixture solutions of rubbing alcohol, vinegar and normal saline, respectively. The analytical flow system is simple with injection of sample (200 or 400 µL) into a stream of water (flow rate: 1.8 mL $min^{-1}$). To avoid absorption of the sample in UV-Vis region, the light source was selected in the near-IR range (890 nm). Linear calibrations ($r^2$ > 0.99) were satisfactorily obtained with extremely high sample throughput of 60 to 200 injections $h^{-1}$. Applications of the method to selected food and pharmaceutical formulations gave results that corresponded well with the comparative methods. This proposed method is reagent-free, environmentally friendly and suitable for in-line quality control in food and pharmaceutical formulations.

*Keywords*: Schlieren effect, flow injection analysis, binary mixture, food and pharmaceutical products, quality control

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# Amine-Functionalized $Fe_3O_4@SiO_2$ as Magnetic Dispersive Adsorbents for the Pre-concentration of Selective Serotonin Reuptake Inhibitor Antidepressants from Aqueous Solutions: Analytical Performance, Sorption Modeling, and Greenness Assessment

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Abstract

This study successfully synthesized, characterized, and applied a magnetic amine-functionalized adsorbent material, modified with an alkaline activator, for the pre-concentration of selective serotonin reuptake inhibitor (SSRI) antidepressant drugs namely escitalopram, fluoxetine, fluvoxamine, paroxetine, and sertraline in water. A range of techniques, including X-ray diffraction (XRD), scanning electron microscopy (SEM), vibrating sample magnetometry (VSM), specific surface area analysis, and Fourier transform infrared spectroscopy (FTIR), were employed to investigate the physical and chemical properties of the synthesized material. Response surface methodology, specifically the $2^7$-run Plackett–Burman and the $2^4$-run Central Composite design, was utilized to explore synergistic factors influencing the magnetic dispersive micro-solid phase extraction method. The optimal conditions for pre-concentrating the target analytes as suggested by model as follows: a sample volume of 15 mL, water temperature of 22 °C, sorbent weight of 0.5 g, effervescent precursor amount of 1 g, solution pH of 11.0, contact time of 7 minutes, and desorption solvent volume of 250 µL. Under these conditions, a high recovery of 90% was achieved, with a desirability value of 0.90. Quantification of the analytes was performed using high-performance liquid chromatography (HPLC) equipped with a diode array detector. The method demonstrated low limits of detection (LOD) and quantification (LOQ), ranging from 0.01 to 0.08 µg/mL and 0.03 to 0.28 ng/mL, respectively. Extraction recoveries at three spiked concentration levels ranged from 79% to 95%, while intra- and inter-day precision showed low variability, with relative standard deviations (RSDs) below 10%. Regeneration studies revealed recovery losses of less than 10% after four cycles of use. The Freundlich isotherm model ($R^2$ >0.990) and the pseudo-second-order kinetic model ($R^2$ >0.980) provided the best fit to the experimental data. Through molecular dynamics simulations, adsorption configurations and energies were systematically calculated to investigate the interaction between SSRI molecules and the adsorbent. Theoretical calculations indicated that adsorption was mainly driven by electrostatic interactions and π–π stacking. Furthermore, the magnetic dispersive micro-solid phase extraction method proved to be environmentally sustainable, achieving an overall AGREEnness score of 0.70, a Blue Applicability Grade Index of 67.5, and a Sample Preparation Metric Sustainability score of 7.37 highlighting its green credentials.

*Keywords*: pharmaceutically active drug, magnetic adsorbent, greenness metric assessment

# Integrated Multidimensional Analytical Strategies for Quality Assessment of Valuable Agarwood (*Aquilaria* spp.)

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Agarwood is an aromatic and valuable wood containing resin, mainly produced by plants of the *Aquilaria* species (Thymelaeaceae family). The formation of resin is induced by the defense response of *Aquilaria* plants to external injuries. The extensive use of agarwood and the depletion of resources make the quality control of agarwood particularly important. Unsystematically identified components in the ethanol extract of *Aquilaria agallocha* were explored by mass spectrometry-guided separation and identification techniques. The multi-modal mass spectrometry integration approach was used to analyze agarwood from multiple perspectives, thereby unearthing the intrinsic components related to quality evaluation. An ambient ionization pre-processing technique combined with miniature mass spectrometry was employed for on-site detection and rapid authentication of agarwood. Multidimensional analytical separation techniques were used to separate the ethanol extract reflecting the resin content to magnify the differences. Meanwhile, based on the technique of combining optical microscopy and mass spectrometry imaging at the spatial level, the compositional distribution of the resin aggregation sites in agarwood slices was visualized. Furthermore, a comprehensive strategy combining targeted and non-targeted methods was adopted to efficiently enrich the aroma of different zones as well as wild Kynam agarwood, which comprehensively revealed the aroma components related to the production area and quality. The gas-liquid microextraction (GLME) technique was further used to capture the volatile target aroma components at different temperatures in real-time and explore the changes in the related odor components. In conclusion, the above research provided data support for the quality control and scientific quality evaluation of agarwood.

*Keywords*: Agarwood, multi-modal mass spectrometry, aroma, quality evaluation

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# **Multi-platform mass spectrometry for in-depth chemical profiling and anti-platelet compound identification in *Panax Notoginseng***

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Mass spectrometry (MS), with its high sensitivity, superior structural elucidation capabilities, and broad applicability, has become an essential tool in natural product research. Our group developed an integrated workflow combining multiple MS techniques to comprehensively characterize the chemical constituents and identify antiplatelet components in the medicinal herb Panax notoginseng (PN). Through integrated analysis of diagnostic ions and neutral losses, 180 ginsenosides were identified. Coupled with mass spectrometry imaging (MSI), the spatial distribution of these ginsenosides was successfully visualized. To resolve common isomeric ambiguities of ginsenosides, complementary dissociation methods—collision-induced dissociation (CID) and electron-activated dissociation (EAD)—were employed, enabling precise determination of sugar substitution sites and linkage sequences on ginsenoside molecules for detailed structural characterization. For non-saponin constituents, a "MS molecular networking–molecular fingerprinting–metabolomics" (3M) strategy identified over 200 compounds, including amino acids, sugars, and nucleosides, with MSI further revealing their spatial distributions. Chemical profile–bioactivity relationship analysis facilitated the discovery and validation of key antiplatelet ginsenosides, such as Rk1 and Rg5. Finally, a quality evaluation method for PN was established using active components as indicators by mass spectrometry analysis. This study highlights the comprehensive utility of mass spectrometry in natural product analysis including constituent identification, bioactivity screening, and quality control, advancing research and development in this field.

*Keywords*: Mass Spectrometry, Mass spectrometry imaging, *Panax Notoginseng*, Ginsenosides, Antiplatelet activity

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# Development of a Simple Analytical Method for *Legionella pneumophila* Using Novel DNA Aptamer-Gold Nanoparticle Conjugates

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Monitoring pathogenic microorganisms in public and environmental waters is important for protecting ecosystems and human health. However, current detection methods such as culture method, qPCR method, and immunoassay require long times, complicated operations, and expensive reagents. In this study, we developed a simple analytical method for *Legionella pneumophila* (*Lp*) as the model of pathogens using DNA aptamer-gold nanoparticle (AuNP) conjugates to determine the *Lp*.

We propose a single-round polymer-enhanced capillary transient isotachophoresis (SR-PectI) technique to obtain *Lp*-binding DNA aptamers [1,2]. The single peak of *Lp*-DNA complexes was separated from randomized DNA library, and was fractionated the single peak area (the dotted area in Fig. 1) (Fig. 1). After fractionation, the *Lp*-binding DNA sequences were determined by next-generation sequencing (NGS) technique. As a result, four DNA aptamers showed higher affinity ($K_d$ = 1.0-3.6 nM) than previous *Lp*-binding DNA aptamers ($K_d$ = 7.6, determined in our experiments) [4].

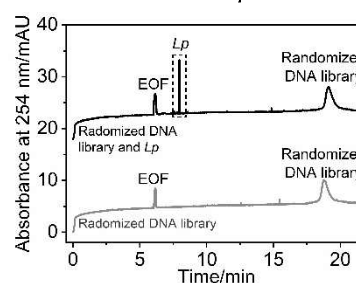
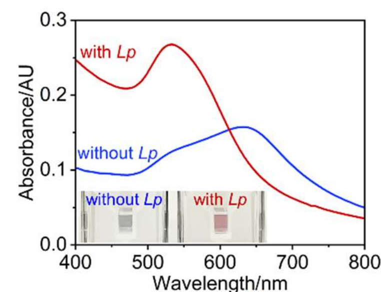



**Fig. 1** Typical electropherograms of randomized DNA library, and the complex of *Lp* and randomized DNA library using PectI. The dotted area is the fractionation area (60 seconds).

The conjugates between our obtained aptamer, which has the highest affinity among the determined aptamers, and AuNPs were prepared (named AuNP conjugates). In the absence of *Lp*, the AuNP conjugates aggregated and turned blue. In the presence of *Lp*, the AuNP conjugates bound to *Lp*, remaining dispersed to give red colour (Fig. 2). By measuring the ratio of the absorbance at 650 nm and 520 nm ($A_{650}/A_{520}$), the number of *Lp* could be analysed. After investigating the aggregation and dispersion conditions of the AuNP conjugates, the calibration curve was obtained and the detection limit was determined to be 89 cfu. The AuNP conjugates could demonstrate the measurements of *Lp* in environmental samples.




AuNP conjugates with and without *Lp*. Inset shows the colour change of AuNPs with and without *Lp*.

In conclusion, novel *Lp*-binding DNA aptamers which have higher than the reported DNA aptamers and a simple analytical method for *Lp* using the obtained *Lp*-binding DNA aptamer were developed. The advantages of our method are rapid (> 1 hour) and easy-to-use (not relying on culture, extraction of DNA and RNA, and PCR) with high sensitivity and selectivity.

*Keywords*: capillary electrophoresis, DNA aptamer, gold nanoparticles, *Legionella pneumophila*

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## Enhancing LiP-MS Structural Proteomics with Multi-Protease Complete Digestion Strategies

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Protein higher-order structure is closely linked to molecular function, making proteome-scale structural analysis essential for understanding how environmental stimuli induce protein conformational changes. Limited Proteolysis-coupled Mass Spectrometry (LiP-MS) combines partial digestion with proteinase K (PK) followed by complete digestion with trypsin and mass spectrometry to facilitate structural proteome analysis. However, the cleavage specificity of trypsin limits peptide diversity and hinders comprehensive identification of PK cleavage sites, restricting the method's ability to detect protein conformational changes across the proteome.

In this study, we developed multiLiP-MS, which employs multiple proteases for complete digestion, generating diverse peptide fragments and improving the detection of PK cleavage sites. HEK293T cell lysates were treated with proteinase K (1:100 enzyme-to-substrate ratio) for 5 minutes at 25 °C, followed by protein denaturation using 5 % sodium deoxycholate. Samples were then digested with one of five proteases (trypsin, LysC, Arg-C, LysargiNase, or V8 protease) and analyzed by nanoLC/MS/MS. The peptides were identified using Spectronaut 18 (Sagan).

We first evaluated the coverage of PK cleavage positions. MultiLiP-MS identified 37,300 PK cleavage positions using five proteases—approximately four times more than conventional trypsin-only LiP-MS. This dramatic increase demonstrates that the diversity in protease specificity significantly enhances PK cleavage site identification. The additional cleavage sites tended to be located near lysine and arginine, the target residues of trypsin digestion, suggesting that PK cleavage sites in these regions could not be identified using trypsin alone. Furthermore, applying multiLiP-MS with trypsin and LysargiNase to rapamycin-treated samples successfully identified residue-level structural changes in the rapamycin target protein FKBP1A, validating the utility of this method for advancing LiP-MS-based structural proteomics.

*Keywords*: LC/MS/MS, proteomics, protein structure, protease

# Evaluating process development strategies through a multi-attribute mass spectrometry approach to minimize disulfide bond-related modifications in monoclonal antibodies (mAbs)

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## Introduction

mAbs rely on the structural integrity provided by interchain disulfide bonds linking the light and heavy chains, as well as intrachain disulfide bonds within the constant or variable domains. These bonds are susceptible to various modifications, including reductive cleavage, trisulfide formation, cysteinylation, and disulfide scrambling, due to their relatively low dissociation energy. Such alterations can affect antigen binding, Fc effector functions, and renal clearance, and may result in aggregation, raising safety concerns. While Protein A purification is commonly used, it faces challenges such as incomplete host cell protein removal and aggregation. We have developed new strategies to enhance mAb properties and minimize disulfide bond modifications. To assess these strategies compared to the conventional method, we extensively applied multi-attribute mass spectrometry.

## Methods

This study explores a modified purification strategy aimed at reducing disulfide bond-related modifications in mAbs. The effectiveness of this strategy is assessed by evaluating disulfide linkages and maintaining the structural integrity and quality of therapeutic mAbs. Peptide mapping using liquid chromatography-mass spectrometry (LC-MS) with Multi-attribute monitoring (MAM) approach was employed, specifically targeting cysteine-stressed and non-stressed IgG1 antibodies to identify potential disulfide bond alterations. To support the LC-MS findings, additional analytical techniques were applied, including size exclusion chromatography (SEC) for protein size distribution, differential scanning calorimetry (DSC) for thermal stability assessment, and polyacrylamide gel electrophoresis (SDS-PAGE) for examining protein purity and aggregation. These complementary methods provided a comprehensive evaluation of the purification strategy's impact on mAb quality and disulfide bond modifications.

## Results

Reduced and non-reduced peptide mapping fragment data, when analyzed using the MAM module, enables the simultaneous assessment of various critical quality attributes (CQAs). The results for in-house synthesized mAbs produced using both methods revealed that both samples had similar intact mass and exhibited similar glycoform profiles, indicating consistent production and similar post-translational modifications.
Non-reduced peptide mapping demonstrated comparable trends in disulfide bond shuffling between the C-mAb (conventional method) and the M-mAb (modified method), suggesting that both samples underwent similar levels of disulfide bond rearrangements. However, when subjected to reduced peptide mapping, a notable difference emerged. The C-mAb showed significantly higher levels of deamidation, suggesting that the modified method was effective in reducing such modifications, which are often associated with reduced stability and altered functionality.
Further analysis using SDS-PAGE indicated that the C-mAb was more prone to disulfide bond-related modifications, particularly under cysteine stress conditions. The M-mAb, on the other hand, showed fewer signs of degradation and aggregation, confirming that the new purification strategy effectively minimized disulfide bond-related alterations. SEC-HPLC provided additional confirmation, where the M-mAb exhibited a more stable profile compared to the C-mAb, with fewer aggregates observed.
Thermal stability assessments through DSC revealed that the M-mAb exhibited a higher melting temperature (Tm) and molar heat capacity, both with and without cysteine stress. This indicates that the M-mAb possesses superior stability, likely due to fewer disulfide bond modifications. In contrast, the C-mAb showed a lower Tm and reduced stability, further highlighting the benefits of the modified purification strategy in enhancing mAb quality. Overall, these preliminary results suggest that the modified purification method significantly improves the stability and integrity of mAbs, reducing disulfide bond-related modifications.

## Novel Aspect

The novel process modification enhances monoclonal antibody stability, utilizing MAM techniques to precisely analyze and reduce disulfide bond modifications.

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# Enhanced-Sensitivity Profiling of Natural Products from TLC Plates Using a Facile Graphite-Based LA-DART-MS Platform

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The isolation and analysis of natural products are crucial to ensure their safety and efficacy, particularly in natural medicine. Thin-layer chromatography (TLC), with advantages in operational simplicity and visual results, is widely employed for natural product detection. Integrating established quality control methods like TLC with ambient ionization mass spectrometry allows targeted characterization of unknown components on TLC plates, markedly enhancing the accuracy and validity of natural products quality assessment. We present a novel graphite-assisted platform to improve the performance of laser ablation direct analysis in real-time mass spectrometry (LA-DART-MS) for TLC plates. This facile technique significantly enhances laser ablation efficiency, boosting MS signal intensity for key compound classes such as flavonoids, alkaloids, volatile oils, and organic acids. To further verify the applicability of this method, we applied this method to the analysis of active components in Chenpi and Qingpi citrus herbs, identifying 14 compounds directly from the TLC plate. When combined with multivariate statistical analysis, the data enabled clear differentiation of Citrus samples based on geographical origin, highlighting its utility for quality control. This work establishes graphite-assisted LA-TLC-DART-MS as a highly sensitive and broadly applicable method for the rapid screening and identification of compounds in complex natural product mixtures.

Keywords: Natural products; Thin layer chromatography; Direct analysis in real time mass spectrometry; Ambient ionization mass spectrometry; Quality control

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# Posters Abstracts

# Prediction of retention time by combining multiple datasets with chromatographic parameter vectorization and transfer learning

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Liquid chromatography-mass spectrometry (LC-MS) is one of the most useful platforms for metabolomics study, but metabolite identification is still a bottleneck in nontargeted metabolomics. The identification of metabolites is usually based on mass spectrometry information, and has a high false positive rate. Retention time (RT) in chromatography can provide information orthogonal to mass spectra, helping improve the accuracy. But, under given chromatographic conditions, the number of compounds with known RTs is small. This data sparsity often leads to insufficient training of machine learning models, seriously affecting the performance of RT prediction.

We propose an effective method (MDL-TL) to address the data sparsity in RT prediction and enhance the RT prediction performance. MDL-TL vectorize chromatographic parameter by autoencoders and Word2Vec, and marks the datasets from different chromatographic experiments by adding the chromatographic parameter vectors into the compound representation. Then MDL-TL combines multiple datasets to pretrain the base model and learn the prior knowledge. For a certain target task, the RT prediction model is obtained by fine tuning the base model.

Experiments on the 14 RPLC datasets and 14 HILIC datasets have shown that MDL-TL outperforms the compared machine learning and deep learning methods (including transfer learning methods) in most cases. Experiments also show that the base model pre-trained with the multiple datasets from chromatographic modes that are the same as the target task usually has a better performance than the model pretrained by combining small datasets from different chromatographic modes. Since MDL-TL incorporates the chromatographic parameter into the RT prediction model, the experiment on the 14 mixed datasets shows that MDL-TL still outperforms the baseline methods in most cases, even using the datasets from different chromatographic modes for pre-training. Further, even seven datasets are combined (total 2240 compounds) for pretraining, MDL-TL can also get a better performance than the compared transfer learning method based on SMRT dataset (containing 80038 compounds) in most cases.

*Keywords*: retention time, chromatographic parameter vectorization, transfer learning, metabolomics

## Automatic solid phase extraction with coated microfibrous sorbent as a front-end to UHPLC – case of determination of xenobiotic residues in surface water

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Automation of multiple steps was used for the sensitive determination of xenobiotic residues in surface waters. The online hyphenated sequential injection analysis instrumentation used surface-modified microfibrous sorbent for the effective solid phase extraction of 1.0 mL of sample into a 100 µL zone as a front-end of reversed-phase ultra-high performance liquid chromatography (SIA-SPE-UHPLC). The SPE column was packed with polypropylene microfibers coated with polydopamine. The freshly prepared column was used for extraction of more than 200 samples in the SIA-SPE-UHPLC method with high reproducibility. The performance of the developed method was demonstrated with the analysis of fifteen river water samples. Our study highlights the advantages of online SPE systems and their contribution to the advancement of water quality monitoring through automation and improved analytical sensitivity.

The authors gratefully acknowledge the financial support of the Czech Science Foundation, project GAČR No 23-05586S.

*Keywords*: online solid phase extraction; sequential injection analysis; ultra-high performance liquid chromatography; water analysis; microfibers

# Efficient enrichment and rapid determination of pyrrolizidine alkaloids by novel microporous organic network extraction coupled with miniature mass spectrometry

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Pyrrolizidine alkaloids (PAs) are hepatotoxic natural toxins found in numerous plants, and their ingestion can lead to severe liver injury. For patients with suspected PA-induced liver damage, the rapid detection of these toxins in biological samples is crucial for accurate diagnosis and timely clinical intervention. This study presents a novel method that combines a microporous organic network (MON) as a selective adsorbent for sample pretreatment with a miniature mass spectrometer for rapid analysis. We demonstrate that this approach enables the efficient enrichment and determination of PAs in complex matrices. Compared to conventional protein precipitation, the MON-based dispersive solid phase extraction (DSPE) significantly enhances detection sensitivity. Furthermore, the MON material exhibited superior performance for PA enrichment and purification over other commonly used adsorbents. The use of miniature mass spectrometry, in place of traditional liquid chromatography-mass spectrometry (LC-MS) systems, offers key advantages in analytical speed and operational simplicity. This integrated strategy holds considerable promise for the rapid, point-of-care screening of PA exposure and the clinical diagnosis of related toxicities.

Keywords: pyrrolizidine alkaloids; miniature mass spectrometry; microporous organic network; urine

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# Highly Sensitive Microchip Electrophoresis of Cationic Analytes by LDMS

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To achieve highly sensitive electrophoretic analysis, in our research group, on-line sample preconcentration by large-volume sample stacking with an electroosmotic flow (EOF) pump (LVSEP)[1], which allows the entire volume of sample introduced into the channel has been applied to microchip electrophoresis (MCE). However, the application of LVSEP has mainly been limited to anionic analytes. For LVSEP analyses of cations, it is necessary to reverse the EOF. To address this issue, polyallylamine-modified microchips, which generates a positive zeta potential on the channel surface, was employed to the analysis of cationic species. However, it was difficult to control the EOF in the polyallylamine-immobilized microchannel, resulting in insufficient enrichment efficiency. In this study, large-volume dual-sample stacking by micelle collapse and sweeping (LDMS)[2], which utilizes micelle collapse by organic solvents, was used to enrich cationic analytes in poly(vinyl alcohol) (PVA)-modified microchannels, generating a stable EOF.

PDMS straight-channel microchips with 150 μm width and 100 μm depth were fabricated, and the channel surface was modified with PVA using a vacuum-drying method[3]. NBD-histamine (HA) was used as a standard cationic analytes. 10 mM MES buffer (pH 5.5), 10 mM sodium dodecyl sulfate (SDS) and acetonitrile (ACN) were employed as a background electrolyte (BGE), a micellar solution and organic solvent for micelle collapse, respectively. In the MCE analysis, a voltage of +1.5 kV was applied, and fluorescence detection was performed with excitation at 488 nm and monitoring at 520 nm.

In the LDMS method, the entire microchannel is initially filled with a sample solution, followed by the injection of a short plug of SDS micelle from the outlet reservoir. By applying a positive electric field, the SDS micelles migrate electrophoretically toward the inlet while capturing and sweeping cationic analytes. By raising the liquid level of the inlet reservoir, an organic solvent is gradually introduced into the channel via pressure-driven flow, which causes the micelle collapse into SDS monomers. When the micelle collapses occur around the inlet reservoir, cationic analytes captured by the SDS micelle were released to create a narrower zone. When NBD-labeled histamine (HA) was used as the model analyte, and the LDMS analysis was performed using a BGE containing acetonitrile (ACN) in the inlet reservoir and 10 mM MES buffer (pH 5.5) in the outlet reservoir, a single sharp-peak was observed. Under conditions where the liquid levels of both reservoirs were equal, the sensitive enhancement factor (SEF) reached to 3000. When the inlet liquid level was raised by 0.25 mm, the SEF was increased to 3500 with significant improvements in the analytical reproducibilities.

Under the initial experimental conditions, it was required as long as 15 min to detect the target peaks. To overcome this limitation, the channel length was reduced from 6 cm to 3 cm. When the liquid level difference between the inlet and outlet reservoirs was varied from 0 to 0.50 mm, the highest peak was obtained at 0.25 mm difference, resulting in the SEF of 1080. The detection time was successfully reduced to *ca.* 6 min, and the peak width was as narrow as 0.4 s. In our enrichment studies for cationic analytes, this was the best SEF and peak width. In the present LDMS condition, the anionic SDS micelles effectively captured the positively charged HA through electrostatic interaction, contributing to sharper peak profiles.

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# Non-invasive diagnostics of Barrett's esophagus - analysis of bile acids in saliva and exhaled breath condensate

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Bile acids (BA) are a group of steroid compounds essential for lipid digestion. However, when the BAs are refluxed into the stomach and the esophagus during the duodenogastroesophageal reflux, they could have a detrimental effect on the esophageal epithelium. This could lead to the development of pathological changes in esophageal tissue, e.g. Barret's esophagus (BE) [1]. The levels of BA in non-invasive samples, such as saliva or exhaled breath condensate (EBC), might serve as a possible biomarker for the BE diagnostics and indicate potential for BE development. For non-invasive sampling, two methods were selected. Saliva sampling was performed by simple spitting. The EBC collection was performed using a lab-developed sampling device. The EBC sampler consists of three serially connected cooled glass tubes, enabling efficient sampling, e.g. a healthy male individual with an approximate lung capacity of 4-5 L can typically collect 100-150 µL of EBC per exhalation. Quantification of 15 bile acids (unconjugated, glycine-conjugated and taurine-conjugated) was performed. The samples were collected as a part of an ongoing clinical study, including patients with gastroesophageal reflux disease (GERD), patients with Barrett's esophagus and healthy subjects. For the purpose of non-invasive diagnosis of BE patients, as well as potential prediction of BE development, we have optimized a preconcentration method utilizing SPE sample treatment and UHPLC-MS analysis of bile acids in the above non-invasive samples [2]. Results show that the ratio for tauro-conjugated bile acids, glyco-conjugated bile acids and total bile acids shows significant statistical difference between patients with BE and healthy subjects ($p < 0.018$, $p < 0.023$, $p < 0.021$) and between patients with BE and GERD ($p < 0.027$, $p < 0.041$, $p < 0.034$). Of all the BA analytes, most promising biomarkers appear to be glycochenodeoxycholic acid (GCDCA) and taurochenodeoxycholic acid (TCDCA). There were no statistical differences between patients with GERD and healthy individuals, suggesting that the ratio pattern is altered specifically in Barret's esophagus.

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*Keywords*: bile acids, saliva, exhaled breath condensate, Barret's esophagus, GERD

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## On-line electrochemical synthesis of fluorescently labeled glycans utilizing a microfluidic chip with electrodes

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Post-translational modifications (PTMs), which involve the covalent attachment of chemical entities to the side chains of modifiable residues, act as molecular switches that allow cells to respond to diverse conditions. PTMs play a vital role in the control of protein activity, stability, and subcellular localization, thereby contributing to intracellular regulation [1]. Microfluidic devices have contributed to several remarkable innovations in both chemistry and biology. The miniaturization of these devices results in reduced reagent consumption, rapid analysis times, and ease of automation. We previously reported a method for PTMs analysis such as glycosylation and phosphorylation that did not require preconcentration by fabricating functional acrylamide gels within the channels of a microfluidic chip [2,3]. However, these methods cannot achieve online fluorescent labeling and require glycans that have been fluorescently labeled in advance. In addition, conventional fluorescent labeling of glycans requires the reaction of glycans with a high concentration of fluorescent reagents for several hours while heating, followed by purification of the excess fluorescent reagent.

Here, we present an online electrochemical synthesis of fluorescently labeled glycans utilizing a microfluidic chip with electrodes (Fig. 1). The microfluidic chip had a microchannel sandwiched between the top and bottom electrodes. A sample solution containing a mixture of fluorescent reagents and glycans of the same concentration was introduced into this microchannel under pressure, and when a voltage was applied to the electrodes, a reductive amination reaction occurred at the cathode, resulting in the production of a fluorescently labeled glycan sample. We report the results of optimizing various conditions using 7-amino-4-methylcoumalin as a fluorescent reagent and maltotriose as a model sample.

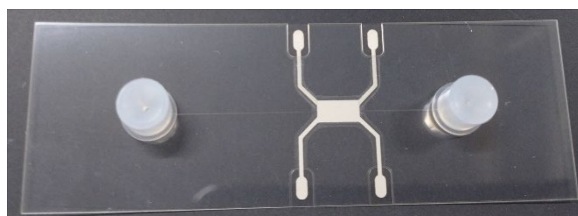

Fig. 1 A photograph of the microfluidic chip with electrodes used in this study.

*Keywords*: electrochemical synthesis, microfluidic chip, glycans, 7-amino-4-methylcoumalin

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# Bioactive Glycan Motif Library Built on Structure-Based Separation for Rapid Profiling of Therapeutic Glycoproteins

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Abstract body (up to 350 words)

The importance of glycosylation towards optimal drug bioactivity in biotherapeutics design is increasingly underscored. In biotherapeutic products, structure, specific modifications, and abundance of glycans can significantly affect the quality and safety of drugs. Therefore, detailed glycomic analysis is a key step in assessing quality control and tuning the glycosylation pathway of therapeutic glycoproteins. However, the inherent structural diversity and complexity of glycosylation significantly makes glyco-analysis extremely challenging. Here, we presented a strategy to facilitate the rapid identification and structural annotation of glycans in biotherapeutics using a catalog of glycans bearing bioactive motifs. Catalogue entries include not only typical glycans found in biotherapeutics, but also atypical glycans such as sialic acid O-acetylation, mannose phosphorylation, polylactosaminylation, NeuGc sialylation, HexNAc sulfation, and galactose-alpha-1,3-galactose. To build this resource, LC-MS/MS-based isomer specific separation and structural elucidation were performed on glycan representative of diverse glycoprotein classes such as monoclonal antibodies, cytokines, and enzymes. Diagnostic fragments were identified to enable rapid rapid and user-independent annotation of MS/MS spectra. Our strategy was successfully applied to structurally characterize glycans from biotherapeutics produced by various cell-based expression systems including CHO, HEK, and SP2/0. This approach enables rapid, yet highly accurate identification of both desired and undesired glycans containing bioactive motifs in various therapeutic glycoproteins, offering a valuable tool for biotherapeutic protein development, quality assurance, and glycoengineering.

*Keywords*: Therapeutic glycoprotein, glycan, structure, LC/MS/MS (up to 5)

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# Metabolomic analysis of tear fluid from patients with diabetic retinopathy using chemical isotope labeling liquid chromatography-mass spectrometry (CIL LC-MS)

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Abstract

Diabetic retinopathy (DR) is a progressive and globally prevalent complication of diabetes leading to vision impairment. Early detection of DR is crucial for successful disease management, aiming to prevent vision loss and related complications in individuals with diabetes mellitus (DM). However, the early diagnosis of DR and monitoring its progression pose significant challenges. This highlights the urgent necessity for discovering reliable biomarkers for intervention strategies and monitoring the prognosis of the disease. Herein, we developed a robust method of combining metabolomics and machine learning (ML) to discover dependable biomarkers for efficient risk stratification in DR. We utilized tear film as a non-invasive sample and employed the chemical isotope labelling (CIL) liquid chromatography-mass spectrometry (CIL LC-MS) technique [1] with a 13C-labelled reference sample as an internal standard for improving separation, ionization efficiency, and quantification accuracy. A total of 822 peak pairs were detected across four channels of CIL LC-MS after blank treatment and data cleansing. This included 212 peak pairs from channel A (amines/phenols), 171 from channel C (carboxylic acids), 308 from channel H (hydroxyl groups), and 131 from channel K (carbonyl metabolites). The detection of these 822 peak pairs suggests a diverse range of metabolites is present in the tear samples. Predictive models based on three ML algorithms, using tear metabolomics data, were extensively tested and compared. These models demonstrated a high degree of accuracy, with the best model - eXtreme Gradient Boosting - achieving a score 89% accuracy in distinguishing patients with DR from healthy control. Additionally, SHAP (SHapley Additive exPlanations) analysis was used to interpret the ML model and identify novel metabolites that have the potential to serve as biomarkers for DR. Our work presents the potential of using the tear metabolomics data to construct an AI-enabled disease diagnostic system that can be applied in real-world clinical settings.

*Keywords*: Metabolomics, Chemical isotope labeling liquid chromatography-mass spectrometry, Diabetic retinopathy, Machine Learning

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## Specific Separation of Halogenated Aromatic Compounds via Molecularly Imprinted Polymers Based on Halogen Bonding

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Endocrine-disrupting chemicals have a strong influence on abnormal thyroid function, and there is an urgent need to identify and legally regulate these chemicals. However, no efficient *in vitro* screening method has been established for TR active compounds, and we strongly require to develop a simple screening method based on an engineering approach.

In our previous study, we succeeded in separating TR active substances from the mixture with TR inactive substances using a column packed with a molecularly imprinted polymer (MIP) via hydrogen bonding. [1] On the other hand, we selectively separated only the TR compounds having thyronine structure, such as triiodothyronine (T3) and thyroxine (T4), and the other active compounds, which have a number of halogen atoms, were not retained on the MIP. Therefore, we newly prepare the MIPs using halogen bonding as main interaction and we used them as packed columns for liquid chromatography (LC) to evaluate the retention selectivity.

In this study, we prepared MIPs and non-imprinted polymers (NIP) which were prepared without template molecules were prepared. 4-vinylpyridine was used as the functional monomer, divinylbenzene as the cross-linker, and a couple of halogenated biphenyls as the template molecules. Cyclohexane was used as the mobile phase, and the alterations in the retention behavior of halogenated aromatic compounds were evaluated by LC. The results demonstrated that the retention behavior differed depending on the numbers and the orientation of the template molecules. It was also found that precise separation using halogen bonding was possible for TR active compounds.

Literature:

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## Quality Control of Synthetic Cyclic Peptides using Two Different Chromatographic Modes

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Abstract body

Peptide pharmaceuticals are one of the new modalities currently attracting attention. PeptiStar, a very young company, was established in September 2017 and began operations in May 2019 as a CDMO (Contract Development Manufacturing Organization) that combines all-Japan technology to efficiently and high-quality manufacture peptide APIs with the latest equipment. In peptide API manufacturing, impurity analysis is important because various impurities are produced as byproducts. Additionally, impurity control is essential in drug development as it affects safety. Particularly for peptides containing non-natural amino acids, the structure becomes complex, and stricter control similar to small molecule drugs is required. Therefore, a lot of time is needed developing analytical methods for impurity analysis. The main separation method used is chromatography, with the reversed-phase high-performance liquid chromatography (RP-HPLC) being the first choice due to its high peak capacity. However, since impurities elute near the main peak, it may not be possible to control its purity with RP-HPLC alone. Therefore, HILIC was selected as another mode, and orthogonality was confirmed using four different cyclic peptides. As HILIC columns, DCpak® P4VP, DCpak® PTZ, and DCpak® PMPC from Daicel Corporation, a joint research partner, were used. This poster introduces the latest regulations important for developing peptide drugs and examples of cyclic peptide analysis using two separation modes.

*Keywords*: Cyclic Peptide, Regulation, Quality Control, RP-HPLC, HILIC

# Separation of IgG fragments utilizing peptidomimetic polymer-modified resins

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Immunoglobulin G (IgG) consists of two antigen-binding regions (Fab) and a constant region (Fc). Their fragments without Fc have attracted attention as therapeutic agents smaller in size than full IgG and retain the antigen binding ability. They have several advantages over full IgG, such as rapid penetration and clearance. However, affinity ligands that can bind to both *κ* and *λ* light chains of Fab have not been developed, leaving challenges for low-cost production. IgG fragments can be genetically engineered to display peptide tags to enable purification by immobilized metal affinity chromatography, but this has the drawback of requiring the inclusion of peptide tag cleavage and removal steps in production [1]. We have previously developed a column for the chromatographic purification of IgG by modifying column packing materials with a polymer that mimics the amino acid composition of Z34C, the IgG-binding peptide derived from protein A, and the structure of histidine (Figure 1a) [2]. The prepared Z34C mimetic column showed pH-dependent retention of IgG like protein A and achieved IgG purification from cell culture supernatants with high purity and recovery by switching the mobile phase pH from neutral to acidic. In this study, we aimed to apply the Z34C mimetic column to the separation of IgG fragments. First, Fab and Fc fragments of IgG were generated by papain digestion of three types of monoclonal antibodies with different light chain classes. And then, the effect of the eluent pH on their retention was investigated. Under neutral conditions, the column showed higher affinity for Fab compared to Fc and was also found to retain both *κ* and *λ* Fab. Then, the separation of IgG fragments from digested IgG samples was performed by switching the mobile phase pH from 6.5 to 5.0 (Figure 1b), and the purity of the separated fragments was evaluated by reversed phase chromatography. As a result, three types of Fab were successfully purified with high purity and recovery, demonstrating that the Z34C mimetic column can be applied as a tag-free, comprehensive method for purifying both *κ* and *λ* Fab fragments.

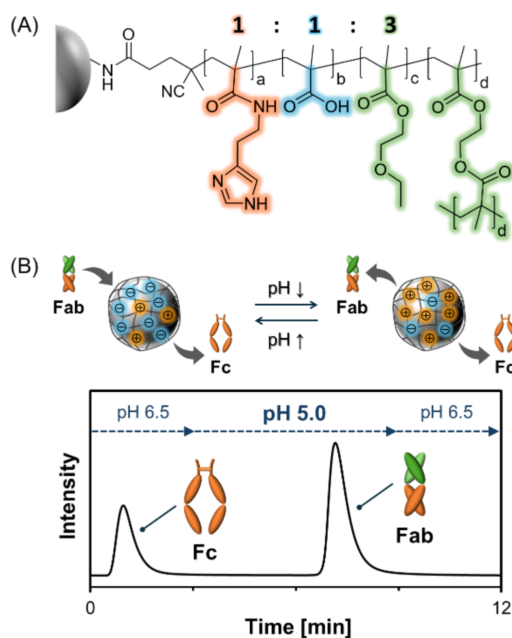


**Figure 1** (a) Molecular structure of the Z34C mimetic polymer. (b) Principles of Fab purification.

*Keywords*: IgG fragment, purification, peptidomimetics, pH-responsive, polymer.

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# Development of alkali resistant reversed phase column packing material based on eggshell utilizing Layer-by-Layer self-assembly

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Porous silica gel is commonly used as a column packing material for reversed-phase liquid chromatography (RPLC) due to its high separation capacity. However, silica gel has the disadvantage that surface silanol residues dissociate in alkaline mobile phases, leading to a decrease in separation efficiency. Therefore, there is a need to develop alkali-resistant column packing materials. We have previously developed alkali-resistant packing material, Eggshell-PMAcO based on eggshell modified with amphiphilic polymer polymaleic acid octadecene (PMAcO)[1]. A column packed with this material enabled the separation of basic compounds using alkaline mobile phase. However, compared to commercial silica gel columns, its retention capacity was relatively low due to the limited amounts of organic components modified on the surface of eggshell. In this study, Layer-by-layer (LbL) method was employed to develop eggshell based column with enhanced retention capacity. Eggshell powder was alternately immersed in anionic PMAcO and cationic polydiallyldimethylammoniumchloride (PDDA) electrolytes to form a multilayer organic membrane. Thermogravimetric analysis confirmed that the amount of organic modification was significantly increased by the LbL method. Scanning electron microscope images showed that the pores derived from the eggshell were retained. The retention of naphthalene was confirmed by HPLC measurements with an analytical column (φ4.6 mm x 150 mm), which showed that as the number of assembled layers increased from 1 to 7, the surface modification amount increased, resulting in stronger retention in reversed phase mode (Fig. 1). Furthermore, the symmetry factor was improved. Further evaluation of column performance associated with increased surface modification via the LbL method, as well as the separation behavior for various compounds, will be discussed in detail in the presentation.

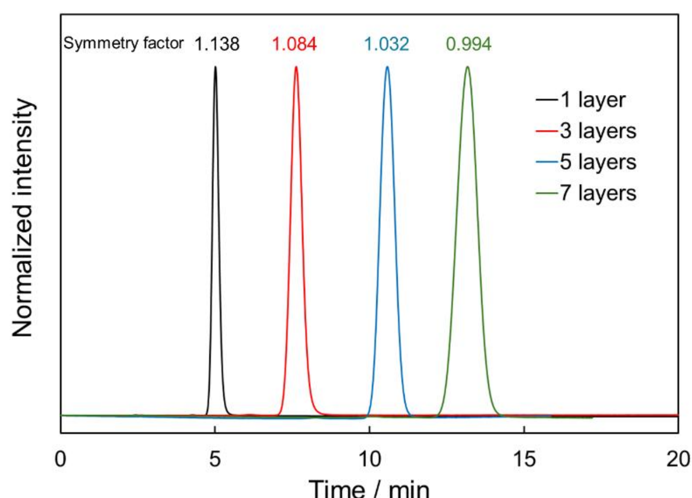


Fig. 1 Chromatograms of naphthalene. Analytical conditions, column temperature: 25 °C; mobile phase: water/methanol=50/50=(v/v); flow rate: 0.5 mL/min; detection: 254 nm; analytes: 0.5 mg/mL naphthalene, injection volume: 2 μL

*Keywords*: HPLC, Layer-by-layer self-assembly, eggshell, alkali resistant, polymer

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# Automatic optimization of gradient profile using AI algorithms on functional food analysis with HPLC

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## Introduction

In general, HPLC method development, enormously time-consuming repeated analysis batch schedule creation is required as well as the chromatographic knowledge and experience in the process of optimizing analytical conditions. Consequently, labor savings through automated method development is paid attention. In this study, fifteen standards mixed solution of catechins, theaflavins, and gallic acid, which were typical functional food components, was employed as a simulated sample, and the AI algorithm equipped in I method development support software LabSolutions MD was utilized. Furthermore, the optimized method was applied to tea leaf analysis.

## Experimental

Ten catechins, four theaflavins, and gallic acid, totally fifteen compounds were subjected to HPLC analysis. First, the gradient profile was optimized by LabSolutions MD using mixed standard solution. The optimized gradient profile was then applied to the analyses of six tea leaves. LabSolutions MD is equipped with a proprietary AI algorithm that automatically searches for the satisfactory conditions by alternately repeating gradient profile improvement then executes analysis under improved conditions. Gradient profiles were automatically searched with a minimum separation criterion of 1.5 for the standard mixture of fifteen compounds. The optimized method was applied to the quantitative analysis of extracts of tea leaves of different types and varieties.

## Results and discussion

The gradient profile that met the criteria (minimum resolution of 1.5) were automatically searched, resulting in significant labor savings. Four green tea samples contained more catechins than those in black tea samples, and the highest concentration of functional component for all four green tea was epigallocatechin gallate, which is expected to inhibit elevated blood glucose in both varieties. In one of green tea samples, two methylated catechins were detected, which have been paid attention for their anti-allergic effects and ability to reduce hay fever. On the other hand, four types of theaflavins were detected in black tea samples. Although one tea sample and one black tea sample were "Benifuki" varieties, the comparison between the two suggests that catechins were converted to theaflavins during fermentation. Above mentioned detailed discussion can be done based to the HPLC results obtained under automatically created and optimized analytical conditions.

*Keywords*: HPLC, AI, artificial intelligence, automatic optimization, functional food

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## Identification of Isomerization in Tryptic Digested Proteins by LC-IM-TOFMS

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Proteins are indispensable materials in biological systems for the structuring of cells and organisms, the catalyzing of metabolic reactions, and so on. In general, proteins in vivo are synthesized from L-amino acids and composed of L-amino acid residues. However, the existence of isomerized proteins containing isomerized D-amino acid and L/D-iso-form residues in vivo has been reported. Such proteins cause age-related diseases such as cataract and Alzheimer's disease. Therefore, the analysis of isomerized proteins is in biological systems is an essential and important task. Here, we focused on liquid chromatography-ion mobility-mass spectrometry (LC-IM-MS), in which the digested-peptides were separated in both LC and IM. In this study, we develop a non-target analysis method for isomeric peptides in LC-IM-TOFMS and the method was applied to tryptic digested proteins.

Synthetic peptides corresponding to the T12 peptide of egg white lysozyme (LZM) with L-, D-, L-iso-, and D-iso-Asp residues were used as the standard peptide sample. Egg white LZM was used as a protein sample. The LZM was dissolved in 50 mM acetate buffer (pH 4.0) and incubated at 60 °C for at least three days to accelerate isomerization. The LC-IM-TOFMS instruments of Synapt G2 HDMS and Cyclic IMS equipt with LC system (Waters) was also used.

First, LC-IM-TOFMS measurements were performed on the synthetic T12 peptide. High dimensional vectors were defined using the MS/MS spectrum intensities of each fragment ion via collision-induced dissociation and the area of the mobilogram with slicing. By the calculation of cos$\theta$ values for the vectors, the identification and localization of isomerized Asp residues was achieved.

The tryptic digested LZM was then analyzed using the aforementioned method. The T12 peptides after the isomerization acceleration resulted in the separation in the time and arrival time planes. The calculation of cos$\theta$ values revealed that the Asp4 residue in the T12 peptide had isomerized to L-iso-form. Additionally, isomerization of the Asp residues in T5 and T8 peptides was observed. The Asn residue in the -Asn-Gly- sequence generally results in isomerization following deamidation (*Anal. Chem.* 2006, 78 (18), 6645-6650). Isomerization of the Ans residue after the deamination was also investigated.

*Keywords*: isomerized protein, non-target, HPLC, ion mobility, mass spectrometry

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## Retention Behavior for Aromatic Compounds with Polyimide Fine Filaments as a Stationary Phase in Reversed-Phase Liquid Chromatography

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HPLC is a popular and also an effective analytical method for the separation of various types of samples complex mixtures. In order to achieve a successful separation, an appropriate choice of the stationary phase from many types of phases developed for modern HPLC separation is particularly important. Recently, polymer-based stationary phases have been introduced as the separation media in chromatographic analysis because of several advantageous features of polymer materials, such as good chemical-resistance, heat-resistance and excellent mechanical strength. Our research group reported the applications of fibrous polymer materials as the separation media in chromatographic methods or the extraction media for sample preparation techniques. As an example, fibrous polyimide was applied to a separation medium in gas chromatography, and a good separation performance was observed. On the basis of the result, the fibrous polyimide enabled the applications as a stationary phase in HPLC method, where the retention behavior of typical aromatic compounds on the polyimide filaments phase has been studied.

Fibrous polyimide-packed column was prepared by longitudinally packing bundles of polyimide filaments (approximately 52800 filaments) into a stainless-steel tube (4.6 mm i.d., 250 mm length), and the column was installed to a typical HPLC system equipped with a UV/Vis detector. As the mobile phase, a mixture of methanol/water was used, and the flow-rate was typically set at 0.50 mL/min.

The retention tendency was studied for aromatic compounds having various alkyl groups on the polyimide-packed column. On typical nonpolar stationary phase, such as octadecylsilica (ODS) phase, a good correlation between the logarithm of retention factors (ln *k*) of the analytes and the corresponding hydrophobicity (log *P*) was obtained. The results showed that the ODS phase has a retention mechanism based on hydrophobic interaction. On the polyimide-fiber phase, however, a different trend was observed for the retention of the analytes with short alkyl functionalities in the molecular structure, when compared to the data obtained on conventional ODS phases. The results clearly demonstrated a possibility of the polyimide filaments as a novel stationary phase having a unique selectivity for various aromatic compounds in HPLC.

*Keywords*: HPLC, stationary phase, polyimide filament, retention behavior

**Development and Separation characteristic evaluation of β-cyclodextrin modified monolithic silica capillary column**

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1. Introduction

The ULTRON AF-HILIC-CD for HILIC column with β-cyclodextrin modified on the silica gel surface has a molecular recognition for the structural isomers. However, the separation power was sometime insufficient due to its weak hydrophilic interactions. Therefore, we had inspired the idea of a novel HILIC column that modifying β-cyclodextrin onto the ULTRON HF-MBS which is a high-resolution capillary column composed monolithic silica with a hierarchical porous structure. In this presentation, we will introduce the characteristic evaluation for the novel HILIC column (Prototype HF-CD) under development and examples of glycan separation.

2. Experimental method

For preparate the Prototype HF-CD, we had prepared a β-Cyclodextrin derivatives has silane reactive groups and diluted that with pyridine. Subsequently, the derivatives solution was passed through the HF-MBS (0.1 mmI.D. x 750 mmL) to react between with the silanol groups on the silica surface. Finally, organic solvents and water had been passed thoroughly through the column for washing, the Prototype HF-CD was obtained.

3. Results and observations

Fig. 1 shows the α(2dG/3dG)-α(α/β) plot to evaluate based on the characterization method for HILIC stationary phases [1] for 34 columns including in the Prototype HF-CD, the HF-MBS, SIL columns and the AF-HILIC-CD.

As results of characteristic evaluation HILIC stationary phase of the ULTRON HF-MBS and the Prototype HF-CD, they showed a tendency to have high molecular structure recognition ability similar to that of the particle type, and the β-cyclodextrin-modified type was more obvious.

That high recognition ability of the Prototype HF-CD was demonstrated in the separation examples 2-AB-labeled glucose homopolymer ladder, Cyclic oligosaccharides and IgG N-Linked Glycans, there were good separations. These chromatograms will be show in the presentation.

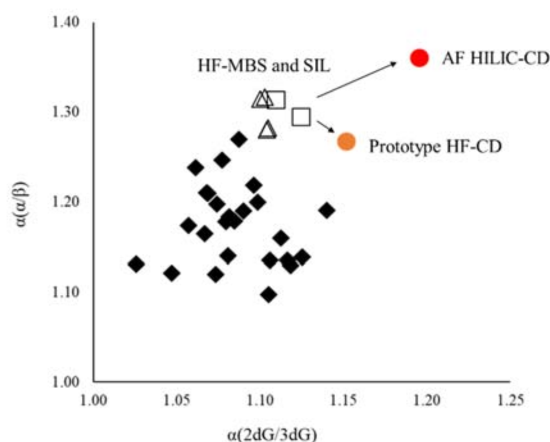

Fig.1 The α(2dG/3dG)-α(α/β) plot for 34 HILIC columns.

*Keywords*: HILIC, β-cyclodextrin, monolithic silica capillary column, molecular structure recognition, Glycan separation

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# Design of Experiments-based optimization of acylcarnitines electrospray ionization process

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Carnitines play a key role in cellular energy metabolism. Acylcarnitines facilitate the transport of fatty acids to mitochondria for their β-oxidation - one of the key cellular processes related to energy metabolism. Carnitine compounds have also been identified as potential biomarkers of cardiovascular diseases. That is why, the aim of this study was to develop a quantitative method for determination of selected medium- and long-chain acylcarnitines in human plasma with the use of HPLC-ESI-QqQ-MS/MS system.

Isolating acylcarnitines from plasma requires the use of an appropriate sample preparation method in order to concentrate the sample and purify the matrix from interfering substances. At the same time, it is necessary to find optimal ionization parameters' settings, so that the resulting method will enable the determination of analytes at the highest possible sensitivity. To achieve this goal in the most efficient way, Design of Experiments methodology was used, which allows for finding the optimal settings of the method parameters in order to obtain the highest possible signal intensity while maintaining the highest repeatability of analyses. Parameters' ranges (capillary voltage: 3000–4500V, nebulizer pressure: 30–50psi, drying gas flow rate: 6–12L/min and drying gas temperature: 280–350°C) were selected based on the literature review and capabilities of apparatus used. Box–Behnken design was chosen because it allows reliable determination of the response surface for studied design space (along with optimal settings) with relatively little experimental work and reagent consumption.

Based on multivariate regression analysis separate models for each acylcarnitine were built. Such an approach enabled prediction of influence of tested parameters on individual response. The most significant variables in the built models were the flow rate of drying gas and its temperature. Although nebulizer pressure and capillary voltage were not found statistically significant, those factors were found to interact with other parameters and affect ionization process. Models built for individual metabolites allowed to conclude all studied compounds would benefit from similar conditions.

With the use of maximize desirability function and assuming that all the discussed compounds are equally important a compromise setting was achieved. The exact suggested parameters settings for positive ionization mode would be gas temperature 350 °C and flow rate of 12L/min. However, overall desirability values hardly changed in higher ranges for studied factors. For example, decrease in gas temperature from 350 °C to 340 °C or 330 °C only slightly reduced the fit (from 0.998 to 0.996 or 0.969).

*Keywords*: acylcarnitines, ionization, design of experiments, optimization, ESI

Acknowledgments

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# Exploration of suitable columns for EPSA measurement using supercritical fluid chromatography and expansion of target compounds

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Abstract body (up to 350 words)

The polar surface area (PSA) is an indicator that represents the area of the polar portion of a molecule's surface, and it is important for evaluating cell membrane permeability in drug structure optimization. PSA, similar to the octanol/water partition coefficient (logP), indicates the polarity and lipophilicity of a molecule. It has been found that there is a good correlation between PSA and experimental data related to membrane permeability. For example, compounds with a very large PSA (over 140 Å²) are empirically known to have low membrane permeability [1].

Since the membrane permeability of drug candidates is directly related to their ability to reach the target site, rapid evaluation at an early stage of development is essential. This evaluation can facilitate the selection of candidate compounds and streamline the development process.

In previous studies, a method for deriving the experimental polar surface area (EPSA) using supercritical fluid chromatography (SFC) has been established [2]. EPSA is calculated based on the retention time of analytes in a calibrated system using compounds with known EPSA values.

In this study, the correlation between retention time and EPSA value was evaluated using various stationary phase columns with different retention selectivity. Good correlation between retention time and EPSA was observed in several polar stationary phases. By optimizing the analytical conditions and using compounds with known polar surface area, it was shown that it is possible to estimate the polar surface area of small molecule drugs and peptide drugs.

By using this new EPSA calculation method using SFC, we can eliminate and promote candidate compounds in the drug discovery phase and streamline the drug development process.

*Keywords*: HPLC, SFC, EPSA

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# Estimation of Surface Area of Gold Nanoparticles Through the Adsorption Amount of Cysteine by Capillary Zone Electrophoresis

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Abstract

The surface area of gold nanoparticles (AuNPs) was estimated through the adsorption of cysteine onto the AuNPs. The AuNPs used were synthesized by a solution plasma process and were polycrystalline. Capillary zone electrophoresis (CZE) was employed to monitor the cysteine adsorption. An AuNP dispersed solution with cysteine adsorption was directly introduced into the CZE system, and a broad peak was obtained in the electropherograms with the dispersed AuNPs. Shot signal for the aggregated AuNPs was not detected in the electropherograms, indicating that the dispersion of the AuNPs was maintained even after the cysteine adsorption. The effective electrophoretic mobility of the AuNPs was reduced by the adsorption of cysteine to the AuNP surface. Any peak attributed to the cysteine was not detected at low concentrations of the cysteine, and the added cysteine was mostly adsorbed onto the AuNPs. An additional peak attributed to the residual cysteine was detected at higher concentrations of cysteine from 100 μmol $L^{-1}$, and a linear relationship was obtained between the concentration of cysteine and the peak area of the cysteine with an x-intercept at 86 μmol $L^{-1}$. This concentration represents the saturation point of surface adsorption onto the AuNPs. The surface area of the AuNP dispersed solution was estimated from the adsorption amount of cysteine and the cross-section area of a cysteine molecule, resulting in an estimated surface area of 0.078 $m^2$ $mL^{-1}$. The estimated surface area was also verified through the adsorption of glutathione onto the AuNP.

*Keywords*: Gold nanoparticles, surface area, cysteine, adsorption amount, CZE

# Towards building a foundation model for automated high-performance liquid chromatography (HPLC) analysis and design

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High-performance liquid chromatography (HPLC) is a critical analytical technique widely employed for chemical separations, yet current method development remains labor-intensive, empirical, and often highly dependent on operator expertise. A central challenge in HPLC analysis and design is accurately predicting the retention time of molecules under specific chromatographic conditions. Recent machine learning approaches have leveraged large retention-time databases from a single standardized column and mobile-phase configuration to build initial models [1], subsequently fine-tuning them for different chromatographic setups to predict their separation capabilities [2]. However, such fine-tuning approaches still demand substantial training data to achieve practical accuracy [3], and the criteria for successful adaptation remain unclear. Foundation models—large, versatile machine learning models pre-trained on extensive datasets, such as GPT for language tasks or AlphaFold for protein structure predictions—present a promising alternative due to their robust generalization and efficient adaptation to related tasks with minimal additional data. To realize a general foundation model for HPLC retention-time prediction, we investigate critical factors influencing efficient model adaptation across various chromatographic conditions. Specifically, we explore scaling laws—previously validated in polymer property prediction [4]—and assess the impact of explicitly incorporating mechanistically relevant chromatographic features, such as particle size and column dimensions. Our approach aims to establish foundational principles guiding efficient, accurate, and broadly generalizable predictions, significantly reducing the experimental workload associated with developing and optimizing HPLC methodologies.

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# The evaluation of a small-capacity polypropylene vial that achieves low bleed and low adsorption

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Many peptides exhibit hydrophobic and ionic adsorption, and the adsorption to vials can impact analytical accuracy. Vials with surface coatings designed to suppress adsorption may sometimes show bleed from the vial, which can compromise the quantitativeness of the analytes. In this context, we have prepared a small-capacity polypropylene (PP) vial, Prototype A, with a special coating on the vial surface that suppresses bleed as well as hydrophobic and ionic adsorption. In this presentation, we will report the results of evaluating the adsorption properties and bleed of various PP vials, including Prototype A, with respect to peptides.

In the evaluation of bleed, we compared Prototype A with other PP vials. The encapsulated solvents included water and a mixture of water and organic solvents. The LC system was the Nexera (Shimadzu), and the mass spectrometer used was the LCMS-2050 (Shimadzu). For the evaluation of adsorption, we compared Prototype A with other PP vials and glass vials. The analytes were peptides, and LC/MS analysis was conducted. The analytical column used was the Shim-pack Scepter series (Shimadzu). The LC system was the Nexera (Shimadzu), and the mass spectrometer used was the LCMS-8060RX (Shimadzu).

In the evaluation of bleed, it was found that Prototype A exhibited bleed levels comparable to or lower than those of other companies' PP vials that claim low adsorption. In the adsorption evaluation, when comparing the peak area values of peptides, many of the other company's vials and glass vials showed no detectable peaks, while Prototype A showed detectable peaks. It is believed that the hydrophilic regions of the peptides adsorbed onto the glass vials due to ion exchange reactions, whereas the hydrophobic regions of the peptides adsorbed onto the other company's PP vials due to hydrophobic interactions. On the other hand, Prototype A is coated with a non-ionic hydrophilic group on the vial surface, which is thought to suppress both ion exchange reactions and hydrophobic interactions, achieving low adsorption. Based on these results, it can be stated that the small-capacity PP vial Prototype A successfully combines peptide adsorption suppression and low bleed through the hydrophilization treatment of the vial surface. (350 words)

*Keywords*: HPLC, adsorption, peptide, vial, bleed

# Application study of online trap column for fast sample cleanup

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Abstract body

In LCMS analysis of biological samples, sample preparation such as protein removal is required to exclude the effects of matrix components. Protein removal methods include denaturation with acids or organic solvents, removal by filters, as well as solid-phase extraction methods and column-switching LC systems.

A reversed-phase chromatography column coated with methylcellulose on the silica gel outer surface is a pretreatment trap column used in column-switching LC systems, which can remove plasma proteins and concentrate small molecule compounds. It demonstrates high reproducibility and durability in small molecule analysis of drugs in plasma. [1-2]

If this protein elimination ability can be used in the pretreatment of other protein-containing samples in the same way, a wide variety of sample preparation can be further speeded up. Therefore, we made prototypes of high-pressure columns (2 mm i.d.×30 mmL.) filled with similar coated silica gel to evaluate protein removal performance.

When the main components of milk protein were injected separately and the eluate from the column was monitored by UV detection, rapid protein elution from the column was confirmed. By increasing the flow rate of the mobile phase, the pretreatment time could be greatly reduced, and it was found that high-pressure columns are effective for efficient protein removal. In addition, a longer length of the pretreatment column increases the pressure but is advantageous for the capture of small molecules. The test sample was injected into the pretreatment column and the elution peak in the gradient after the pretreatment process was monitored by UV to confirm the retention behaviour. A recovery test with the addition of caffeine to a coffee beverage containing milk found that caffeine was captured in the pretreatment column without co-eluting with protein.

In the measurement of coffee beverages by automatic pretreatment using a column-switching system, each quantitative value was equivalent to that of corresponding offline protein removal sample, but the samples were not diluted, allowing for high-sensitivity measurements or low-volume sample injections. It also provided the effect of cleaning up coexisting ingredients.

*Keywords*: HPLC

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# Automated scale-up workflow from analytical to Preparative SFC

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In drug discovery and development laboratories, the synthesis, screening, and purification of target compounds are essential processes. During synthesis, impurities often accompany the main compound, making purification critical for precise analyses. Preparative reversed phase liquid chromatography (RPLC) and supercritical fluid chromatography (SFC) are commonly used methods for purification. However, these methods require chromatographic expertise and significant time investment, which hinders their widespread adoption. Thus, enhancing the efficiency of these techniques is crucial for researchers, particularly for medicinal chemists.

SFC offers advantages such as improved separation through appropriate column selection, yielding high purity products. It also reduces solvent consumption and increases productivity for preparative purification. However, developing effective preparative SFC methods remains challenging, as non-experts often perform these tasks, leading to less frequent use compared to RPLC.

This study investigates an automated scale-up workflow utilizing SFC-MS. The workflow features an automated function that recommends optimal purification columns through systematic screening and generates ideal preparative conditions based on analytical SFC-MS results. We employed three types of columns with varying retention selectivity for separating low molecular weight pharmaceuticals, enabling effective purification of diverse compounds.
An algorithm for calculating preparative gradient conditions from retention times obtained in screening analysis was developed using seven test compounds for each column.

We will present the details of the algorithm for calculating preparative gradient conditions from retention time obtained in screening analysis and, examples of its application to the SFC preparation of small molecule pharmaceuticals containing impurities.

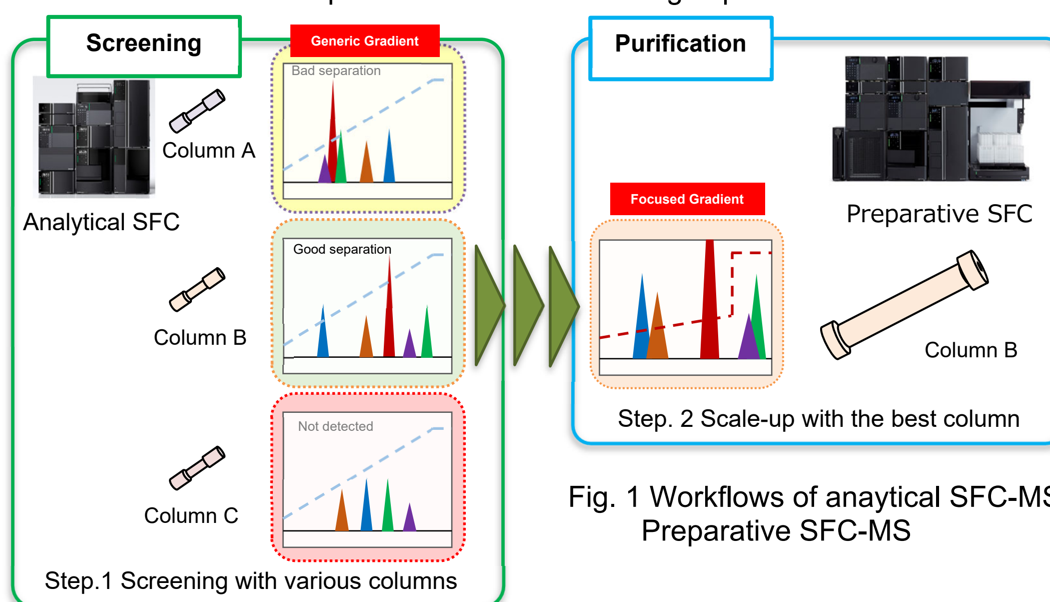


Fig. 1 Workflows of anaytical SFC-MS to Preparative SFC-MS

*Keywords*: SFC, purification, scale-up

# PRIMARY SUTRUCTURAL ANALYSIS OF PEPTIDES WITH MODIFIED AMINO ACIDS AND CYCLIC PEPTIDES WITH DISULFIDE BONDS

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Traditionally, the majority of pharmaceuticals have been small molecules produced by chemical synthesis. In recent years, the number of macromolecular drugs (e.g., antibody drugs and biopharmaceuticals) that use proteins as active ingredients have been increasing along with the development of biotechnology. In addition, as a new type of pharmaceutical product, middle molecular drugs, which combine the advantages of both small molecular drugs and antibody drugs, have been attracting attention. Peptide therapeutics are one of these new classes of middle molecular drugs. Peptide therapeutics can be manufactured by chemical synthesis like small molecular drugs, which is relatively inexpensive and can be produced in large quantities. They can easily penetrate cell membranes and act on the intercellular targets because they are also smaller in molecular size than macromolecular drugs. A peptide drug is designed to be pharmaceutically effective by well-considered its amino acid composition, sequence, and steric structure.

For structural analysis of peptides, it is common to use mass spectrometers and search engines using genome databases as in protein analysis. But some types of peptides are difficult to analyze using this method. The experimental result may differ from the mass number from genome database when the amino acid sequence of a peptide with modified amino acids is analyzed by a mass spectrometer, resulting in unreliable experimental data. In addition, it is very complicated and difficult to identify amino acid sequences using mass spectrometers without using databases.

We report peptide analysis using a protein sequencer with Edman degradation and a matrix-assisted laser desorption/ionization time-of-flight mass spectrometer, MALDI-TOF MS, to identify the amino acid sequence of a peptide containing a modified amino acid, or the steric structure of a peptide with two disulfide bonds.

*Keywords*: protein sequencer, HPLC, peptides, PTH-amino acids

References

# Multidimensional Chiral HPLC Analysis of Lysine and Its Metabolites in Human Urine

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Lysine (Lys) is an essential amino acid metabolized in mammals *via* the pipecolate and saccharopine pathways to pipecolic acid (PA) and 2-aminoadipic acid (2-AAA), respectively. These compounds are known to exist in both D- and L-forms owing to their chiral structures. Recent findings have revealed the presence of D-amino acids in mammals, highlighting their potential physiological roles and utility as biomarkers. However, due to the extremely low abundance of D-amino acids and interference from various coexisting biological substances, studies distinguishing their enantiomers *in vivo* are limited. Therefore, highly selective analytical methods are crucial for the precise evaluation of trace amounts of amino acid enantiomers in biological samples.

In this study, we developed a multidimensional HPLC system combining reversed-phase, anion-exchange, and enantioselective separations for the selective analysis of Lys, PA, and 2-AAA enantiomers in human urine. Urine samples were diluted with water and derivatized with 4-fluoro-7-nitro-2,1,3-benzoxadiazole (NBD-F) at 60 °C for 2 min. The reaction was terminated by the addition of an aqueous trifluoroacetic acid solution, and an aliquot was subjected to the multidimensional HPLC. Fluorescence detection of NBD-labeled amino acids was performed at 530 nm with excitation at 470 nm.

NBD-labeled amino acids were first separated by a reversed-phase column (Singularity RP18, 1.0 × 250 mm), then purified using an anion-exchange column (Singularity AX, 1.5 × 250 mm), and finally resolved on an enantioselective column (Singularity CSP-001S, 1.5 × 250 mm) for enantiomer separation. This system enables baseline separation for all target amino acids, with resolution values exceeding 2.5. In human urine samples, all target enantiomers were detected, with %D values of 6.8 % for Lys, 72.1 % for PA, and 1.3 % for 2-AAA. Additionally, slight fluctuations in the %D value of 2-AAA were observed over the course of the day, whereas the %D values of Lys and PA remained relatively stable throughout the day. These results demonstrate the applicability of multidimensional HPLC with pre-column fluorescence derivatization for trace-level chiral analysis in complex biological matrices and support further investigations into the physiological significance of the enantiomeric distribution of lysine and its metabolites.

*Keywords*: Multidimensional HPLC, amino acids, enantiomer separation

# Advanced Strategies for High-Efficiency Extraction, Ultra-Sensitive Detection of PFAS from Groundwater with Novel Adsorbents Using Fluorous Affinity

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Abstract

Per- and polyfluoroalkyl substances (PFAS) are widely used synthetic chemicals persisting in the environment and have been frequently detected in groundwater, raising concerns over their potential health impacts. However, accurate enrichment and separation of PFAS, particularly short- to medium-chain compounds, remain analytically challenging due to their high polarity and diverse chemical structures [1]. In Japan, recent nationwide surveys have identified multiple PFAS-contaminated regions, highlighting the urgent need for analytical methods with broad applicability and high selectivity.

Following our prior investigation on fluorous affinity and fluorinated stationary phases in high performance liquid chromatography (HPLC) [2], we aimed to improve PFAS retention by increasing the fluorine surface density on silica supports. A high-density fluorinated silica gel was synthesized using trimethoxy(1H,1H,2H-heptadecafluorodecyl) silane (F18-C10-silane) under optimized grafting conditions. FTIR analysis indicated intensified and broadened absorption around ~1200 $cm^{-1}$, consistent with successful high-density grafting of perfluoroalkyl chains. BET analysis showed reduced surface area (from 324.5 $m^2/g$ to 16.1 $m^2/g$) and pore size (to 9.5 nm), indicating partial pore filling. These results suggested that dense fluorinated grafting alters surface properties, forming a microenvironment conducive to F–F interactions and PFAS selectivity. The HPLC column packed with the modified material exhibited improving retention for mono- to hexa-fluorinated aromatic PFAS. Compared to both non-fluorinated C10-silica gel and our previous fluorinated phases, the new stationary phase enhanced retention strength. It also enabled efficient separation of perfluorinated esters (C5–C13) using purely organic mobile phases. These esters showed substantial retention on the new stationary phase, while non-fluorinated analogues exhibited almost no retention. Notably, this selective separation was achieved without aqueous modifiers, underscoring the material's strong fluorous affinity and enhanced fluorophilic interactions enabled by increased surface fluorination.

These findings demonstrate that fluorinated surfaces can serve as effective platforms for capturing a wide range of PFAS. Current efforts aim to optimize mobile phase conditions for real groundwater samples and support comprehensive PFAS analysis for future in vitro and in vivo toxicity studies. This approach may help connect environmental monitoring with health risk evaluation, supporting effective PFAS risk management.

*Keywords*: HPLC, PFAS, stationary phase, fluorous affinity, separation performance

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# A NOVEL PIM SAMPLING PROBE FOR ELECTRIC FIELD-ENHANCED DRUG EXTRACTION FROM BIOLOGICAL FLUID

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## Abstract

The analysis of biological fluids in clinical laboratories remains a critical yet challenging task, primarily due to the complexity of sample preparation and the potential for analyte loss during multi-step protocols. To overcome these limitations, we present a novel, low-cost, and disposable sampling probe that enables solvent-free, electric field-driven extraction of drug analytes directly from biological matrices. This innovative probe integrates a polymer inclusion membrane (PIM) as both the extraction and storage medium, streamlining the entire sample pretreatment process. By leveraging electrophoretic transport, the system facilitates efficient analyte isolation, concentration, and retention within the PIM. The proposed approach significantly reduces handling steps, enhances analyte recovery, and offers a practical solution for point-of-care or high-throughput clinical analysis [1]. The sampling probe comprises a non-conductive glass rod, a copper wire, and a PIM fabricated at the end of the glass rod. The glass rod is immersed in a homogeneous membrane solution containing an optimized composition of cellulose triacetate (CTA) as the base polymer, 2-nitrophenyl octyl ether (2-NPOE) as the plasticizer, and 1-butyl-3-methylimidazolium trifluoromethylsulfonate ([BMIM] [Otf]) as the ionic carrier, resulting in a 90 µm-thick PIM on the sampling probe [2]. The developed probe electrokinetically extracted ofloxacin from human serum, and human plasma. The practicability and reliability of the electric field enhanced-extraction were evaluated by HPLC-FLD to quantify the desorption of extracted ofloxacin. Under optimized conditions, a quantification limit of 20-200 ng/ml was achieved for the two biological fluid.

**Keywords:** Polymer inclusion membrane (PIM), Sample preparation, Electrokinetic extraction

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# LC-MS-Based Comprehensive Lipidomic Analysis of Plant-derived Exosome-Like Nanoparticles from Flower Petals

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Recently, plant-derived exosome-like nanoparticles (PELNs) have been in the spotlight as sustainable and biocompatible nanocarriers for pharmaceuticals and cosmetics. They boast benefits such as cost-effective production, stable supply, resistance to gastrointestinal digestion, and favorable skin and blood-brain barrier permeability. However, detailed knowledge of their lipid composition remains limited. In this study, apoplast-derived exosome-like nanoparticles from various fresh flower petals, such as rose (RANa: Rose Apoplast-derived Nanoparticles), were isolated using SEC after sequential filtration, without tissue disruption or ultracentrifugation. Characterization of the particles by NTA and TEM revealed particles with an average diameter of 170–220 nm at concentrations of up to $1.2 \times 10^9$ particles/mL.

Chloroform-free lipid extraction was performed for RANa, and comprehensive lipidomic analyses were conducted using LC-ESI-OAD-Q-TOF (Shimadzu LCMS-9050) and LC-ESI-QqQ-MS/MS (Shimadzu LCMS-8045). LC separation was performed using an Imtakt Unison UK-C18 MF for LCMS-9050-based non-targeted lipidomic analysis; a Shim-pack Velox C18 for LCMS-8045-based TG MRM analysis; and a Phenomenex Kinetex C8 for LCMS-8045-based phospholipid MRM analysis. The oxygen attachment dissociation (OAD) technique enabled non-targeted lipidomic analysis with precise localization of double bonds. This allowed identification of lipids, including ceramides and other species not detectable by Shimadzu MRM library methods, with data processed and annotated using MS-DIAL. In contrast, for targeted lipid quantitation using LCMS-8045, a triple quadrupole system of was operated under ESI conditions, employing multi reaction monitoring (MRM) based on a pre-validated lipid library. The triglyceride (TG) and phospholipid MRM libraries enable quantitation across 60 registered TGs and major phospholipid classes, including lysophospholipids, phospholipids, and sphingomyelins. OAD-based analysis revealed a total of 528 lipid species were contained in RANa, with the composition was dominated by unidentified lipids (54%), with TG (17%), DG (11%), Cer (12%), phospholipids (1%), and others (6%). The proportion of neutral lipids versus ceramides (40%) was higher than typically observed in PELNs. TG and DG species were rich in unsaturated fatty acids, while ceramides displayed structural diversity such as Cer-HS, Cer-NS, AHexCer, and Hex2Cer.

These findings suggest that RANa exhibits characteristics of solid lipid nanoparticles (SLN) or nanostructured lipid carriers (NLC), with stability for over two months at room temperature and potential benefits such as barrier improvement, antioxidant activity, and enhanced penetration. This study provides the first detailed lipidomic profile of rose petal-derived PELNs and supports their potential application as high-value cosmetic and pharmaceutical ingredients. In this presentation, we will also compare the lipid profiles obtained from OAD-based non-targeted analysis and MRM library-based targeted quantitation to highlight their complementary strengths and differences.

Keywords: LC-MS, lipidomics, PELNs, flower-derived extracellular vesicles, rose

# Development of molecularly imprinted polymers recognizing folic acid in aqueous systems for the separation of folic acid-modified liposomes

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In this study, we develop a molecularly imprinted polymer (MIP) that recognizes folic acid in aqueous condition for the separation of folic acid-modified liposomes.

Liposomes are nanoscale vesicles composed of biocompatible phospholipids and are commonly used as drug carriers. They can efficiently encapsulate and deliver both hydrophilic / hydrophobic drugs. According to these properties, folic acid-modified liposomes have attracted attention as a means of enabling targeted delivery to cancer cells that overexpress folate receptors. Although folate receptors are present in normal cells such as the placenta and intestines, they are localized at the apical surface of polarized epithelial cells, making them difficult to access from ligands in the bloodstream. In contrast, they are often exposed on the cell membrane of cancer cells. By exploiting this characteristic, liposomes modified with folic acid on their surface can selectively and efficiently deliver drugs to cancer cells. However, multifunctional liposomes require multiple processes, such as the introduction of imaging molecules and modification of target ligands, leading to complex manufacturing processes. As a result, batch-to-batch variability is likely to occur, making it difficult to uniformly control surface properties and particle size thereby limiting reproducibility and efficacy *in vivo*. These manufacturing challenges hinder scale-up and are one of the major factors preventing targeted liposome formulations from reaching clinical application.

This study aims to address the challenges of ensuring uniformity in particle surface properties and particle size by applying molecular imprinting technology to develop separation equipment that can selectively separate liposomes based on the recognition of folic acid. To achieve the objective, we focus on molecularly imprinted polymer (MIP). During polymerization for MIPs, template molecules are incorporated into the crosslinked polymer to construct selective binding sites. However, liposomes are structurally unstable in organic solvents and may cause structural damage. Therefore, in this study, we are optimizing the conditions for preparing MIPs that can selectively recognize and separate folic acid under aqueous conditions, with a view to applying them to liposomes.

# Temperature Responsive Mixed Mode Chromatography for Effective Separation of Ionic Biomolecules and Proteins

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Temperature-responsive chromatography have been investigating for analysis and separation of the various types of biomolecules [1]. We developed two types of temperature-responsive mixed-mode chromatography for the effective separation of ionic biomolecules and proteins. The temperature-responsive mixed-mode chromatography column was prepared by packing poly(*N*-isopropylacrylamide) (PNIPAAm)-modified beads and poly(2-acrylamido-2-methylpropane sulfonic acid) (PAMPS)-modified beads in various compositions (Fig.1A). The PNIPAAm-modified silica beads and PAMPS-modified silica beads were synthesized via surface-initiated atom transfer radical polymerization of NIPAAm and AMPS, respectively. The elution behavior of cold remedy medicines and monoamines from the prepared mixed-mode column was observed. The effective separation of these analytes was achieved by altering the composition of the PNIPAAm and PAMPS beads. Additionally, we developed another type of mixed-mode column using a mixed polymer brush composed of PNIPAAm and poly(*N,N*-dimethylaminopropyl acrylamide) (PDMAPAAm) as the ligand in the stationary phase (Fig.1B). The elution behavior of various acidic proteins from the prepared beads was also observed. Several proteins were adsorbed onto the column at elevated temperatures because of the enhanced electrostatic interaction of exposed PDMAPAAm and the increased hydrophobic interaction resulting from the dehydration of PNIPAAm. Using the developed mixed-mode column, a mixture of proteins was separated using a step temperature gradient. These results indicate that the developed temperature-responsive mixed-mode chromatography is a useful separation tool for ionic biomolecules and proteins.

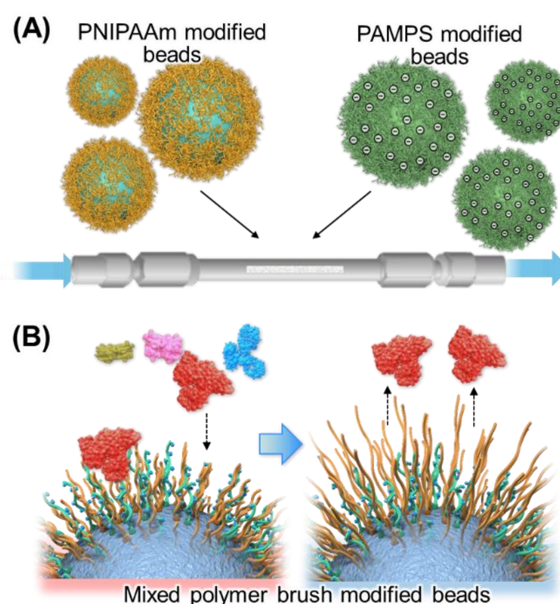


Fig.1 Temperature-responsive mixed mode chromatography using (A) mixture of two types of beads and (B) mixed polymer brush modified beads.

*Keywords*: Mixed mode column, Bioseparation, Electrostatic interaction, Protein Separation

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# A Rapid and Efficient Screening Method Development of Secondary Metabolites using LC-Raman

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Natural products have been played a key role in drug discovery due to their unique structures and diverse bioactivities [1]. However, owing to the complexity of their resources such as the microbial cultures and plant extracts including plenty of bioactive compounds, screenings for natural products have been time-consuming and laborious [2]. Although recent utilization of liquid chromatography-mass spectrometry (LC/MS), nuclear magnetic resonance (NMR) and genome techniques to the efficiency improvement of screenings, rapid screening and structural characterization remains challenging [3].

Raman spectroscopy is a non-destructive and non-contact analytical technique that enables rapid characterization of chemical structures. In addition, it works in aqueous environments. Therefore, liquid chromatography-Raman (LC-Raman) spectroscopy, Raman spectroscopy combined with sample separation by liquid chromatography (LC), has the higher potential to facilitate the compounds in the complex biological materials to be characterized. In this study, we especially introduce the screening for nitrogen-containing compounds in actinomycete cultures since they have a potential to be various drugs.

The secondary metabolites of actinomycetes isolated and cultured from soybean roots were used for the screening. First, the strains containing nitrogen-containing compounds were selected by Raman spectra of their chloroform extracts. Second, LC-Raman screening of the selected strains was conducted to identify the nitrogen-containing compounds using Raman spectra database. In this screening, after the separation by LC, the extracts were fractionated on well plates at every constant interval and dried to obtain LC-Raman spectra.

The compound eluted at the retention time of 8 minutes in the *Streptomyces* sp. extract was confirmed a nitrogen-containing compound exhibiting the Raman bands between 3100 and 3500 $cm^{-1}$ corresponding to NH stretching. The LC-Raman spectra of the compound agreed well with that of nocardamine [4], a bioactive compound. Raman and LC-Raman spectroscopy realized the rapid identification and characterization of the compounds by providing their structure information in the early stages of the screening.

*Keywords*: HPLC, Raman spectroscopy, Screening, Secondary metabolites

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## Functional Polymer Modified Interfaces for Cell Separation

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Regenerative medicine, which involves cell transplantation for the treatment of intractable diseases, has garnered significant attention. Current research focuses on temperature-responsive polymer-modified cell culture dishes and cell separation materials, where polymer-modified interfaces regulate cell adhesion for applications involving cell sheets and separations [1]. This study examined polymer-modified interfaces for the temperature-dependent control of cell adhesion (Fig. 1). Air plasma was applied to a glass substrate (24 mm × 50 mm, thickness 0.02 mm) to activate its surface, followed by humidification at 60% for 2 h. Chloromethylphenyl ethyl trimethoxysilane (CPTMS), an initiator for atom transfer radical polymerization (ATRP), was dissolved in toluene and reacted with the glass substrate. Functional polymers were grafted onto the glass surface via ATRP, utilizing $CuCl_2$ as a catalyst, tris[2-(dimethylamino)ethyl]amine ($Me_6TREN$) as a ligand, and ascorbic acid as the reducing agent. The polymer chain length was modulated by altering the monomer concentrations. The state of the polymer modification was assessed through contact angle measurements, and the molecular weight was determined using gel permeation chromatography (GPC). NIH/3T3 cells were seeded at 37°C on polymer-modified glass within a 35 mm dish and incubated. Subsequently, the temperature was reduced to 20°C to facilitate cell detachment. Variations in the contact angle after each modification step confirmed the success of the reactions. The molecular weight was controllable by adjusting the monomer concentration. Under optimal polymer chain length conditions, cells adhered at 37°C and detached at 20°C (Fig. 2). At 37°C, polymer dehydration induces hydrophobicity and cell adhesion, whereas rehydration at lower temperatures results in cell detachment from the surface. Alterations in the composition of the culture medium also influenced cell adhesion behavior, suggesting that both polymer state modification and medium composition can effectively regulate cell adhesion and detachment.

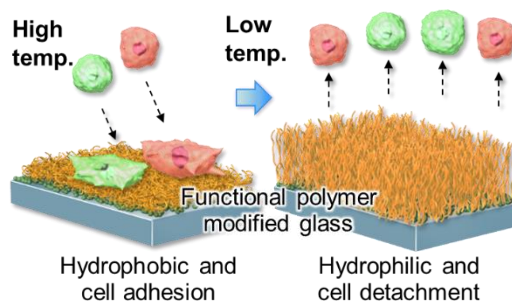


Fig.1 Temperature-modulated cell adhesion and detachment using functional polymer modified interfaces.

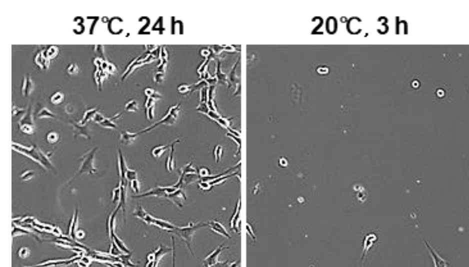


Fig.2 Cell adhesion and detachment on functional polymer modified interfaces.

*Keywords*: Functional Interface, Cell separation, Temperature-responsive chromatography

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# Evaluation of the behavior for fluorous affinity using HPLC

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Per- and poly fluoroalkyl substance (PFAS) are resistant to hydrolysis, photodegradation, microbial degradation, and metabolism, and are known as forever chemicals. Among these compounds, perfluorooctanoic acid (PFOA) and perfluorooctane sulfonic acid (PFOS) in particular have been used in a variety of applications in the past, and due to their resistance to degradation, they have been cited as causing environmental pollution and adverse effects on human health. They are likely to dissolve in water due to water solubility and are anticipated to persist in the environment for extended periods because of their high chemical stability, water, non-volatility, and non-biodegradability. To prevent environmental pollutions of PFAS, the development of selective separation mechanisms for PFAS at the disposal stage is necessary.

On the other hand, there are still unresolved aspects regarding the behavior of fluorine. As an example of fluorine behavior, it is known that organic fluorine compounds are not mixed with water layers and/or organic layers but instead mix with other organic fluorine compounds. Meanwhile, in high performance liquid chromatography (HPLC), organic fluorine compounds can be separated using a fluorine-modified column under reverse-phase conditions. In this separation, the fluorous affinity may contributes and the detailed mechanism has not evaluated [1]. In this study, we develop a new fluorine-modified column to elucidate the behavior of unresolved fluorous affinity (fluorine-fluorine interaction). We are also aiming to further improve the separation efficiency of fluorinated compounds using the newly developed column.

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# Development and Optimization of a Novel Silica-Titania Monolithic Stationary Phase for Capillary Liquid Chromatography

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Silica monoliths possess a dual-pore structure consisting of mesopores and macropores. This unique structure, characterized by high porosity and large specific surface area, enables high-efficiency separations at high flow rates. However, silica-based monoliths have a major drawback of low resistance to basic conditions. Due to its low resistance to basic conditions, the monolithic structure may collapse when basic compounds as mobile phase or sample are used [1, 2, 3].

To improve this drawback, we aimed to develop a basic-resistant silica-based monolithic column by introducing titanium, *i.e.* a material that has high pH stability, during the monolith synthesis process. In addition, capillary columns can contribute to reducing solvent waste and enable the use of expensive or novel mobile phase additives, thereby expanding the range of mobile phase compositions and reducing environmental burden [3, 4].

In this study, titanium tetraisopropoxide, *L*-lactic acid, and water were mixed at room temperature and stirred until a clear solution. We defined the resulting solvent as “titania”. A separate precursor solution was prepared by mixing 0.01 M acetic acid, polyethylene glycol (Mw = 10,000), urea, titania as well as tetramethoxysilane, and was stirred under ice-cooling. This mixed solution was filled into a pretreated capillary (0.320 mm I.D. × 0.450 mm O.D.), and both the capillary and remaining solution in the vial were heated *via* the sol–gel method. After heating, the white powder formed in the vial was also packed into the capillary.

Under various combinations, the effect of heating conditions during the sol–gel method on the phase separation behavior of the silica–titania monolith inside the capillary was evaluated. The basic-resistance of the white powder formed in the vial was tested using Tris–HCl and sodium carbonate buffer solutions, proving its durability of up to pH 11. Furthermore, chromatographic evaluation of the packed columns using various acetonitrile (ACN) concentrations revealed that the column could be operated under both reversed-phase as well as hydrophilic interaction liquid chromatography (HILIC) separation modes.

*Keywords*: Capillary LC, monolithic stationary phase, silica monolith, titania, sol-gel method

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# Development of a separation platform for biopharmaceuticals using a spongy-like polymer

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Abstract body

Recently, the focus of drug development has shifted from small-molecule compounds to macromolecular biopharmaceuticals and is further expanding to therapies that leverage extracellular particles and even whole cells. As therapeutic modalities diversify and grow more complex, separation and purification still account for roughly half of manufacturing costs, making faster development and cost reduction pressing issues for end users. In parallel, MS-based proteomics is indispensable for the analysis of these biotherapeutics, yet there is a strong demand for sample-preparation techniques that can rapidly process minute samples. Against this backdrop, we have begun developing an ultraporous, sponge-type monolithic polymer (“sponge monolith”) with high flexibility and flow-through characteristics.

The sponge monolith is produced by melt-kneading thermoplastic polymers to yield a flexible, porous separation matrix. It features very large through-pores (～µm) that provide exceptionally high permeability compared with conventional media, and it is composed of bioinert polymers. The matrix surface carries reactive epoxide groups, enabling facile immobilization of diverse protein ligands and functional moieties. Furthermore, by adding functional microparticles—such as hydrophobic chromatographic beads or titanium dioxide—during melt-kneading, hybrid materials with enhanced performance can be fabricated. Using this material, we achieved high-throughput separation and purification of antibodies and extracellular particles such as viruses and exosomes, as well as whole cells [1-6]. The sponge monolith has also been validated as an effective sample-preparation medium for low-input omics analyses, including proteomics [7]. In this presentation, we describe the performance of the sponge monolith and discuss its future prospects as a universal separation platform for the life sciences and pharmaceutical development.

*Keywords*: Liquid chromatography, spongy-like polymer, biopharmaceuticals, proteomics.

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# Liquid Chromatographic Separation of H/D Isotopologues Enabled by Aromatic π Interactions

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## Abstract

Deuterium (D), an isotope of hydrogen (H) containing an additional neutron, is approximately twice as heavy as ordinary hydrogen. Although deuterated and hydrogenated molecules exhibit similar chemical properties, they can be readily distinguished by mass spectrometry (MS) and nuclear magnetic resonance (NMR) due to the deuterium isotope effect. This phenomenon is widely investigated in organic synthesis, biosynthesis, and pharmaceutical development; however, efficient chromatographic separation of hydrogen/deuterium isotopologues remains limited. Liquid chromatography (LC), based on analyte partitioning between stationary and mobile phases, is highly sensitive to weak intermolecular interactions. Our previous studies demonstrated fullerene-modified stationary phases capable of enhancing π-π stacking, spherical recognition, and dipole-induced dipole interactions; however, the isotope effect in LC was not systematically examined.

In this work, we developed a graphene nanosheet-decorated silica stationary phase to harness graphene's strong π interactions for isotopologue separation. Graphene dispersion was prepared by sonicating graphite flakes in dichlorobenzene (DCB), settling for one week, and centrifuging to obtain the supernatant. Amino-functionalized silica ($SiO_2{\cdot}NH_2$) was reacted with 4-azido-2,3,5,6-tetrafluorophenyl succinate (PFPA·NHS) in toluene under dark conditions, followed by microwave-assisted decoration with graphene nanosheets. As shown in Figure 1, scanning electron microscopy confirmed uniform graphene coverage on the silica particles. When packed into an LC column and operated under normal-phase conditions, the material achieved clear separation of hydrogenated and deuterated benzenes. Benzene exhibited longer retention than Benzene-D6, consistent with stronger π-π interactions between graphene and protiated aromatics.

This study demonstrates a graphene-based stationary phase as an effective platform for investigating isotope effects and achieving isotopologue separation in LC. The findings provide new insight into isotope-surface interactions and offer potential applications in analytical chemistry, isotope labeling studies, and pharmaceutical analysis.

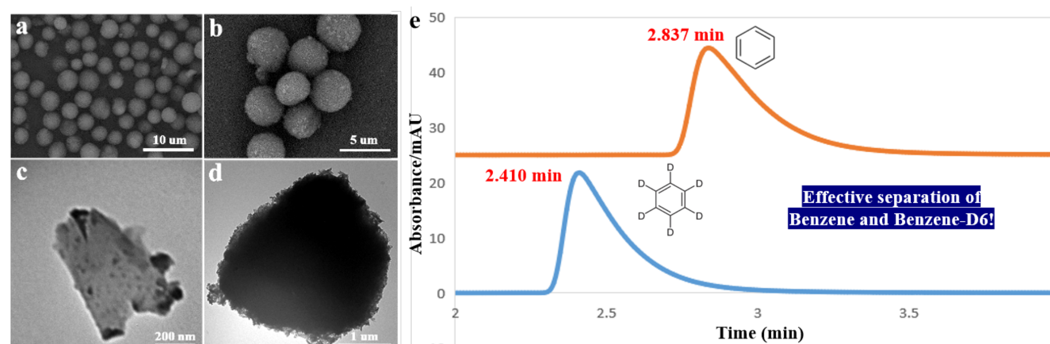


**Figure 1.** SEM images of (a) $SiO_2{\cdot}NH_2$ and (b) graphene-decorated $SiO_2{\cdot}NH_2$-PFPA·NHS; TEM images of (c) graphene nanosheets and (d) graphene-decorated $SiO_2{\cdot}NH_2$-PFPA·NHS; (e) chromatograms of benzene and benzene-D6 with hexane as the mobile phase.

*Keywords*: HPLC, H/D isotopologues, graphene, π-π interactions, microwave

## Development of Dendrimer-type Monolithic Capillary Stationary Phases for Mixed-mode Chromatography

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Monolithic stationary phases offer several advantages, including high fluid permeability, high mass transfer and their ease of preparation in a fused silica capillary [1]. Mixed-mode chromatography combines multiple separation modes to allow for simple and efficient separation of complex or practical samples. Dendrimers are molecules with a structure in which side chains regularly branch off from a core, and the number of functional groups at the ends increases exponentially with the number of branches (*i.e.*, generations) [2]. In this study, dendrimer-type monolithic stationary phases were synthesized by repeatedly reacting 1,4-butanedioldiglycidyl ether (BDDE) and ammonia on the surface of monolithic stationary phases. Anion exchange chromatography, reversed-phase chromatography, and hydrophilic interaction chromatography (HILIC) were performed to evaluate the mixed-mode function of each generation.

To obtain anchoring sites for the chemical grafting of the polymer at the inner wall of a fused silica capillary (0.32 mm I.D.), 3-(trimethoxysilyl) propyl methacrylate was reacted with silanol groups inside the capillary. Glycidyl methacrylate and ethylene dimethacrylate were dissolved in a porogen consisting of a mixture of ethanol, 1,4-butanediol, and pure water. 2,2'-azobisisobutyronitrile was dissolved in the mixture and subsequently sonicated for 5 min. The resulting mixture was subsequently introduced into the pretreated capillary and sealed at both ends. The capillary was immersed in a water bath at 60˚C for 24 h to allow for polymerization. Subsequently, the stationary phase was reacted with ammonia in the water bath at 80˚C for 1 h. Here we call the resulting stationary phase 0th generation. Subsequently, the stationary phase was reacted with BDDE in the water bath at 80˚C for 3 h and reacted with ammonia in the same way to obtain 1st generation. To obtain higher generations of the stationary phase, the reactions with BDDE and ammonia were repeated.

The retention times of polycyclic aromatic hydrocarbons decreased with increasing generations of the dendrimer-type stationary phases. This result indicates that the hydrophobicity of the stationary phases decreases as the generation of dendrimer-type stationary phases increases. The retention times of nucleobases were small values in each generation, while these of inorganic anions increased with increasing generations of dendrimer-type stationary phases.

*Keywords*: dendrimer, organic polymer monolith, mixed-mode chromatography, stationary phase

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# Examination of Analytical Conditions for Synthetic Peptides

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In recent years, medium-molecule drugs have gained attention as promising new therapeutics. Peptide drugs, such as insulin and GLP-1, are representative examples of medium-molecule drugs. Peptides used in peptide drugs exhibit various properties depending on their amino acid composition, sequence, and conformation. Liquid chromatography is commonly employed for the structural analysis and quantification of peptides; however, challenges such as adsorption and carryover often arise during peptide analysis. In this study, we investigated analytical conditions with a focus on addressing these issues in columns and vials.

Semaglutide, used as the evaluation sample, was purchased from AA Blocks. A stock solution of 1 mg/mL was prepared by dissolving 5 mg of Semaglutide in 5 mL of 1% formic acid/acetonitrile (75:25, v/v). This stock solution was utilized for column evaluation, while a diluted solution of 0.0001 mg/mL was prepared for vial adsorption evaluation.

To evaluate adsorption effects on column materials, analyses were performed using three types of Shim-pack Scepter C18-120 columns (Stainless Steel type, PEEK-lining type, and bioinert coating type) from Shimadzu. The LC system used was Nexera (Shimadzu). For vial adsorption evaluation, TORAST-H PP (Shimadzu GLC) vials were compared with two other types of low-adsorption vials from different vendors.

In the column adsorption evaluation, the PEEK-lining type and bioinert coating type columns demonstrated similar peak area intensities. In contrast, the Stainless Steel type column exhibited a 10% lower peak area intensity compared to the PEEK-lining type and bioinert coating type columns, suggesting adsorption on the metal surface.

For vial adsorption evaluation, changes in peak area intensity were measured from the time the sample was placed in the vial to 2 hours later. Among the three vials tested, the peak area of vendor A's vial decreased by 25% over 2 hours, vendor B's vial decreased by 48%, and the TORAST-H PP vial showed only a 9% decrease. These results confirmed that the degree of adsorption varies depending on the vial type.

In the poster presentation, we will also discuss the evaluation of carryover effects on columns and equipment. (335 words)

*Keywords*: HPLC, peptide, GLP-1, adsorption

# Development of Novel Organic Reaction Field for The Compounds with Catechol Structure using Triptycene Based Polymer

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Triptycene has characteristic paddle-wheel structure and the unique space surrounded by aromatic rings, called the internal free volume (IFV), which is known to be suitable for adsorption of specific molecules. Although triptycene-based polymer has been used as adsorbent materials by taking advantage of this space, the molecular recognition ability in the IFV has not been clarified in detail and there are few examples of the applications utilizing this property.

In organic synthesis, it is common to introduce the protecting group to prevent unwanted side reactions. However, the protecting step gives complicated reactions and decreases the yield. On the other hand, in our previous study, we used triptycene-based polymer as the stationary phase to evaluate the specific interactions between the aromatic rings in polymer and analytes, and we found that triptycene-based polymer exhibited selective adsorption toward catechol. Therefore, we consider the idea of applying triptycene-based polymer as a solid-phase material for the reaction involving the compounds with catechol structure.

In this study, triptycene-based polymers were synthesized by the solvent knitting method [1] and packed into the HPLC column to investigate adsorption characteristics and elution conditions by HPLC measurement. As a result, compounds with catechol structure were selectively adsorbed onto the polymer briefly. The compounds were strongly retained when acetonitrile was used as the mobile phase and easily eluted by adding formic acid. Furthermore, we employed dopamine in an acetylation reaction with and without the polymer. As a result, monoacetylated compounds were obtained in the presence of the triptycene-based polymer, whereas triacetylated compounds were mainly produced in the control experiment without the polymer.

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# Development of Multi-Dimensional HPLC Systems for the Analysis of Fermentation Related D-Amino Acids in Food/Beverage Samples

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Amino acids (AAs) play key roles in nutrition, adulteration, and quality assessment of natural products. Although L-enantiomers of AAs are found in foods and beverages as the predominant isomers, their D-form antipodes are also present in some of the edible materials. Specifically, the D-form of alanine (Ala), aspartic acid (Asp), glutamic acid (Glu) and serine (Ser) have been reported at relatively high levels (compared with those of other D-amino acids) in fermented products, and are considered to be associated with physiological functions relevant to human health through the daily diet. However, the amounts of these D-AAs are low in most cases, and are often accompanied by complex interferences present in the real-world samples, making their accurate determination challenging. To address this limitation, multi-dimensional HPLC systems coupled with fluorescence detection has been developed to achieve highly selective and sensitive analysis, and applied to the determination of D-amino acids in food/beverage samples.

The sample preparation procedure for 4 chiral amino acids (8 enantiomers) involved dilution of the sample in an aqueous solution, followed by pre-column fluorescence derivatization. To the diluted sample, Na-borate buffer (pH 8.0) and an acetonitrile (MeCN) solution of 4-fluoro-7-nitro-2,1,3-benzoxadiazole (NBD-F) were added. After heated at 60 °C for 2 min, 0.2 % aqueous trifluoroacetic acid (TFA) is added, and subsequently introduced into the multi-dimensional HPLC system. Fluorescence detection was performed with excitation and emission wavelengths of 470 nm and 530 nm, respectively.

For the development of the two-dimensional HPLC system, 7 Pirkle-type columns (Singularity CSP-003S, -013S, -403S, -603S, -001S, -019S, -021S, 1.5 mm × 250 mm) were tested for separating D-AAs and their L-counterparts in the second-dimension. As a result, the Singularity CSP-603 column (having *N*-(3,5-dinitrophenylaminocarbonyl)-L-diphenylalanine in the chiral recognition site and connected to the aminopropyl silica via a γ-aminobutyric acid linker) exhibited the most effective separation performances. Using mixed solutions of MeCN and methanol (50:50, v/v) containing 0.15–0.60 % formic acid, separation factors of 1.54–2.35 and resolutions of 4.44–11.22 were obtained for Ala, Asp, Glu and Ser enantiomers. Integrating a reversed-phase column (Singularity RP18, 1.0 mm × 250 mm) and the Singularity CSP-603S column in the first and second dimensions, a two-dimensional HPLC system was developed and several liquor samples were analyzed. As a result, D-Ala, D-Asp, D-Glu and D-Ser were detected and relatively high levels of D-AAs were found in 2 Thai wines produced from mangosteen and Syrah grape. Further investigations on their origins and physiological significance are expected.

*Keywords*: Multi-dimensional HPLC, amino acid, chiral separation, fermented foods

**Development of a Two-Dimensional Chiral LC-MS/MS System for the Determination of Alanine, Aspartic Acid and Serine Residues in Proteins Exposed to Stress Conditions**

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Recently, the presence of D-aspartic acid (Asp) and D-serine (Ser) residues is clarified in the proteins under age-related disease conditions along with the progress of analytical techniques. The isomerized amino acid residues have also been found in proteins exposed to various stresses conditions, drawing attention to the environment surrounding the proteins as a factor of epimerization. However, the conditions and mechanisms of isomerization remain mostly unclear, and determination of the D-amino acid residues under various degradation conditions is expected. In the present study, chiral analysis of amino acid residues was performed using our analytical platform combining the separation by a two-dimensional LC system and detection by a tandem mass spectrometer in the proteins stored under high temperature and basic pH conditions.

Protein samples were hydrolyzed in the gas phase with 6 M $^2HCl/^2H_2O$. After the hydrolysates were dried up, the residues were dissolved in water to obtain amino acid aqueous solutions. The amino acids were reacted with 4-fluoro-7-nitro-2,1,3-benzoxadiazole (NBD-F)/acetonitrile, then the reaction was terminated by adding an aqueous solution containing 0.2 % trifluoroacetic acid. A portion of the reaction mixture was analyzed by a two-dimensional LC-MS/MS system.

In the first dimension, the target amino acids were separated as their NBD derivatives in approximately 100 min using a microbore ODS column (Singularity RP18, 1.0 x 250 mm). In the second dimension, a narrowbore Pirkle-type column (Singularity CSP-013S having L-diphenylalanine in the chiral selector, 1.5 x 250 mm) was used, and practically sufficient chiral separations ($Rs \geq 2.44$) were obtained for all target NBD-amino acids. The method was applied to analyze D-amino acid residues in the proteins stored under various stress conditions. When ovalbumin (OVA) was dissolved in K/Na phosphate buffer (pH 7.4) and stored at 37 ºC for 2 weeks, a clear D-form peak was observed only for Ser (%D = 3.0). In case of OVA dissolved in K/Na borate buffer (pH 9.6) and stored at 37 ºC for 2 weeks, the %D of Asp and Ser increased up to 1.2 and 10.8. On the other hand, high %D values (1.3 for alanine, 15.3 for Asp and 30.3 for Ser) were observed when OVA was stored at pH 9.6 and 60 ºC for 2 weeks. These results indicate that isomerization of amino acid residues occurs when proteins are exposed to high temperature and basic pH conditions, and further investigations to clarify isomerization mechanisms and biological significance are expected.

*Keywords*: Enantiomer separation, D-amino acid residues, protein degradation, 2D LC-MS/MS

# Development of a Two-Dimensional HPLC System for the Determination of Alanylalanine Stereoisomers in the Plasma of Mice with Renal Dysfunction

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By the recent advances in analytical techniques, the presence of several D-amino acids and peptides containing D-amino acids has been demonstrated in mammals including humans. Especially, D-alanine (Ala) has been found in various mammalian species and reported to be associated with renal dysfunction. Additionally, the presence of dipeptides containing D-Ala in real-world biological samples has also been reported. Notably, the dipeptide AlaAla, composed of two Ala residues, has been shown to exist as its DD-form in the bacterial peptidoglycan. Therefore, chiral analysis of AlaAla stereoisomers and elucidation of their contents in mammals (the host of microflora) were expected. For analyzing trace levels of stereoisomers with similar physicochemical properties in complicated biological matrices, two-dimensional (2D) HPLC combining reversed-phase and chiral separations is an effective methodology. In the present study, a 2D-HPLC system has been developed, and applied to the enantioselective determination of Ala and AlaAla amounts in the plasma of mice with renal dysfunction.

The plasma samples were deproteinized by adding 20 volumes of methanol (MeOH), and a portion of the obtained supernatant was dried under reduced pressure. The residue was re-dissolved in water, then amino acids and dipeptides were reacted with 4-fluoro-7-nitro-2,1,3-benzoxadiazole (NBD-F). The reaction mixtures were subjected to the 2D-HPLC system with fluorescence detectors (Ex. 470 nm, Em. 530 nm).

For the first dimension, an ODS column (Singularity RP18, 1.0 x 250 mm) was used and the concentration of acetonitrile (MeCN) in the mobile phase and column temperature were examined. By using 7 % MeCN 0.01 % trifluoroacetic acid in water (50 ºC), the two NBD-AlaAla diastereomers and NBD-Ala were sufficiently separated (*R*s≥2.21). These 3 targets were also separated from other proteinogenic amino acids. For the second dimension, 4 enantioselective columns having different amino acids (Singularity CSP-001S having L-leucine, 008S having L-2-naphthylalanine, 013S having L-diphenylalanine and 019S having L-Ala, 1.5 x 250 mm) were tested. As a result, the Singularity CSP-013S column showed the superior separations for NBD-D/L-Ala, NBD-DD/LL-AlaAla and NBD-DL/LD-AlaAla. By using the mixtures of MeCN/MeOH (25/75, v/v) containing 0.015–0.1 % formic acid as the mobile phases, and target analytes were enantioseparated within 40 min (*R*s≥2.29). The developed 2D-HPLC system was applied to the analysis of plasma samples obtained from mice with renal dysfunction. As a result, D/L-Ala and DD-AlaAla were clearly observed, and the amounts of DD-AlaAla of mice with renal dysfunction were higher than those in control mice. Further investigations of Ala and AlaAla stereoisomers to clarify their tissue distributions and associations with disease are in progress.

*Keywords*: AlaAla, stereoisomer, 2D-HPLC, chiral separation, renal dysfunction

## Three-Dimensional HPLC Analysis of Glutamic Acid Enantiomers in Mouse Testis and Related Tissues

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Recent progress of analytical methods enables the determination of D-amino acids (enantiomers of widely observed L-amino acids) in higher animals including mammals. Among them, D-glutamic acid (Glu) was reported to be localized in the testis of some animals (mouse and Japanese kuruma prawn) and considered to be involved in the reproduction system. Although the determination of D-Glu in the testis and related tissues is expected, the amounts of D-Glu in mammalian tissues are extremely low and interfered with uncountable intrinsic substances. Therefore, a highly selective and sensitive analytical method is essential and in the present study, a rapid three-dimensional (3D) HPLC system has been developed and applied to the determination of Glu enantiomers in mouse testis and related tissues.

Mouse tissues were deproteinized with methanol (MeOH), and a portion of the supernatant was dried under reduced pressure. The residue was dissolved in water and amino acids were derivatized with 4-fluoro-7-nitro-2,1,3-benzoxadiazole (NBD-F) under alkaline conditions (pH 8.0). The mixture was heated at 60 °C for 2 min, and 0.2 % trifluoroacetic acid (TFA) in water was added to terminate the reaction. NBD-amino acids were detected by their fluorescence emission at 530 nm with excitation at 470 nm.

For the development of the 3D-HPLC system, the stationary and mobile phases were examined. As the stationary phases for the first and second dimensions, two reversed-phase columns with different retention characteristics, Singularity RP18 (1.0 × 150 mm) and Sunniest Biphenyl (1.0 × 150 mm), were tested. To solve the mobile phase compatibility issues, the retention of a stationary phase used in the second dimension must be stronger than that in the first dimension under the same mobile phase conditions. Adopting 12.5 % MeCN 0.05 % TFA aqueous solution as a mobile phase, NBD-Glu was eluted at about 20 min using both Singularity RP18 and Sunniest Biphenyl columns. On the other hand, adopting 20 % MeOH 0.05 % TFA aqueous solution as a mobile phase, NBD-Glu was eluted at 20 min using a Singularity RP18 column and at 35 min using a Sunniest Biphenyl column. Therefore, a Singularity RP18 column (20 % MeOH 0.05 % TFA in water) was selected for the first dimension, and a Sunniest Biphenyl column (12.5 % MeCN 0.05 % TFA in water) was selected for the second dimension. For the third dimension, a Singularity CSP-013S column (1.5 × 150 mm) was selected as the stationary phase, and a mixture of MeCN and MeOH (25/75, v/v) containing 0.4 % formic acid was used as the mobile phase ($Rs$ = 2.65 for Glu enantiomers, separated within 20 min). By using this 3D-HPLC system, determination of Glu enantiomers was performed in the mouse testis and related tissues. In the testis, the %D value was 0.15 and a distinct peak of the D-form was observed. On the other hand, in the related tissues (caput epididymis, cauda epididymis, vas deferens and seminal vesicle), the amounts of D-Glu were trace. Further studies to evaluate the amounts of D-Glu in the testis and related tissues of various animal species, and to clarify the biological significance are ongoing.

*Keywords*: 3D-HPLC, amino acid, enantiomer separation, D-Glu, testis

# Evaluating process development strategies through a multi-attribute mass spectrometry approach to minimize disulfide bond-related modifications in monoclonal antibodies (mAbs)

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## Introduction

mAbs rely on the structural integrity provided by interchain disulfide bonds linking the light and heavy chains, as well as intrachain disulfide bonds within the constant or variable domains. These bonds are susceptible to various modifications, including reductive cleavage, trisulfide formation, cysteinylation, and disulfide scrambling, due to their relatively low dissociation energy. Such alterations can affect antigen binding, Fc effector functions, and renal clearance, and may result in aggregation, raising safety concerns. While Protein A purification is commonly used, it faces challenges such as incomplete host cell protein removal and aggregation. We have developed new strategies to enhance mAb properties and minimize disulfide bond modifications. To assess these strategies compared to the conventional method, we extensively applied multi-attribute mass spectrometry.

## Methods

This study explores a modified purification strategy aimed at reducing disulfide bond-related modifications in mAbs. The effectiveness of this strategy is assessed by evaluating disulfide linkages and maintaining the structural integrity and quality of therapeutic mAbs. Peptide mapping using liquid chromatography-mass spectrometry (LC-MS) with Multi-attribute monitoring (MAM) approach was employed, specifically targeting cysteine-stressed and non-stressed IgG1 antibodies to identify potential disulfide bond alterations. To support the LC-MS findings, additional analytical techniques were applied, including size exclusion chromatography (SEC) for protein size distribution, differential scanning calorimetry (DSC) for thermal stability assessment, and polyacrylamide gel electrophoresis (SDS-PAGE) for examining protein purity and aggregation. These complementary methods provided a comprehensive evaluation of the purification strategy's impact on mAb quality and disulfide bond modifications.

## Results

Reduced and non-reduced peptide mapping fragment data, when analyzed using the MAM module, enables the simultaneous assessment of various critical quality attributes (CQAs). The results for in-house synthesized mAbs produced using both methods revealed that both samples had similar intact mass and exhibited similar glycoform profiles, indicating consistent production and similar post-translational modifications.

Non-reduced peptide mapping demonstrated comparable trends in disulfide bond shuffling between the C-mAb (conventional method) and the M-mAb (modified method), suggesting that both samples underwent similar levels of disulfide bond rearrangements. However, when subjected to reduced peptide mapping, a notable difference emerged. The C-mAb showed significantly higher levels of deamidation, suggesting that the modified method was effective in reducing such modifications, which are often associated with reduced stability and altered functionality.

Further analysis using SDS-PAGE indicated that the C-mAb was more prone to disulfide bond-related modifications, particularly under cysteine stress conditions. The M-mAb, on the other hand, showed fewer signs of degradation and aggregation, confirming that the new purification strategy effectively minimized disulfide bond-related alterations. SEC-HPLC provided additional confirmation, where the M-mAb exhibited a more stable profile compared to the C-mAb, with fewer aggregates observed.

Thermal stability assessments through DSC revealed that the M-mAb exhibited a higher melting temperature (Tm) and molar heat capacity, both with and without cysteine stress. This indicates that the M-mAb possesses superior stability, likely due to fewer disulfide bond modifications. In contrast, the C-mAb showed a lower Tm and reduced stability, further highlighting the benefits of the modified purification strategy in enhancing mAb quality. Overall, these preliminary results suggest that the modified purification method significantly improves the stability and integrity of mAbs, reducing disulfide bond-related modifications.

## Novel Aspect

The novel process modification enhances monoclonal antibody stability, utilizing MAM techniques to precisely analyze and reduce disulfide bond modifications.

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