



30TH INTERNATIONAL SYMPOSIUM ON MICROSCALE BIOSEPARATIONS

PÉCS, HUNGARY APRIL 27 – MAY 1, 2014



BOOK OF
ABSTRACTS



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PLENARY LECTURES

PL-1**SEPARATION SCIENCE – A FIFTY YEAR PERSPECTIVE AND THE FUTURE**

Karger, Barry L.

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With the receipt of the Beckman Medal, it is appropriate to look back on my 50 year career in bioanalytical chemistry and to look forward to the next 5 to 10 years (not possible 50 years ahead). My focus has been separation science, not just the principles but also the application to solve real problems. When I began my career, chromatography was where analytical chemists conducted research, first in GC and then this new field of high pressure liquid chromatography. Electrophoresis in a slab gel format was the domain of the biologists. When column operation became available (CE), I moved into this field and, along with a few others, was involved in developing technology for the Human Genome Project. Since then a key development has been the rapid advance of MS which continues to this day. While CE had a major impact on DNA separations, its wide applicability has been held back by a lack of effective coupling to MS. This is changing, and it is likely that CE/MS will have an impact in the coming years, both in a column and microfluidic format. Other areas in the coming period include nanoscale separations where new physical principles can be used to advantage, e.g. slip flow. I will discuss these new developments from the context of what I learned over my career. The most important principle is to understand the problem and goal to which your technology will be applied. Impact is what matters, and your technology is only as good as the problems it is able to solve. These points will be discussed in my presentation.

PL-2**SINGLE MOLECULE ELISA IN EXTENDED-NANO FLUIDIC DEVICE**

Kitamori, Takehiko

Department of Applied Chemistry, School of Engineering, The University of Tokyo,

One of the most promising applications of microfluidics is immunoassay, which is expected to be personalized and mobile in the near future as a clinical and social application in the aged society. We proposed micro ELISA system using a microfluidic device in the early period of microfluidics [1], and had already made it in practical and commercial use. Our scientific and technological curiosity has already gone into immunoassay in extended-nano fluidic system. Ultimate smallness of extended-nano fluidics makes it be ultimate small volume analysis which ranges from femto to even atto liter level. These values are several orders smaller than single cell volume. Creating methodology of analysis in this volume sector is really challenging and will give a big breakthrough to the bio and medical research fields.

We fabricated extended-nano channels into glass microchips, and succeeded in fluidic control at 10-100 nm in size. Detection at this size and volume is quite difficult, and therefore, we developed a differential interference contrast thermal lens micro detection (DIC-TLM), and we also succeeded in optical readout of non-fluorescent molecules in an extended-nano channel which was smaller than even optical wavelength. We have already developed three main methodologies for extended-nano fluidics, that is, fabrication, control and detection, and we applied them to develop extended-nano chromatography and immunoassay at atto liter and femto liter scale. One of the indispensable breakthrough technologies for these analytical applications is low temperature bonding method for glass substrates and covers. Surface modification on extended-nano channels is able to survive during the bonding processes in case the bonding temperature is at room temperature or a bit higher.

An ELISA system was realized in an extended-nano fluidic device. Depth of the channel was 200 nm, while the width was 3 μm . The length of the capture antibody immobilized local area was 135 μm . Antigen-antibody reaction volume was almost 100 femto liter. In addition to this device, we prepare several different dimensions devices. This ultrathin liquid layer is much smaller than diffusion length of target antigen protein and the target antigen proteins collide several 1000 times at the capture antibody immobilized surface. Therefore, the target antigen proteins may be perfectly captured by the immobilized capture antibody, and chemical amplification by enzymatic reaction and ultrasensitive readout by DIC-TLM may be able to detect the captured antigen. Actually, we succeeded in detecting countable numbers and even single target antigen protein by referring to Poisson distribution of the obtained signal.

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PL-3**USING MICROSCALE SEPARATIONS AND MASS SPECTROMETRY TO UNDERSTAND THE BIOLOGICAL MECHANISMS OF DISEASES**

Yates, III J.R.; Pankow, S.; Bamberger, C.; Wang, Y.; Fonslow, B.; Han, X.

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A component to understanding biological mechanisms in disease involves identifying the proteins expressed in cells as well as their modifications and the dynamics of perturbed processes. Several major technologies, but especially mass spectrometry, have benefited from large-scale genome sequencing of organisms. The sequence data produced by these efforts can be used to interpret mass spectrometry data of proteins and thus enables rapid and large-scale analysis of protein data from experiments. Advances in multi-dimensional separations as well as mass spectrometry have improved the scale of experiments for protein identification. Quantitative mass spectrometry can be used to study biological processes such as protein-protein interactions, development or the effects of gene mutations on pathways. Recent studies on the loss of function mutant form of the Cystic Fibrosis Transport Regulator ($\Delta F508$) as it progresses through the folding pathway will be presented. Through the study of protein-protein interactions, we are beginning to understand the critical interactions regulating pathways for export or destruction. What is clear from these studies is that protein complexes are mixtures of different protein proteoforms that require new approaches to sort them out to establish their functional roles. We will show recent data using a CE system to investigate protein complexes.

PL-4**LC-MS OF PROTEINS USING SLIP FLOW CAPILLARIES**

Wirth, Mary J.; Wu, Zhen; Zhang, Ximo

Purdue University

Slip flow is a nanofluidic phenomenon that enhances flow rates for submicrometer particles in reversed-phase liquid chromatography. The phenomenon enables 0.5 μm particle diameters to be used with present commercial instruments, thereby giving higher peak capacities than conventional capillary UHPLC. Capillaries of 75 μm i.d. and 4.5-cm in length, packed with C18-modified 0.5 μm silica particles, exhibited ten-fold flow enhancements due to slip flow. This gives stable nanospray for LCMS with a reasonable separation length. We show that complex protein mixtures are separated with peak capacities on the order of 750 for intact proteins. The physical narrowness of the peaks provides for high sensitivity by virtue of maintaining a high concentration of protein in the eluting zones. The peaks widths are a few seconds, which is a time-frame compatible with high resolution mass spectrometers.

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PL-5

PUTTING ARTEFACTS TO WORK: TURNING DNA SEPARATION PROBLEMS INTO ULTRA-PORTABLE AND SENSITIVE LAB-ON-CHIP NA DETECTION SOLUTION

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Descroix, Stephanie¹; Malaquin, Laurent¹

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We shall present a new label-free DNA detection method with direct electronic read. It uses a nonlinear electrohydrodynamic phenomenon discovered by our group when trying to separate by capillary electrophoresis large DNA molecules: when subjected to high electric fields, suspensions of large charged macromolecules in microchannels, such as long DNA molecules, create "giant" dynamic concentration fluctuations [1], which ruin size fractionation [2]. These fluctuations are associated with large conductivity heterogeneities. More recently, we demonstrated the possibility to detect these heterogeneities using a contact-mode local conductivity detector. In order to decouple the detection electronics from the high voltage excitation one, an original and simple "doubly symmetric" floating mode battery-operated detection scheme was developed. A wavelet analysis is then applied to unravel from the chaotic character of the electrohydrodynamic instabilities a scalar signal robustly reflecting the amplification of DNA [3]. We also present recent and unpublished results, combining this method with isothermal Rolling-Circle-Amplification of DNA, which naturally yields molecules from hundred thousand to several mega base pairs. This allows for the development of very compact, low cost and energy-efficient chips for the specific and ultra-sensitive detection of DNA. Applications for portable devices in various fields of medicine, environment and biotechnology will be discussed.

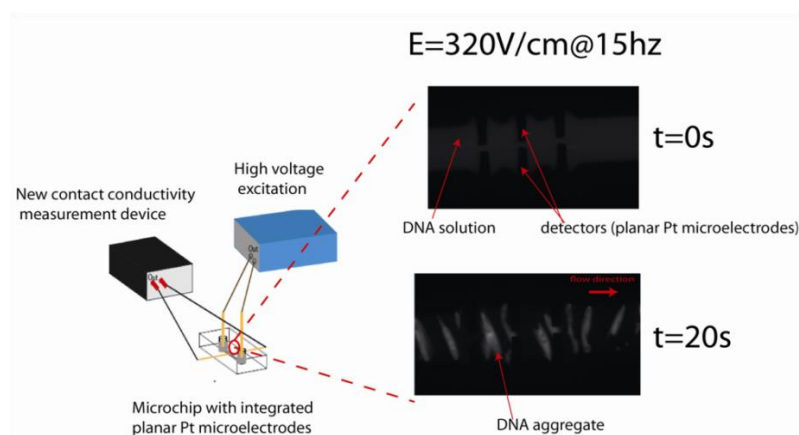


Fig 1: Principle of detection (left) and image of the electrodes setup (upper right) and labelled DNA aggregates (lower right)

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PL-6

IMPROVING PATIENT OUTCOME BY STRATEGIC TECHNOLOGY DEVELOPMENT IN THE HEALTHCARE SECTOR

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Global Healthcare is under pressure to meet the demands from patients to reach an improved efficiency in Cancer-, Neurodegenerative-, Diabetes and Cardiovascular diseases. Societies have difficulties in managing an increasing burden of healthcare costs. New drug characterization assays are central in providing evidence to the specificity and selectivity of drugs. Targeted drug development requires mode of drug action mechanistic studies in order to get approval from the FDA. Disease presentation and clinical sample collection are key strategic resources that need to be invested in order to understand disease mechanisms and to design the next generation diagnostics and treatments for Cancer patients. The technological developments provide a novel tool to achieve such highly detailed imaging by using Imaging Mass Spectrometer. In the first human study undertaken, we have been able to provide evidence of drug localization within targeted pathophysiology regions. In addition, we present data on the occurrence of, IRESSA, TARCEVA and Tiotropium within lung after dosing exposure, revealing specific localizations in tissue compartments at 10- μ m spatial resolution that reaches sub-cellular levels. Mass spectrometry imaging provides an effective means to measure the distribution of drug molecules in tissue samples. By comparing drug ion distribution patterns in tissue sections, we observed that following exposure, IRESSA, TARCEVA and Tiotropium parent ions and fragment ions were dispersed in a the central airways into the lung parenchyma and pleura. To meet hospital demands, Biobanks are developed in order to integration a collection of clinical samples from health and disease that provides human information to aid the unmet need of patients. Interfacing Biobanlks to modern healthcare developments becomes intimately linked to archiving patient samples in large scale studies, building biobank archives. These developments are seen within current application of Personalized Medicine strategies of matching specific disease genotypes and phenotypes with specific pharmaceutical drug treatment has shown tremendous efficacy in several important diseases. The expansion of this treatment paradigm into other diseases has led to new thinking considering the diagnosis and treatment of disease, including "Precision Medicine".

PL-7**WHY DO WE LOSE IT? ANALYTICAL INSIGHTS INTO HUMAN NEUROMUSCULAR DEGENERATION**

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No matter what we do, eat or practice – sooner or later we all will start losing our abilities to control our bodily functions, maintain muscle control and innervations. Most of us will grow old and degrade by natural causes, while some of us unfortunately will be affected by fast or slow progressive diseases or even have accidents with traumas that will initiate dramatic degenerative effects. Amyotrophic lateral sclerosis (ALS) is the most common type of motor neuron disease in adult patients and is characterized by progressive muscle paralysis. However, the clinical tools for ALS diagnostics do not perform well enough and their improvement is needed. We have recently demonstrated that a shotgun proteomic approach coupled with relative quantitative dimethyl labeling (DML) method can analyze the global changes in the human skeletal muscle proteome from ALS patients. Very limited amount of muscle biopsy taken from randomized ALS patients and healthy controls were used for comparison. The underlying objective of our study was to identify potential protein biomarkers related to the development of ALS but also to be able to get in detail structural characteristics of protein modifications that are initiated by disease. These proteins are involved in several biological processes including muscle development and contraction, metabolic process, enzyme activity, glycolysis, regulation of apoptosis and transport activity. In order to eliminate a risk to confuse ALS with other denervation, muscle biopsies of patients with postpolio syndrome and Charcot-Marie-Tooth disease (negative controls) were compared to those of ALS and control samples. In parallel we have been monitoring patients from the intensive care unit (ICU) that after accidents and trauma ends up in assisted ventilation or respirator treatment. These patients experience dramatic muscle denervation and degeneration already after a few days of treatment. We have identified a number of different factors behind this phenomenon that also reveals new knowledge about the natural aging muscle.

KEYNOTE LECTURES (KN)

AND

ORAL PRESENTATIONS (L)

ORDER OF SESSIONS

KN-01

A LASER PRINTED, ROTATION-DRIVEN MICRODEVICE (RDM) IN POLYESTER WITH PASSIVE VALVE FLOW CONTROL FOR PROTEIN QUANTITATION AND CELL COUNTINGOuyang, Y.¹; Thompson, B.¹; Begley, M.²; Landers, James P.^{1,2,3}¹*Department of Chemistry, University of Virginia*²*Department of Mechanical Engineering, University of Virginia*³*Department of Pathology, University of Virginia*

The ever-increasing application of Lab-on-a-Chip (LOC) technology to real-world problems has elevated interest in these analytical devices. As the development becomes more application-driven and the functionality shown to be robust, flow control and cost-effective manufacturability of microdevices from inexpensive commercial-off-the-shelf materials becomes critical.

We have shown the utility for using polyester (overhead transparencies) as the substrate and printer toner to generate rotation-driven microdevices (RDMs) for a variety of analytical processes including DNA extraction, PCR and electrophoretic separation. We followed this by demonstrating that CD-like PeT microchips could be fabricated for fluidic transport controlled by centrifugal forces and valving by laser-printed hydrophobic valves. By simply using rotation speeds that generate enough centrifugal force, fluids could be mobilized through open channels and, at higher speeds, through hydrophobic valves. In a PeT RDM with two fluidic layers fabricated by laser ablating the microfeatures in the polyester sheet and then laminating using the toner for bonding, we show that a simple colorimetric protein assay can be carried out in an automated manner. In doing so, we highlight the ability to integrate a substantial number of fluidic control elements to achieve a basic diagnostic functionality (i.e., protein quantification). The functionality requires fluid metering, mixing, and aliquoting, and thus, represents an effective demonstration of fundamental operations required for a broad range of other applications. One of these involves cell counting via DNA quantitation based on the extent of magnetic bead aggregation as the optical signal. A bi-directional rotating magnetic field (RMF) was critical to effective magnetic actuation of the PeT RDM for multiplexed cell counting. Enumeration of white blood cells in the human blood samples was demonstrated with the RDM with reasonable accuracy for clinical applications (CV=10%). Fusing the cost-effective and versatile PeT RDM with a simple bi-RMF, we present a promising strategy for automation and multiplexing of limited blood count chemistries.

L-01-1**CAPILLARY ELECTROPHORESIS FOR MINIATURIZATION ENZYMATIC ASSAYS ON KINASES OF SIGNALING PATHWAYS**

Nehmé, Reine¹; Nehmé, Hala¹; Benedetti, Hélène²; Routier, Sylvain¹; Morin, Philippe¹

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L-01-2**THE COUPLING BETWEEN THE OPTIMAL EXPERIMENTAL CONDITIONS FOR THE PURIFICATION OF PROTEINS AND
THE FORMULATION OF A NOVEL TYPE OF ANTIBIOTICUM
WITH AN EXTREMELY HIGH SELECTIVITY**

Hjertén, Stellan

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Upon a review of all scientific papers I have published and of an article by Sober and Petersen I found that an extremely high resolution of proteins could be achieved by a combination of only two separation methods: anion-exchange chromatography (or free zone electrophoresis) and hydrophobic-interaction chromatography. These two (three) methods are based on the parameters negative net surface charge and net surface hydrophobicity of proteins (and viruses and bacteria). Accordingly, the same parameters may be expected to characterize the surface structure of both proteins, viruses and bacteria. This conclusion gives us immediately the information about the surface properties of a novel class of antibiotics for selective capture (= the inactivation) of pathogens: Their net surface charge should be the same as that of the pathogen and their net surface hydrophobicity somewhat higher than that of the pathogen for its selective capture of the pathogen. Two animal experiments confirm this conclusion.

KN-02**STRATEGIES FOR IMPROVING DETECTABILITY OF CAPILLARY ELECTROPHORESIS**

Kanemori, Koichi; Ota, Hiroya; Kawai, Takayuki; Naito, Toyohiro; Kubo, Takuya; Otsuka, Koji

Kyoto University

The analysis of carbohydrates is one of the important issues in biological/clinical diagnoses since carbohydrates are important in biological functions. Among various techniques for analyzing carbohydrates, capillary electrophoresis (CE) is advantageous due to its ability to achieve a rapid analysis with low consumption of samples. In conventional CE of carbohydrates, however, a troublesome derivatization of a sample is often required for the detection and/or separation since most of carbohydrates have no UV absorbance and no charge. To overcome this drawback, we focused on the affinity CE analysis using quinolineboronic acids (QBAs) which form complexes with cis-diol compounds like most carbohydrates. In a background solution (BGS) containing QBAs, the complexation provides the variations of both the fluorescence intensity and apparent electrophoretic mobility of the complex from those of native carbohydrates, resulting in both the label-free fluorescence detection and selective separation of carbohydrates. The variations of the emission-spectra of QBAs by the complexation were measured with adding sorbitol into QBA solutions. As a typical result, the emission intensity of 5-isoQBA was increased upon adding sorbitol. To investigate the effects of the complexation and pH of the BGS on the mobility of 5-isoQBA, the effective electrophoretic mobilities of 5-isoQBA and its complex with sorbitol were measured with varying pH. Since the complexation of 5-isoQBA with carbohydrate gave a negative charge to the neutral carbohydrate at pH 6.0–12.0, the electrophoretic migration of the complex was attained without any pre-column derivatization. Nine carbohydrates, including sorbitol, mannitol, galactitol, catechol, galactonic acid, arabinol, xylitol, meso-erythritol, and mannose, were analyzed in a phosphate buffer containing 5-isoQBA, which indicated the successful detection and separation of these carbohydrates without any derivatization.

The combination of an on-line sample concentration technique, large-volume sample stacking with an electroosmotic flow pump (LVSEP) [1, 2], with mass spectrometry (MS) in CE was investigated to improve the detectability. Constructing LVSEP-CE-MS has been difficult due to the lack of the outlet reservoir, since LVSEP requires a BGS from the outlet side of the capillary for pumping out the sample matrix. In this study, the sheath liquid poured into the electrospray ionization (ESI) interface was employed as the BGS, where the sheath liquid was successfully introduced into the capillary from the ESI interface. To evaluate the analytical performance of the system, three aromatic sulfonic acids were analyzed. As a result, the analytes were 75–120-fold concentrated in LVSEP-CE-MS compared to conventional CE-MS.

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SENTAN (JST); KAKENHI (MEXT) No. 24350039.

L-02-1**PROTEIN IMPRINTED MATERIALS FOR THE TARGET PROTEIN CAPTURE
IN THE PROTEOMIC SAMPLE**

Yang, Kaiguang; Li, Qinran; Liu, Jinxiang; Li, Senwu; Zhang, Lihua; Liu, Jianxi

*National Chromatographic R&A Center, Key Laboratory of Separation Sciences for Analytical Chemistry,
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With the recent development of proteome techniques, the interest in finding biomarkers from human plasma for clinical diagnosis has gained new momentum. However, the wide dynamic range of proteins in abundance, over 10¹⁰, brings great challenges to discover low abundance proteins with significant biological functions [1]. Therefore, in proteome study, it is indispensable to deplete high abundance proteins to identify low abundance ones. Antibody techniques have been proven the most effective methods [1]. However, with the consideration of stability, robustness and cost, the development of artificial antibody, molecularly imprinted materials, might be a state-of-the-art solution.

Three generations of protein imprinted materials were developed in our study for proteomics application. In the first generation, porous protein-imprinted monolithic polyacrylamide materials was prepared by 3-D imprinting strategy and applied to extract the target proteins from complex protein mixtures by affinity chromatography [2]. In the second generation, one new approach, combining metal-coordination with surface imprinting technology, was developed to prepare protein-affinity materials, which showed higher specific recognition ability towards the target proteins [3]. The other new approach in the second generation, hierarchical imprinting, was developed and applied for high abundance protein imprinting [4.] In the third generation, the epitope of the target protein was applied to overcome the difficulty in obtaining the pure protein as the template. Meanwhile, environmental-friendly polymer self-assembly technology, instead of polymerization, was applied to fabricate the materials. In conclusion, molecular imprinting would be a promising method for the proteomic study.

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L-02-2**APPLICATION OF MICRODIALYSIS IN PRE-CLINICAL PHARMACOKINETIC STUDY**

Lu, Chia-Ming¹; Lin, Lie-Chwen²; Tsai, Tung-Hu¹

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²National Research Institute of Chinese Medicine

Objectives: Microdialysis is a real-time and continuous sampling technique for small molecules. The basic principle of microdialysis is a passive diffusion following Fick's law, and the driving force is a concentration gradient allowing analytes to pass through a semi-permeable membrane. Based on the perspective of clinical pharmacology, only the protein-unbound fraction of a drug is available for absorption, distribution, metabolism and elimination, as well as delivery to the target sites for pharmacodynamic actions.

Methods: Blood, brain and bile microdialysis systems consisted of a microinjection pump, a microfraction collector and the appropriate microdialysis probes. The dialysis probes for blood (10 mm in length), brain (3 mm in length) and bile (7 cm in length) were made of silica glass capillary tubing arranged in a concentric design. For enterohepatic circulation study, the bile duct of the donor rat was cannulated proximal to the liver and the other end of tube was inserted through the bile duct into the duodenum of the recipient rat. Lamivudine is an antiretroviral drug which is used as one of the example to investigate protein-unbound form lamivudine in the rat blood and liver, and subsequently to examine its pharmacokinetics in rat.

Results: The regional brain distribution, the portion of drug that passes through the blood-brain barrier, and hepatobiliary excretion can be defined by the area under the curve (AUC) ratio of blood-to-brain (AUC_{brain}/AUC_{blood}) and blood-to-bile (AUC_{bile}/AUC_{blood}). Furthermore, the pros and cons of microdialysis are discussed, including the detailed surgical techniques in animal experiments from rat blood, liver and bile duct for the analysis of protein-unbound drug. The pharmacokinetic data of lamivudine demonstrated that the area-under concentration versus time curve (AUC) of lamivudine in blood and liver were 531.97 ± 37.60 and 682.49 ± 195.81 min $\mu\text{g/mL}$ after lamivudine administration (10 mg/kg, iv), the elimination phase half-life ($t_{1/2\beta}$) were 41.44 ± 4.19 and 38.26 ± 4.91 min, respectively. The results suggest that lamivudine provides an even distribution in blood and liver after drug administration.

Conclusion: The strategy of multiple sampling in a single animal largely reduces the use of experimental animals and receives more reliable data for pre-clinical pharmacokinetic study.

KN-03**EXTENDED ELECTROSPRAY NEEDLE FOR CE-MS SEPARATIONS**

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With the recent instrumental developments, capillary electrophoresis coupled to mass spectrometry (CE-MS) seems to be gaining momentum especially for analyses of complex biological samples. On-line CE/MS coupling is almost exclusively practiced with the electrospray ionization. Based on the fluidic connections of the separation capillary to the electrospray emitter, the interface designs are typically divided into two categories, i.e., the sheath and sheathless arrangement, with additional subdivisions such as coaxial design, liquid junction arrangement, etc. For the flow rates above 100 nL/min, electrospray is considered to be concentration sensitive. Thus, the sensitivity of the CE-MS setup reflects the instrument's ability to deliver the separated zones into the electrospray needle in the lowest possible volume of the spray liquid. In the simplest case the electrospray tip can serve also as the CE separation capillary without the need for any additional interface. In such an arrangement the CE separation column must be of sufficiently low internal diameter to provide stable and efficient spray and, in principle, only one high voltage power supply can be used, driving both the CE separation and the electrospray ionization. Under suitable conditions, with regard to the capillary size and background electrolyte conductivity, sufficient electric field strength for the electrophoretic separation can be established by the electrospray current passing through the CE column. This work describes the practical limits of this approach with respect to the internal diameter and length of the CE column, the composition and conductivity of the BGE and the applied separation/spray voltage. Several examples of CE/MS analyses of biomolecules will demonstrate the practical potential and limitations of this direct CE/MS connection.

L-03-1**DETECTION OF MONOALKYL CARBONATES BY CE-MS/MS**

do Lago, Claudimir L.; Vidal Denis T. R.; Francisco, Kelliton J. M.; Bezerra, Vagner

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Monoalkyl carbonates (MACs) or hemiesters of carbonic acid (HECAs) are an emerging class of substances with potential interest in biological processes. Although the knowing about existence of MACs dates back almost a hundred years, evidences that compounds of this class can be formed spontaneously from an alcohol and bicarbonate – in conditions similar to the biological systems – arose only recently [1, 2]. Previous studies with capillary electrophoresis with capacitively coupled contactless conductivity detection (CE-C⁴D) allowed the determination of mobility, diffusion coefficient, and ionic radius of several HECAs as well as kinetic and thermodynamic constants for the formation reaction in aqueous medium [1-3]. In a subsequent study, electrospray ionization mass spectrometry (ESI-MS) was introduced as an alternative technique [4]. The present contribution deals with the use of CE-ESI-MS/MS, which improves the limit of detection and selectivity.

The background electrolyte (BGE) was ammonium propionate (pH 8.7), which allows separation and detection of the MACs in the counter EOF flow. The ESI sheath liquid was ammonium propionate in water/methanol 1:1. As previously observed [4], mild ESI conditions favors the transfer of MACs to the gaseous phase. The selectivity was improved by MS/MS detection in the MRM mode by monitoring the transition of M^- to $[M - CO_2]^-$ at natural collision (0 eV) with N_2 . Due to the possible decomposition of the MACs during the CE run because of the Joule heating, a new thermostating case for the CE capillary was created.

Although loss of 44 u at low collision energy is a striking feature of the MACs compared to other carboxylates, the coupling with CE is even more advantageous in the detection of MACs because it gives an orthogonal dimension in cases as, for example, the solution prepared with allyl alcohol and $NaHCO_3$. Both monoallyl carbonate and $Na(OH)(HCO_3)^-$ have the same m/z 101. However, Na^+ migrates as a cation and, thus, the isobaric cluster is not formed during the detection of the CE peak of monoallyl carbonate. The separation of a mixture of MACs of ethanol, 2-propanol, 1-butanol, and 3-pentanol was also demonstrated. The increase of sensitivity compared to CE-C⁴D allowed for the first time the detection of monogeranyl carbonate: a MAC of a fatty alcohol that is a carbonate counterpart of the geranyl pyrophosphate (an important intermediary in the biosynthesis of sterols).

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L-03-2**RETHINKING THE PROTEOMICS STRATEGY FOR LC/MS - SCALING FOR OPTIMAL RESULTS**

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Nano-LC-MS is often used in Proteomics experiments, due to the high sensitivity of nanospray and optimal use of the small sample amount. Sample loadability using enrichment columns and ease of operation - however remain a challenge.

In recent years, UHPLC has improved speed and resolution in chromatography using sub-2-micron particles and increased pressure. Applying this approach in nano-LC-MS however, does not always lead to improved results for the Proteomics experiment. Also, electrospray ionization development has reduced the sensitivity gap between nanospray and standard electrospray, which brings up the question whether it is time to rethink the optimal setup for Proteomics experiments, especially when developing methods for routine applications.

KN-04**APPLICATION OF CORE-SHELL SILICA BASED CHIRAL STATIONARY PHASES IN NANO-LC AND CEC**

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Recently chromatographic stationary phases (SPs) silica-based on core-shell material have been successfully experimented and adopted for both chiral and achiral separations utilizing high-performance liquid chromatography (HPLC). The success in using such columns stands in the properties of the silica particles enabling fast analysis and high efficiency mainly due to the better homogeneity of the particles and the faster mass transfer (mobile and stationary phase). The structure of the phase is represented by a solid core with a very thin layer outside responsible for the interactions with analytes.

Based on the literature data, it seems that core-shell particles can offer valuable results especially when analyzing large molecules. However, recently, such SPs have been also applied for the separation of small molecules, e.g., chiral compounds of pharmaceutical interest.

In this communication the main advantages in using superficially porous (core-shell) particles in liquid chromatography will be briefly introduced. Afterwards the separation of chiral compounds by nano-liquid chromatography (nano-LC) and capillary electrochromatography (CEC) employing core-shell particles containing (adsorbed) cellulose derivatives will be illustrated. The main experimental parameter influencing efficiency and chiral resolution will be discussed.

L-04-1

CAPILLARY ELECTROPHORETIC CHIRAL ANALYSIS AND DETERMINATION OF HELIX INVERSION BARRIER OF HELQUATS USING SULFATED CYCLODEXTRINS AS STEREOSELECTORS

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Acad. Sci. CR, Inst. Org. Chem. & Biochem.

Helquats (helical *N*-heteroaromatic dications) represent a new class of functional organic molecules [1]. They were introduced as a structural link between helicenes and viologens (paraquat and diquat). Combining molecular characteristics of both these classes of compounds, chirality of helicenes and electrochemical properties of viologens, helquats possess potential to be applied in molecular electronics and catalysis. In the relatively simple synthetic procedure, helquats were obtained as racemates containing *M*- and *P*-helical forms [1, 2]. However, pure enantiomers are necessary for investigation of their properties depending on helical chirality. For monitoring of preparative resolution of racemic mixtures of helquats, a new capillary electrophoresis (CE) method was developed [3, 4].

Enantiomers of a series of 34 helquats comprising 5, 6 and 7 fused rings in the helical backbone were resolved by CE in acidic 22/35 mM sodium/phosphate background electrolyte, pH 2.4, using randomly or single isomer sulfated α -, β - and γ -cyclodextrins (CDs) as chiral selectors. Non-covalent interactions between dicationic helquat enantiomers and sulfated CDs provided negatively charged complexes, which were separated as anions at reversed voltage polarity (-12 kV) in untreated or hydroxypropylcellulose coated fused silica capillaries 50/375 μ m id/od, 300/400 mm effective/total length, and detected by UV-absorption at 200 nm.

Evaluation of CE analyses of the whole set of 34 helquats showed that at least one of the sulfated CDs provided baseline separation of all 34 helquats. Concerning the capability of the individual randomly sulfated CDs for helquat enantioseparation, the S- γ -CD separated completely 28 helquats (82% success rate), followed by S- β -CD with 22 helquats (65%) and S- α -CD with 16 resolved helquats (47%). From the single isomers sulfated CDs, the best results were obtained using 2,3-diacetyl-6-sulfo- β -CD with 27 resolved helquats (79%).

Additionally, CE was applied to determine helix inversion barrier of helquats, i.e. activation energy of mutual interconversion of *M*- and *P*-helquat helical forms. The configurationally labile helquats could not be resolved but enantiomers of more hindered helquat structures were separated by CE and their helix inversion barrier was found to be in the range 106 – 120 kJ/mol.

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L-04-2

COMPARATIVE ENANTIOSEPARATION OF β -BLOCKERS WITH TWO SULFATED β -CYCLODEXTRIN DERIVATIVES IN AQUEOUS AND NON-AQUEOUS CE WITH SPECIAL EMPHASIS ON ENANTIOMER AFFINITY PATTERNS

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A series of eight chiral β -blocker drugs, acebutolol, atenolol, carazolol, carteolol, carvedilol, propranolol, sotalol and talinolol, have been enantioseparated using two single component anionic β -cyclodextrin (CD) derivatives, namely heptakis (2,3-di-O-methyl-6-sulfo)- β -cyclodextrin (HDMS- β -CD) and heptakis (2,3-di-O-acetyl-6-sulfo)- β -cyclodextrin (HDAS- β -CD), in aqueous and non-aqueous capillary electrophoresis (CE). The main objective of this work was to study the influence of the nature of substituents (methyl or acetyl) in positions 2 and 3 on the CD derivatives and of the electrophoretic medium (water or methanol) on enantioselectivity and enantiomer affinity patterns (EAPs) for these structurally related compounds. The EAPs towards these two CDs were found to be opposite to each other in both aqueous and non-aqueous media for atenolol and talinolol while opposite EAPs towards the two CDs were only observed in aqueous CE (ACE) for carteolol and sotalol, and in non-aqueous CE (NACE) for acebutolol, carazolol and propranolol. On the other hand, opposite EAPs with a given CD derivative were also observed when the aqueous background electrolyte (BGE) was replaced by the methanolic BGE in some cases: for carazolol, carteolol, carvedilol and propranolol in the presence of HDMS- β -CD and only for acebutolol with HDAS- β -CD. The more pronounced tendency to changes in EAP with the BGE composition in the presence of HDMS- β -CD might be related to the fact that this CD derivative generally leads to lower affinities and enantioselectivities compared to HDAS- β -CD. The possible molecular mechanisms leading to these changes in EAP are being studied in both aqueous and non-aqueous BGEs using rotating frame nuclear Overhauser effect (1D ROESY) NMR spectroscopy [1-2].

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KN-05**QUALITATIVE AND QUANTITATIVE ANALYSIS OF PROTEIN MODIFICATIONS USING SHEATHLESS CAPILLARY ELECTROSPRAY MASS SPECTROMETRY (CESI-MS)**

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Traditionally, LC-ESI-MS and/or MALDI-MS are the analytical techniques of choice for the analysis and quantification of post-translationally modified proteins and peptides. However, it is well documented that analysis of especially phosphopeptides using LC-ESI-MS is not without challenges. Poor ionization efficiency, low separation efficiency and neutral losses etc. often impedes detection and characterization.

Thus, we evaluated the capability of CESI-MS for the separation and analysis of various common protein modifications using a PTM standard provided by the ABRF Proteomics Standards Research Group (sPRG). The PTM standard, consisting of 70 different modified peptides including 29 phosphopeptides, was analyzed with both CESI- and LC-ESI-MS. The results were compared with respect to the number of identified peptides and separation characteristics. The application of acidic conditions and an uncoated capillary result in a moderate suppression of the EOF. When working in positive mode (+30kV), a low flow rate towards the MS can be obtained and also the positively charged peptides migrate towards the MS. Applying this system, di-, tri-, and tetra-phosphorylated peptides, which are less positively or even negatively charged can be fully separated from all other positively charged peptides.

In the second part of the study we investigated core and linker histones, small highly basic proteins occurring in all higher eukaryotes in multiple subtypes. All histones are subjected to extensive post-translational modifications that are known to be significant for gene expression and DNA repair. Both the complexity and unique physical and chemical property of this protein family is a challenging task requiring the most advanced separation techniques and sophisticated instrumentation available. Although CESI-MS detection of histone modifications is limited by the mass loading capacity of the system, it has the advantage of providing a rapid overview of the modification status of intact proteins, allowing fast and reproducible comparisons between treated and untreated cells or different cell phases.

Finally, CESI-MS was used to characterize and quantify phosphoproteins and phosphopeptides present in a highly complex sample, the proteome of yeast. Stable isotope labeling in cell culture (SILAC) was used for relative quantification of two yeast proteomes. Protein extracts of these two strains were mixed and enzymatically digested using Lys-C. The resulting peptides were pre-separated by RP-HPLC and were analyzed by CESI-MS using a neutral capillary. A total of 1244 phosphorylation sites could be assigned and 1371 phosphorylated peptides quantified. 50 phosphopeptides were found to be differently regulated relative to the unphosphorylated protein. The work presented here shows the first quantitative phosphoproteomic approach that combines stable isotope labeling by amino acids in cell culture (SILAC) with CESI-MS separation.

L-05-1

**IN-SPRAY SUPERCHARGING OF INTACT PROTEINS BY CAPILLARY ELECTROPHORESIS-MASS SPECTROMETRY
USING A SHEATH LIQUID INTERFACE**Bonvin, Grégoire; Rudaz, Serge; Schappler, Julie*University of Geneva*

Capillary electrophoresis (CE) hyphenated with electrospray ionization (ESI) mass spectrometry (MS) is a suitable technique for the analysis of intact proteins. The main configuration to realize this coupling is the sheath liquid interface, which is characterized by the addition of a make-up liquid providing the electric contact as well as the appropriate flow and solvent composition for ionization and evaporation. One main advantage of this interface is that the composition of the sheath liquid can be adjusted to optimize the ionization without affecting CE selectivity and efficiency. In the case of protein ionization, this feature is particularly interesting to modulate their charge-state distribution (CSD).

In this study, we report for the first time the addition of supercharging reagents to the sheath liquid in CE-ESI-MS experiments of intact proteins to increase the maximum charge ($Z_{\max}$) of multiply-charged gas-phase ions, while maintaining acceptable separation performance. Various supercharging agents were tested (2-nitrobenzyl alcohol, 3-nitrobenzyl alcohol, 4-nitrobenzyl alcohol, and sulfolane) with different background electrolytes (BGE). Their impact was estimated for three model proteins (human insulin, human growth hormone, hemoglobin A0) exhibiting different properties in terms of size, structure, and flexibility. Their influence on the global sensitivity and CE performance for each protein was also assessed.

First, the impact of the main CE parameter on proteins' CSD was studied. Lower charge states were obtained when the pH of the BGE was increased, which indicated that although the sheath liquid plays an important role in protein ESI, the CSD is also driven by the BGE pH, which determines the protein charge state in solution prior to its ionization by electrospray. Then, the addition of supercharging reagents to the sheath liquid was successfully implemented to further modify the proteins' CSD. Among the supercharging reagents tested, 3-nitrobenzyl alcohol (*m*-NBA) and sulfolane were selected as appropriate additives because they presented the highest volatility avoiding clogging issues. Both compounds permitted a shift toward higher charge states in acidic condition. The addition of *m*-NBA permitted to fully protonate the strongest basic amino acids (*i.e.*, Arg, Lys, and His), whereas sulfolane exhibited a more effective supercharging effect compared to *m*-NBA, enabling the protonation of less basic amino acids. Under basic conditions, the tested reagents did not succeed in supercharging proteins and sulfolane even suppressed ionization for the most flexible protein. This result suggested that proteins that are negatively-charged in solution could not experience in-spray supercharging. As expected, the addition of supercharging reagents to the sheath liquid had no deleterious impact on the CE performance, regardless of the pH. The sensitivity remained globally unchanged for every proteins and supercharging additives, while in some cases sulfolane led to a sensitivity improvement.

This work has not been presented in international congress yet. It was submitted to *Analytica Chimica Acta* and is currently under minor revisions.

L-05-2

"PLUG-AND-PLAY" C18-SILICA MONOLITHIC MICROCARTRIDGES FOR THE ANALYSIS OF NEUROPEPTIDES BY ON-LINE SOLID PHASE EXTRACTION CAPILLARY ELECTROPHORESIS

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Capillary electrophoresis (CE), like many other microanalytical techniques, has poor concentration sensitivity due to the limited sample volume that can be loaded onto the capillary. Several strategies have been investigated in order to improve CE sensitivity. Today, the use of on-line solid phase extraction capillary electrophoresis (SPE-CE) is widely recognized as a powerful approach to overcome this major drawback [1-3]. In SPE-CE, an extraction microcartridge or analyte concentrator placed near the inlet of the separation capillary contains a sorbent which retains the target analyte, enabling a large volume of sample to be introduced. After rinsing to eliminate non-retained molecules, the retained analyte is eluted in a smaller volume of appropriate solution, resulting in sample clean-up and concentration enhancement, before the electrophoretic separation.

In this study, C18-silica monoliths were synthesized as a porous layer in open tubular capillary columns to be cut later into microcartridges for the analysis of neuropeptides by SPE-CE-UV and SPE-CE-MS [4]. First, several types of C18-silica monolithic (MtC18) microcartridges were used to analyze standard solutions of five neuropeptides (i.e. dynorphin A (1-7), substance P (7-11), endomorphin 1, methionine enkephalin and [Ala]-methionine enkephalin). The MtC18 sorbents, were especially selective against endomorphin 1 (End1) and substance P (7-11) (SP (7-11)). The best results in terms of sensitivity and inter-microcartridge reproducibility were achieved using the microcartridges obtained from a 10 cm open tubular capillary column with a thin monolithic coating of large through-pore size (1-5 μm). Run-to-run repeatability, microcartridge durability, linearity ranges and LODs were studied by MtC18-SPE-CE-MS. As expected due to the improved selectivity, the best LOD enhancement was obtained for End1 and SP (7-11) (50-fold with regard to CE-MS). Finally, the suitability of the methodology for the analysis of biological fluids was tested with plasma samples spiked with End1 and SP (7-11). Results obtained were promising because both neuropeptides could be detected at $0.05 \mu\text{g}\cdot\text{mL}^{-1}$, which that was a value close to the LOD for standard solutions ($0.01 \mu\text{g}\cdot\text{mL}^{-1}$).

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L-05-3**CE- ICP/MS FOR SCREENING PROTEINS TARGETED BY URANIUM**

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During the last decade, metalloproteomics has received increasing attention due to its widespread impact in toxicology, metabolomics, and neurophysiology. Once incorporated into an organism, metal selection by a protein is the result of multiple steps. Therefore, analytical approaches have to cope with this complexity and allow the analysis of metal speciation in complex samples. These difficulties are exemplified by uranium, which plays an important role in the nuclear fuel cycle. Its toxicity is chemical rather than radiological, and it accumulates in the skeleton and kidneys [1]. However, the mechanisms involved in both transport in blood and delivery to target organs remain unknown. Hence, identification and characterization of uranium transport proteins is key to develop efficient prevention and detoxification approaches.

In our research, Capillary Electrophoresis (CE) hyphenated with Inductively Coupled Plasma Mass Spectrometry (ICP/MS) was used to combine the less intrusive, high separation efficiency of CE with the capacity to sensitively detect multiple elements of ICP/MS. Additionally, a UV detector was installed to enable the visualization of proteins during the analysis. This method addresses two main challenges: 1) achieving high resolution separation and 2) maintaining the integrity of the uranium-protein complexes during the analysis. Specific electrophoretic buffers create working conditions close to physiological conditions and reduce adsorption of uranium onto the silica and the consequent dissociation of the protein-uranium complexes during the separation.

This method was used for the simultaneous characterization of the interaction between uranium and several proteins in the presence or absence of physiological competitors, e.g., carbonate, a strong complexing agent of uranium in blood. A mixture containing the main proteins in serum was analyzed, and the results were compared to affinity constants determined from individual proteins. Moreover, the interaction of uranium with calcium phosphate-binding proteins was investigated to provide information regarding the accumulation of uranium in bones.

In conclusion, a reliable and precise analytical method for uranium speciation was established. Although the silica on capillary surface can compete with target proteins for binding to uranium, the method offers valuable information about the interaction process between uranium and various proteins. This method could be further developed to study the speciation of uranium in more intricate biological samples.

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KN-06

RECENT STUDIES ON ENANTIOMER SEPARATION MECHANISMS IN CAPILLARY ELECTROPHORESIS

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Capillary electrophoresis (CE) is important techniques for analytical separation of enantiomers. It is clear that even only at the expense of higher peak efficiency CE may allow to observe enantioseparations for certain chiral analyte-selector pairs where the separation power of HPLC is insufficient for achieving this goal. In addition, chiral CE offers almost unlimited possibility from the viewpoint of adjustment of separation factor. Together with aforementioned conceptual advantages CE offers some favorable technical characteristics for achieving high separation selectivity. Thus, chiral stationary phases (CSPs) in HPLC contain commonly limited and predefined amounts of a chiral selector, whereas the concentration of a chiral selector is easily variable and just limited by the solubility (for charged selectors also with Joule heating) of a chiral selector in a CE buffer. In addition, the combination of two or more (chiral) selectors is technically much easier and not associated with instrumental difficulties in CE compared to column-coupling in HPLC. Again, two columns are coupled with given amounts of the chiral selectors in HPLC whereas the ratio of chiral selectors in a combination can be easily optimized in CE. Thus, chiral CE offers really enormous flexibility from the viewpoint of the adjustment of the separation selectivity. This in combination with the inherently high separation efficiency makes chiral CE a very powerful technique for separation of enantiomers. In addition, CE offers enormous possibilities for investigation of fine (stereochemical) mechanisms of non-covalent intermolecular interactions. The most important conceptual advantages of CE for the investigation of non-covalent interactions together with the high efficiency is the cumulative character of this technique. The combination of these two may allow observing the fine (enantioselective) effects in selector-selectand interactions which can be easily overlooked when non-cumulative techniques are used, or even with cumulative techniques that offer moderate or low efficiency. Several non-separative analytical techniques, such as nuclear magnetic resonance (NMR) spectroscopy, can be also used to investigate non-covalent interactions. A main conceptual difference between NMR spectroscopy and separative techniques like chromatography and CE is that in NMR spectroscopy no spatial separation of interacting species occurs as a consequence of those potential interactions. Therefore, the results of each interaction are not accumulated and the final result is that of a single selector-selectand interaction. Contrary to this, every increment of a single selector-selectand interactions resulting in different distribution of components between two phases (or one phase and one pseudophase) (spatial separation) sums up, justifying any final selectivity achieved by separative techniques. NMR spectroscopy complements CE well for studies aimed to better understanding of enantioselective non-covalent interactions in the liquid phase by providing the information about the stoichiometry, association constants and structure of selector-selectand complexes. The main topic of this presentation is a combined use of CE and NMR spectroscopy for better understanding of mechanisms of noncovalent interactions between cyclodextrins and chiral drugs exemplified with talinolol, propranolol and ketoprofen.

L-06-1**STEREOSPECIFIC CAPILLARY ELECTROPHORESIS ASSAYS FOR METHIONINE SULFOXIDE REDUCTASE ENZYMES**

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Under conditions of oxidative stress, L-methionine (L-Met) residues in peptides and proteins are easily oxidized to L-methionine sulfoxide (L-Met(O)) by reactive oxygen species. L-Met(O)-containing proteins accumulate during ageing and may play a role in degenerative diseases. Protein-bound as well as free L-Met(O) can be reduced by methionine sulfoxide reductase (Msr) enzymes, a group of thiol oxidoreductases, protecting cells against oxidative damage. Because of the chirality of the sulfoxide moiety, L-Met(O) exists as the pair of diastereomers, i.e., L-methionine-(S)-sulfoxide (L-Met-S-(O)) and L-methionine-(R)-sulfoxide (L-Met-R-(O)). For the reduction of the diastereomers, stereospecific Msr enzymes exist. MsrA enzymes reduce free and protein-bound L-Met-S-(O) while MsrB enzymes reduce protein-bound L-Met-R-(O) with little affinity for free L-Met-R-(O). The latter is reduced by methionine-(R)-sulfoxide reductase (fRMsr) [1, 2].

The present contribution will report the development and application of validated offline and in-capillary assays for the analysis of the stereospecificity of Msr enzymes. The separation of dabsyl-L-Met(O) diastereomers and dabsyl-L-Met in a successive multiple ionic-polymer coated capillary using a 35 mM phosphate buffer, pH 8.0, containing 25 mM sodium dodecyl sulfate as background electrolyte and an applied voltage of 25 kV was employed for an offline assay for the analysis of the reduction of free L-Met(O) by Msr enzymes. β -Alanine was used as internal standard. Following enzyme incubations, derivatization of L-Met(O) and the product L-Met was performed with dabsyl chloride. The stereospecificity of human Msr enzymes including the Michaelis-Menten kinetic data could be determined.

An in-capillary assay used Fmoc-L-Met(O) as Msr substrate. Separation of the diastereomers as well as the product Fmoc-L-Met was achieved in a successive multiple ionic-polymer layer coated capillary using a 50 mM Tris buffer, pH 8.0, containing 30 mM sodium dodecyl sulfate as background electrolyte and an applied voltage of 25 kV. 4-Aminobenzoic acid was employed as internal standard. An injection sequence of incubation buffer, enzyme, substrate, enzyme and incubation buffer was selected. Following optimization of mixing time and mixing voltage the assay was subsequently applied for the analysis of stereoselective reduction of Fmoc-L-Met-S-(O) by human MsrA and of the Fmoc-L-Met-R-(O) by human MsrB.

An assay employing a peptide substrate was developed for Lys-Ile-Phe-Met(O)-Lys-DNP. Separation of the diastereomers and the reduced product was obtained in fused-silica capillary using 50 mM Tris-HCl, pH 8.0, containing 12 mg/mL sulfated β -cyclodextrin as background electrolyte. The assay was applied to study the reduction of L-Met(O) by Msr enzymes.

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L-06-2

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ENANTIOSEPARATION OF UNUSUAL AMINO ACIDS

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Unusual amino acids are those amino acids that are neither found in proteins assembled during protein biosynthesis nor generated by posttranslational modifications. The unusual amino acids are found mostly in plants and microorganisms and arise as intermediates or as the end products of metabolic pathways.

In recent years, as key components of numerous bioactive molecules, pharmaceuticals and peptidomimetics, unusual amino acids (especially newly synthesized conformationally constrained α -, β - or γ -amino acids) become increasingly important. Determination of the biologically active conformation of peptide hormones is an important goal in modern biology. Since most peptide hormones are highly flexible molecules with numerous conformations possible under physiological conditions, one useful approach involves the introduction of conformational constraints. In this approach secondary structural features such as α -helix, β - and γ -turns, *etc.* are built into the peptides in order to stabilize specific structures.

The wide-ranging utility of unusual amino acids has led to considerable interest in their enantioselective syntheses, which require analytical methods for a check on the enantiopurity of the final products.

Direct HPLC methods for the enantioseparation of unusual secondary α - and β -amino acids were carried out on different types of selectors. Recently developed *Cinchona* alkaloid-based chiral stationary phases, Chiralpak ZWIX(+)TM and ZWIX(-)TM exhibited highly efficient separation ability toward unusual secondary α -, β^2 - and β^3 -amino acids. Due to their zwitterionic nature, the chiral selectors in principle allow the enantiomeric separation of a remarkably broad scale of ionizable chiral analytes, ranging from acidic to basic and zwitterionic compounds.

The separations were carried out in polar-ionic mode applying methanol/acetonitrile mobile phase containing different acid and base additives (formic acid, acetic acid, phosphoric acid, ammonia, ethylamine, diethylamine, triethylamine and propylamine). The different amines and acids greatly influenced not only retention but selectivity and resolution too. The separation was optimized by variation of methanol/acetonitrile and acid/base ratio. Of the acids acetic acid, while from the amines triethylamine proved to be the most promising with respect to enantioseparation. In some cases direct correlation was observed between the structure of analytes and chromatographic parameters.

Experiments were performed at constant mobile phase compositions in the temperature range -5 - 55 °C in order to study the effects of temperature, and thermodynamic parameters were estimated from plots of $\ln k$ or $\ln \alpha$ versus $1/T$. Some mechanistic aspects of the chiral recognition process are discussed with respect to the structures of the analytes. It was found that the enantiomeric separations were in most cases enthalpically-driven, but entropically-driven separation was also observed. The sequence of elution of the enantiomers was determined in most cases.

Acknowledgements

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L-06-3**CHIRAL ANALYSIS OF AMINO ACID NEUROTRANSMITTERS AND NEUROMODULATORS**

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Both excitatory and inhibitory amino acid neurotransmitters have important role in the function of the central nervous system (CNS). Glutamate and aspartate are the primary excitatory transmitters, while glycine is the main inhibitory transmitter besides gamma-amino butyric acid (GABA). It was also found that low amounts of D-enantiomers of some amino acids are present in the CNS and their neuromodulator functions are under intensive studies. Various analytical methods have been reported for quantifying amino acid enantiomers. Capillary electrophoresis is especially useful for separation of small polar compounds available in limited sample volume. However, the detection of amino acids is problematic as they lack strong UV absorption. Their sensitive detection thus requires fluorescence derivatization, allowing the application of laser-induced fluorescence (LIF) detector.

First we studied the role of aspartate in neurotransmission during learning and memory formation, thus its concentration along with glutamate was needed to be determined in brain microdialysate samples. Three commonly used fluorescent tags have been tested for labeling the analytes. Our results suggest that contrary to the excellent intrinsic sensitivity of LIF detection, limit of quantification about 100 nM can be reached, because of the fair efficiency of the derivatization reaction at low analyte concentration. Consequently the LOQ is about one to two orders of magnitude higher than the limit of detection.

D-Amino acids are gaining increasing interest as modulators of neurotransmission, especially during neuronal development and neurogenesis, suggesting their involvement in neuronal plasticity and functional adaptation of the nervous system. Although the presence of several D-amino acids in mammalian brain has been demonstrated, the most significant role of D-serine and D-aspartate as modulators of NMDA-glutamate receptors has been described.

CE-LIF methods for chiral separation and quantification of fluorescently labeled amino acids, among them D-aspartate and D-serine have been developed. Following derivatization the amino acids of interest are negatively charged, thus a positively charged amino-cyclodextrin: 6-monodeoxy-6-mono(3-hydroxy)propylamino- β -cyclodextrin was chosen as chiral selector. Unlike some neutral cyclodextrins this selector was found appropriate for chiral resolution of the studied amino acids. In case of achiral co-migrations dual cyclodextrin system was applied to adjust the selectivity of the separation.

The applicability of the method has been shown for the analysis of D-amino acids in brain tissue samples, and microdialysates of experimental animals, as well as extracts of cell culture. Differential distribution of D-serine and D-aspartate in the brain areas has been demonstrated. In the chicken brain these D-enantiomers were most abundant in the medial striatum of six-hour old chicken, where their concentration was 21 and 18 nmol/g wet tissue, respectively. Their concentration has shown considerable age-dependence. D-Glutamate has not been detected in the brain samples. In the microdialysates 50 nM change in the concentration of D-serine could be measured with appropriate accuracy. However, the method lacks the sensitivity for quantitative determination of D-aspartate in these samples, where about one tenth of the extracellular analyte concentration is present due to the efficiency of the sample collection.

KN-07**COMPARATIVE GLYCOPROFILING OF HIV GP120 IMMUNOGENS BY CAPILLARY ELECTROPHORESIS AND MALDI-MS**

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Glycoprofiling of proteins with multiple glycosylation sites is a challenging task, not only because of the sugar components in a given oligosaccharide can have different positions and linkages, but also the occupancy and microheterogeneity nature at the different sites. High performance capillary electrophoresis is one of the frequently applied techniques for detailed glycosylation analysis. The use of fluorophore labeling assures very high sensitivity, especially with laser induced fluorescence (LIF) detection. Targeted exoglycosidase digestion with sialidase, fucosidase and other sugar specific enzymes is a powerful additional tool to obtain precise structural information (linkage and position) of complex glycosylation patterns. MALDI-MS is on the other hand, a useful orthogonal method for rapid semi-quantitative glycan profiling of material limited samples.

The HIV Envelope glycoprotein (Env) is a particularly perplexing molecule from a glycan analysis perspective. Env is also the sole target for neutralizing antibodies and is of key interest in the field of rational immunogen design. The HIV surface subunit, gp120, contains most of the antigenic surface of the molecule, and the moderate efficacy results of recent HIV vaccine trials has revitalized interest towards the use of soluble gp120 constructs as vaccine immunogens. Env is nearly 50% carbohydrate by mass with 20 to 30 N-linked glycosylation sites, and the glycosylation profile has been shown to influence both antigenicity and immunogenicity. Additionally, several key epitopes have glycan components as some of the most broadly neutralizing antibodies are glycan dependent. Therefore, glycosylation profiles of gp120 immunogens are a key variable for the elicitation of an effective humoral response against HIV. Such effects have already been observed due to differences in the degree of sialylation of gp120 subunits from different expression systems.

In this presentation, global N-glycosylation patterns are compared for gp120 constructs from various isolates. Capillary electrophoresis with laser induced fluorescence detection combined with the use of exoglycosidase digestion steps was used to analyze the peak distribution in the glycosylation profiles and to elucidate the structures. Because of apparent co-migration of several structures, MALDI-MS was also applied to verify the molecular masses of the components identified in the samples. This comparative study revealed that while glycoform distributions are relatively invariant between similar isolates, gp120 construct modifications, such as gD tagging in recent RV144 trials, can substantially alter the glycosylation profile.

L-07-1

QUANTITATIVE PROFILING OF GLYCANS RELEASED FROM PROTEINS USING STABLE-ISOTOPE-CODED GLYCAN LABELING AND SHAPE-SELECTIVE HPLC HYPHENATED TO MS

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Introduction

Among all post-translational modifications, glycosylation is the most common one and plays crucial roles in various biochemical processes including cell adhesion, protein folding, receptor binding and signal transduction. Protein glycosylation analysis is still challenging due to the diversity of glycan structures regarding linkage position, branching, length and composition of the polysaccharide chains. Common diseases like cancer and inflammation are known to be associated with aberrant protein glycosylation profiles. The in-detail characterization of these glycan profiles and structures is required for improving our understanding of disease-related changes in their expression level as well as in their specific biological functions.

Experimental

Capillary HPLC was hyphenated to high resolution mass spectrometry (MS) for the characterization of oligosaccharide structures attached to proteins. Glycan Reductive Isotope Labeling (GRIL) of the oligosaccharides released from the proteins was chosen as labeling strategy aimed for improving accuracy and reproducibility of relative glycan quantitation. *N*-linked glycans were released enzymatically and derivatized by ¹²C₆/¹³C₆-aniline; *O*-linked glycans were released chemically by a one-step reaction covering beta-elimination and derivatization with d₀/d₅ 1-phenyl-3-methyl-5-pyrazolone (PMP). Porous graphitized carbon (PGC) as well as zwitterionic (ZIC)-HILIC surfaces were used as stationary phases for the size- and shape-selective separation of the labeled *N*-glycans. In an alternative approach, following the classical bottom-up proteomic stream, the site specificity of glycosylation was monitored when working on the level of glycopeptides.

Results

Using the described isotope labeling methods an improved relative quantitation of the different glycan variants could be attained. Aniline labeled *N*-glycans were separated according to size and linkage positions by means of ZIC-HILIC as well as by PGC surfaces, allowing so the relative quantitation of isobaric isomer variants. Different abundances of these glycan variants were found when comparing different model glycoproteins.

Conclusion and Outlook

Strengths and weaknesses of the different experimental/methodological approaches will be discussed in terms of sensitivity and type of structural information to be gained.

The described methodology is aimed at analyzing glycosylation of proteins secreted from cell cultures. In an *in vitro* setting the change of glycosylation pattern upon inflammatory stimulation of the cells will be monitored for establishing "pure" inflammation-associated glyco-signatures.

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L-07-2

LECTIN AFFINITY ENRICHMENT IN COMBINATION WITH MICROCHIP CAPILLARY GEL ELECTROPHORESIS FOR SENSITIVE AND SELECTIVE GLYCOPROTEIN ANALYSIS

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Glycosylations play a crucial role in a variety of processes including recognition events, immunology, and cancer biology. For gaining a better understanding of the biological function of a glycoprotein or to control the integrity of an engineered product, it is thus of importance to find new bioanalytical methods with high reliability and low time consumption. A very suitable platform is offered with the 2100 Bioanalyzer (Agilent Technologies) that enables microchip capillary gel electrophoretic (MCGE) analysis with high sensitivity comparable to and sometimes even exceeding results from conventional sodium dodecyl sulphate - polyacrylamide gel electrophoresis (SDS-PAGE) after silver staining [1].

Since method specific parameters are to date only available for unmodified proteins, a detailed evaluation for well-known model glycoproteins using MCGE was performed with respect to precision of sizing, accuracy of quantitation, and detection sensitivities. The results showed high reproducibility in quantitation and sizing comparable to those of unmodified proteins. With an increasing degree of glycosylation, however, the molecular weights determined by MCGE deviated from mass spectrometry and SDS-PAGE derived values. Investigating the dynamic range for on-chip staining in comparison to covalent fluorescent labeling prior to separation resulted in higher sensitivities for the latter.

For targeted analysis of glycoproteins in complex biological samples the combination of MCGE with preceding affinity enrichment is required. After covalent labeling of the sample the glycoproteins were specifically enriched with lectins covalently linked to tosylactivated magnetic beads (Life Technologies), eluted with buffers containing the competitive sugar, and analyzed by MCGE. In order to effectively diminish unspecific interactions, binding and elution conditions were optimized using again the set of model glycoproteins and non-glycosylated β -galactosidase as negative control. For validation of the method, human blood serum as a complex biological sample was analyzed using the developed strategy. Confirming selective enrichment the same sample was also separated by SDS-PAGE and glycoproteins were identified by tryptic *in-gel* digestion and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS). Serum was used either pure or after depleting the twelve most abundant proteins (Top 12 spin columns, Pierce) leading in both cases to the identification of merely glycoproteins next to serum albumin. The approach was further applied to the only fairly studied glycoproteome of the cellulose degrading fungus *Trichoderma atroviride* resulting in the identification of two proteins – one of which, β -galactosidase, is known to have several putative N-glycosylation sites in *Trichoderma reesei* [2]. For both experiments it was observed that lectin enrichment shows unspecific interactions (protein binding) of the beads with the investigated biological sample.

Taking the possible occurrence of unspecific interactions into account for data interpretation, the developed lectin affinity enrichment combined with MCGE proved to be a rapid and reliable method for selective and sensitive glycoprotein analysis.

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L-07-3

SIALIC ACID LINKAGE ANALYSIS ON GLYCOPEPTIDES USING CE-ESI-MS/MS

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A growing body of evidence suggests that the linkage of sialic acids on glycoproteins may become an important marker of disease progression [1]. A plausible explanation for this phenomenon is that sialic acids are often found at the end of the glycan chain, serving as a binding site for human lectins, toxins and pathogens. The prostate specific antigen (PSA), a marker for prostate cancer, is known to contain many different glycans at a single N-glycosylation site (N₆₉) and as such it represents a good model for studying the relationship between glycosylation and health status [2].

A combination of capillary electrophoresis (CE) and high resolution time of flight (TOF) mass spectrometry (UHR-QqTOF maXis Impact) was chosen as an analytical platform for this study. CE is known for its high separation efficiency and sensitivity, especially in combination with the innovative sheathless ESI interface from Beckman Coulter.

Commercially acquired PSA was reduced, alkylated and digested with trypsin prior to analysis. With our current setup we were able to identify 35 glycan compositions with a minor amount of PSA (1 ng protein weight). We found three clusters of either non-, mono- or di-sialylated glycopeptides in our electropherogram. Strikingly, we observed that within the clusters that contain at least one sialic acid, we detect several different peaks with the same glycan compositions, demonstrating isomer separation which appeared to be caused by the separation of differentially linked (α 2,3 and α 2,6) sialic acid containing glycopeptides. This was supported by linkage-specific derivatization of enzymatically released N-glycans, where the α 2,3-linked sialic acids are converted into a lactone while α 2,6-linked sialic acids form an ethyl ester. Furthermore, we confirmed α 2,3 sialic acid linkages using a linkage specific sialidase. Overall, we identified about 65 different glycopeptides on a single N-glycosylation site.

We are currently investigating if the pKa values of the differentially linked sialic acids differ from each other and whether this might explain the separation. To the best of our knowledge this sialic acid linkage-specific separation has hitherto not been described in literature. Therefore, we believe that this CE-ESI-MS/MS platform not only has great potential for studying PSA in prostate cancer research but also in other glycoproteomic workflows to study disease related alterations in glycosylation.

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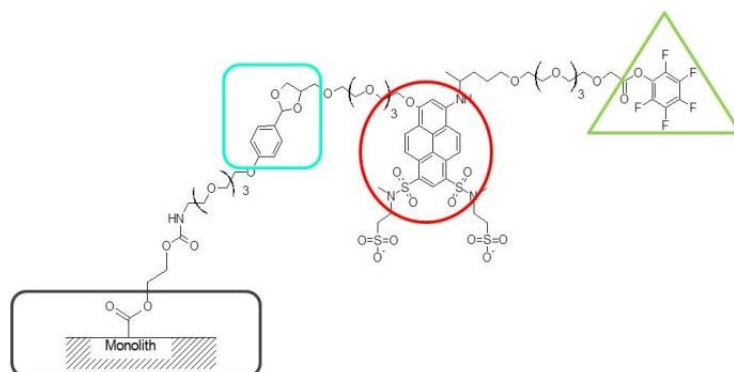
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KN-08

METHOD AND SOLID PHASE REAGENT FOR LABELING OF ANALYTES

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Analyte labeling (e.g., with a fluorescent tag) is straightforward when the analyte is monofunctional, but difficult when the analyte is multifunctional (e.g., proteins). Labeling is fast, when the concentration of the analyte is high, but slow when the analyte is present in a dilute solution. In order to mitigate both of these difficulties, we designed a labeling method called **SCALER** (short for **S**imultaneous **C**apture, **L**abeling and **E**fficient **R**elease) that relies on a new **Solid Phase Labeling Reagent (SPLR)** to integrate and optimize all steps of the labeling process. The design and use of **SCALER** will be demonstrated via the fluorescent labeling of amino-group containing analytes present in dilute solutions. Implemented in a pipette tip, the amine-reactive **SPLR** consists of four elements: (i) a hydrophilic monolith support, (ii) cleavable tethers anchored to the monolith and connected to (iii) a fluorophore that carries (iv) an amine-reactive arm (structure shown below). The hydrophilic monolith is synthesized from 2-methoxyethyl acrylate (MEA) and 2-hydroxyethyl acrylate (HEMA) as monomers and ethyleneglycol dimethacrylate as crosslinker. By varying the mole ratio of MEA and HEMA, the surface concentration of the hydroxyethyl groups serving as anchors for the cleavable tethers can be tuned as desired to regulate the surface density and spacing of the cleavably anchored reactive fluorophores. The hydrophilic tether incorporates an acid-cleavable 1,3-dioxolane ring formed from 4-hydroxybenzaldehyde and glycerol. The glycerol side of the 1,3-dioxolane ring is attached to position 6 of an 8-amino-1,3-disulfonamido pyrene fluorophore via an ether linkage. The amine-reactive group is implemented as a pentafluorophenol activated carboxylic acid group attached to the 8-amino group of the pyrene core of the fluorophore. The terminal groups of the disulfonamide substituents in the 1- and 3- positions of the pyrene core can be strong electrolytes, weak electrolytes or nonelectrolytes according to the needs of the separation and/or detection method used for the analysis of the labeled analyte (in our case, they are alkylsulfonate groups facilitating CE separation of the labeled analytes). This **SPLR** is used in the **SCALER** method that consists of the following sequence of steps. First, a dilute solution of an amino-group containing analyte in a pH 8 bicarbonate buffer is aspirated (repeatedly) through the **SPLR**-containing pipette tip to exhaustively remove the analyte from solution and immobilize (accumulate) it on the **SPLR**. Then, all of the remaining reactive pentafluorocarboxylate ester groups on the **SPLR** are converted to amides by a highly concentrated solution of an auxiliary quencher amine. This renders them unreactive and prevents their attachment to an already labeled analyte. Next, the excess quencher solution is washed off and the anchored fluorophores – attached to either analyte or quencher molecules – are cleaved off, in under 10 minutes, by a few microliters of a pH 5 acetic acid – acetate buffer. The collected effluent is then analyzed using the desired separation and detection method. All elements of the **SPLR** can be easily modified to suit the analyte/ analytical method.



L-08-1**EXTRACTING INFORMATION FROM THE IONIC STRENGTH DEPENDENCE OF THE ELECTROPHORETIC MOBILITY
USING THE 'SLOPE-PLOT'**

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The effective mobility (μ_{ep}) is the main parameter characterizing the electrophoretic behavior of a given solute. It is well known that μ_{ep} is a decreasing function of the ionic strength for all solutes. Nevertheless, the decrease depends strongly on the nature of the solute (small ions, polyelectrolyte, nanoparticles). Different electrophoretic modelings from the literature can describe this ionic strength dependence. However, the complexity of the ionic strength dependence with the solute characteristics and the variety of the analytical expressions of the different existing models, make the phenomenological ionic strength dependence difficult to apprehend. In this work, the ionic strength dependence of the effective mobility was systematically investigated on a set of different solutes (small mono and multicharged ions, polyelectrolytes and organic/inorganic (nano)particles). The phenomenological decrease of the electrophoretic mobility with the ionic strength was experimentally described by calculating the relative electrophoretic mobility decrease per ionic strength decade (S) in the range of 0.005 – 0.1 M ionic strength. Interestingly, a graphical representation, called the 'slope-plot', displaying S as a function of the solute electrophoretic mobility at 5 mM ionic strength allows defining different zones that are characteristics to the solute nature. In short, small ions occupy the lower part of the slope-plot and the nanoparticles and particles the upper part. Polyelectrolytes occupy a relatively localized zone from the upper left to the middle part of the graph.

This new graphical representation offers a convenient and easy way to distinguish solutes according to their chemical nature (small ions, nanoparticles, polyelectrolytes). It also gives phenomenological S values, which represents the percentage of relative mobility decrease per ionic strength decade, and that can be directly used by experimentalists. Such approach should be highly valuable to (i) identify or confirm the nature of a given solute; (ii) help in optimizing separation of mixtures containing different kinds of solutes by modification of the BGE ionic strength. Examples of applications of the slope-plot for the characterization of polymeric drug delivery systems will be given. Knowing the importance of nanoparticles in recent industrial applications and their relative risks and regulations, this new approach may also contribute in defining simple experimental protocols for the identification of nanoparticles in industrial products.

L-08-2

CURRENT CHALLENGES IN DEVELOPMENT OF RETENTION TIME ALIGNMENT ALGORITHMS

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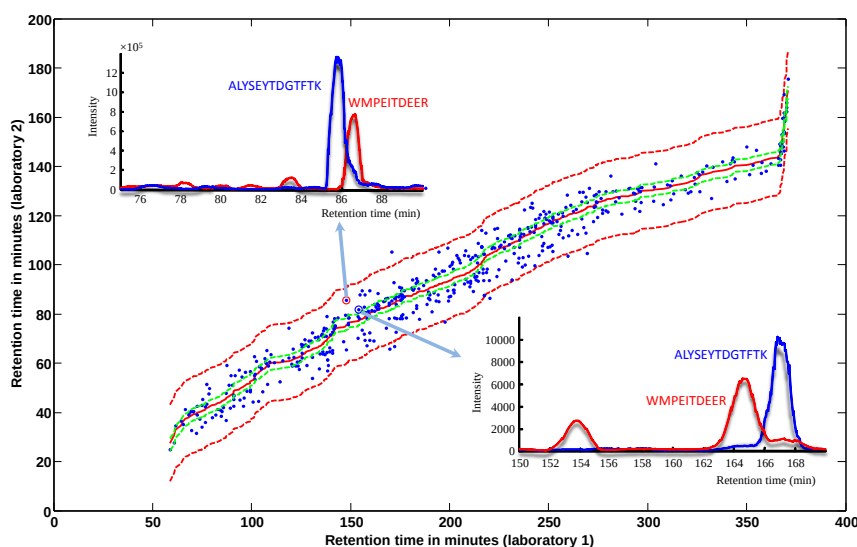
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Retention time alignment [1-3] is an important supporting step in quantitative pre-processing of LC-MS(/MS) chromatograms preparing the raw data for statistical analysis in differential profiling experiments. Inaccurate retention time alignment may introduce error in identifying corresponding peaks in multiple chromatograms for peaks, where partially or entirely single stage information (retention time and mass to charge ratio) is available. Accuracy of time alignment depends on multiple properties of the LC-MS(/MS) chromatogram set such as molecular complexity, concentration variability and distribution of compounds along the retention time and mass to charge ratio dimensions. Slight orthogonality in separation between chromatogram introduce an uncertainty to identify the absence or corresponding compounds in multiple chromatograms [4]. This presentation will cover how current retention time alignment approaches can deal the different properties of LC-MS(/MS) datasets and which quality control methods are available for users to assess the global and local accuracy of retention time alignment.



Effect of orthogonality on identifying corresponding peaks in two chromatograms of the same sample acquired in two laboratories.

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L-08-3

PSEUDO-ISOTACHOPHORESIS VS. PH-MEDIATED STACKING USING HYDRODYNAMIC SAMPLE INJECTION METHOD IN MICELLAR ELECTROKINETIC CHROMATOGRAPHY

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Capillary electrophoresis (CE) in recent years has become one of the most important separation techniques utilized all over the world. Despite many advantages CE suffers from relatively low detection sensitivity when compared to chromatographic techniques. To overcome this inconvenience a number of on-line preconcentration strategies have been developed [1]. However, only few can be applied in biological fluids analysis due to abundance of salt in these samples. Among the on-line preconcentration techniques pseudo-isotachophoresis (p-ITP) and pH-mediated stacking (PMS) were reported to be efficient in preconcentration of high ionic strength samples [2, 3]. Comparison of these two techniques and the implication on sample preparation procedure was investigated. Moreover, a hybrid technique was developed and examined.

Capability of signal enhancement effect of p-ITP and PMS techniques was investigated for twenty biogenic amino acids. The compounds of interest were derivatized using 2,4-dinitrofluorobenzene reagent. Separations were performed in uncoated fused silica capillaries (50 μm x 80 cm) using background electrolyte (BGE) composed of 140 mM SDS and 20 mM TRIS (pH 8.60) after application of high voltage (20 kV). p-ITP technique was applied through organic solvent and salt addition to samples while PMS was performed by electrokinetic injection of strong acid (0.1 M HCl) after sample hydrodynamic injection. Combination of p-ITP-PMS techniques was obtained by organic solvent and salt presence in sample and electrokinetic injection of HCl subsequently after large volume sample injection.

Conducted experiments have shown advantages of both investigated techniques. Impact of derivatization mixture components on applicable preconcentration mechanism was emphasized. Developed p-ITP-PMS technique based on hydrodynamic sample injection was proved to be efficient in high ionic strength samples preconcentration with significant advantages resulting from combination of these two techniques.

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KN-09**UNUSUAL BEHAVIOR OF DNA IN A UNIFORM ELECTRIC FIELD**

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Identical molecules move with identical velocities when placed in a uniform electric field within a uniform electrolyte. Here we report on finding that homogeneous DNA does not obey this fundamental rule. While most DNA moves with similar velocities, a fraction of DNA moves with velocities that vary within a multiple-fold range. The size of this irregular fraction increases several orders of magnitude when exogenous counterions are added to the negatively charged DNA. The irregular fraction decreases several orders of magnitude when the counterions are removed from DNA by dialysis against deionised water in the presence of a strong electric field (the electric field promotes the dissociation of the DNA-counterion complexes, while dialysis facilitates irreversible partitioning of counterions and DNA). These results suggest that: (i) DNA can form very stable complexes with counterions, (ii) these complexes can be dissociated by an electric field, and (iii) the observed non-uniform velocity of DNA is caused by electric-field induced slow dissociation of these stable complexes. We further investigated whether removal of counterions by electrodialysis against deionized water could elicit any novel DNA properties or behaviors. Counterintuitively, when deprived of counterions, DNA precipitates from the solution into amorphous aggregates. The aggregates remain stable even when the electric field is turned off, but readily re-dissolve when counterions are re-introduced. The explanation of the new phenomenon still needs to be developed. Our findings help to better understand a fundamental property of DNA: its interaction with counterions. In addition, these findings suggest a practical way of making electromigration of DNA more uniform: removal of strongly-bound DNA counterions by electro-dialysis against deionized water.

L-09-1

**MODULATION OF ELECTROOSMOTIC FLOW AND CAPILLARY SURFACE PROPERTIES
IN CAPILLARY ELECTROPHORESIS USING CHARGED POLYMER COATINGS**

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Capillary electrophoresis (CE) is one of the most powerful techniques for the separation of biomolecules. However, the separation efficiency of proteins in CE is often compromised by their tendency to interact with the silanol groups on the surface of the inner capillary and by an uncontrolled electroosmotic flow, thus an effective regulation of the surface is necessary to obtain efficient and reproducible separations.

Recently, we have introduced a polymeric film whose conformation on the surface is easily controlled by the variation of the surrounding buffer pH and ionic strength [1]. The pH dependence is achieved through synthesis of the surface coating through copolymerization of *N,N*-dimethylacrylamide (DMA) with weak acrylamido acids and bases that go under the trade name of Immobilines. Herein, we report on the synthesis of novel hydrophilic polymeric coatings that can modulate the electroosmotic flow (EOF) value and the properties of the capillary walls. The novelty of these poly(DMA)-based copolymers relies on the simultaneous presence of chemically reactive groups and silane groups in the backbone, which results in highly stable films through a process that combines phisi- and chemisorption. In particular, the polymers contain the following functionalities: i) a DMA segment that self-adsorbs on the capillary walls by weak non covalent interactions (such as hydrogen bonds or Van der Waals interactions) ii) a pending silane hydrolizable monomer, 3-(trimethoxysilyl) propyl methacrylate (MAPS), which promotes the condensation of the polymer with surface silanols iii) a chemically reactive monomer, glycidyl methacrylate (GMA), whose oxirane groups strengthen the stability of the film through the formation of covalent bonds with silanol groups and iii) ionizable monomers (Immobilines) that confer charges to the polymer chain in a precise pH dependent manner. In fact, the incorporation of ionizable monomers confers the polymer chain a positive or negative charge, whose density depends on the buffer pH, and the copolymerization of both weak acrylamido acids and bases yields an amphoteric coating that adopts a net positive or negative charge again by simply changing buffer pH. The presence of several Immobilines with different pK_a values allows the synthesis of several different polymers with similar characteristics, but conferring different properties to the capillary walls. Furthermore, the content of these ionogenic monomers can be adjusted allowing a perfect control of the surface charge density when the capillary wall is bathed in a given buffer pH. As a consequence, the separation of alkaline or acidic protein is improved in different pH conditions because the interaction with the surface walls is prevented by electrostatic repulsion.

The coating procedure is another main advantage of these polymers: it is very simple and fast as it consists in the adsorption of a diluted aqueous solution of the pre-synthesized polymer on the capillary walls and can be performed directly online into the CE instrument.

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L-09-2

EFFICIENT PRECONCENTRATION AND SEPARATION OF AMYLOID PEPTIDES: TOWARD A MINIATURIZED DIAGNOSTIC TOOL

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The neuropathological hallmarks of Alzheimer disease (AD) are amyloid plaques, mainly composed of beta-amyloid 1-42 (A β 1-42) and neurofibrillary tangles containing hyper phosphorylated Tau. To date, only the analysis of A β 1-42, total tau and phospho-tau-181 in Cerebrospinal Fluid (CSF) is routinely used for the diagnosis [1]. Although these three biomarkers have been well-established and validated internationally, the assays alone are not sufficient to diagnose with certainty this disease and the diagnosis still rely on a combination of cognitive and imagery tests. Moreover, as the neurodegeneration is estimated to start 20 to 30 years before the first clinical symptoms become apparent [2], the major challenge is to diagnose the disease at its earliest stages. Several groups have recently demonstrated the diagnostic power of the simultaneous quantification of A β 1-38, A β 1-40, and A β 1-42 in CSF [3]. In particular, we reported a promising ratio combination producing a high discrimination between AD and non demented patients [4]. Besides these conventional A β peptides, Wilfang group proposed the N-truncated A β 2-42 as a candidate biomarker [5].

Our aim was therefore to develop a microchip for the full separation and quantitation of the C and N truncated variants of A β peptides from biological fluids. We intended to improve not only the diagnostic specificity by the multibiomarkers approach but also its precocity by attaining sensitivities compatible with their measurement at early stages of the AD.

In this presentation, we will present the way we achieved a high resolution of homologous and structurally very close amyloid peptides into one simple and commercial glass microchip and also describe different strategies, we developed, for the preconcentration of the peptides from biological fluids. Prior to their separation, peptides were derivatized with Fluorophores 488 as described previously [4]. We compared the performance of several coatings, including PEO and EpDMA in terms of pH stability, analysis reproducibility, resolution attained between A β 1-38, 1-40 and 1-42, and recovery (to measure their possible adsorption to the channel wall). EpDMA which provided the best performances was applied to the separation of different relevant mixtures of A β peptides and to CSF samples spiked with these peptides. Besides the separation, two pre-concentration approaches were evaluated, the first one based on isotachopheresis and the second on immunocapture using a novel fluidized bed. The ITP combined to an electrokinetic injection allowed to detect the underivatized A β 1-40 at lower concentrations than ever previously achieved using UV detection (50 nM). Besides, we also proposed a novel strategy to immunoextract and preconcentrate specifically A β peptides using a microfluidic magnetic fluidized bed we recently developed [6]. Here, the design of the microfluidic device was optimized in order to compensate for the decaying intensity of the magnetic field. Using functionalized magnetic beads, preconcentration factor around 1000 were achieved for standard and fluorescent A β peptides reaching the concentration level expected in CSF (1 nM).

The overall results open promising outlook to set a new sensitive and more discriminating miniaturized diagnostic tool of AD.

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L-09-3

NUMERICAL MODELING OF MICROFABRICATED CE-ESI-MS NEBULIZER INTERFACE

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A novel microfluidic capillary electrophoresis – electrospray mass spectrometry interface (CE-ESI-MS) design is investigated by means of computational fluid dynamics (CFD) simulation. The device features two narrow channels for nebulizer gas. The physical object is simulated in 2D axial symmetry layout in order to reduce computational time. The model is based on the turbulent form of the Navier-Stokes equation assuming a compressible one phase flow with same physical characteristics as nitrogen at 293.15 K. Since the flowing media is compressible, its density is corrected with the function of pressure. The velocity field distribution and the pressure drop are calculated by numerical methods. It is concluded that that CFD simulation is an appropriate tool to develop new microfabricated nebulizer designs.

ESI interfaces in CE-MS play various roles: i) physically position the CE capillary close to the MS orifice, ii) close the electrical circuit for both the CE and ESI sides and iii) support the proper droplet formation required for creation of free, gas phase ions [1, 2]. To this end, numerous tools have been developed during the past decade aiming to achieve high selectivity and sensitivity at a simple and low cost manner. Predominantly, these tools were developed without deeper theoretical considerations. To speed up this endeavor, CFD modeling techniques will be introduced in this presentation. CFD is a simulation tool to help quickly achieve an optimal design at low cost with a minimum number of actual experiments. Albeit, modeling and simulation are primarily considered as design tools, they can also be used to support experimental data interpretation [3]. The demonstration will focus on the investigation of the pressure and velocity field distribution in order to discover unexpected dead-volume problems inside the microchip.

The schematic representation of the device along with the modeled domain of interest, which is meshed for finite elements, is shown in Figure 1.

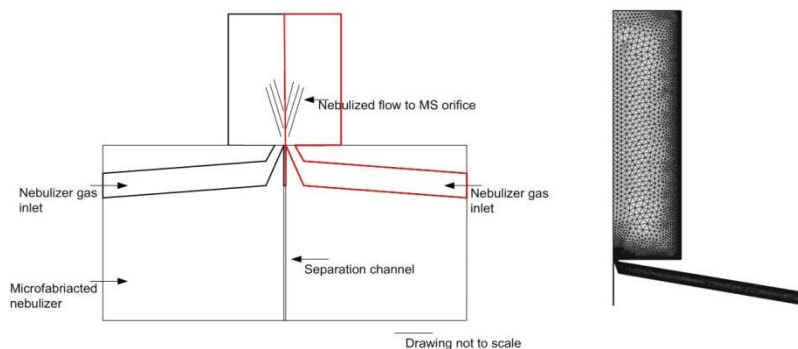


Figure 1. Scheme of the microfabricated nebulizer and the modeled domain of interest (the red part on the right hand side)

Based on the preliminary calculated Reynolds number, which indicates the flow characteristic, the fluid dynamics is highly turbulent. The turbulent fluid flow is chaotic and its trajectory is nearly unpredictable. The flow characteristic is determined by inertial forces. A commonly applied approach, k- ϵ turbulent model, is applied to estimate the kinetic energy dissipation. This model solves two variables: k; the turbulent kinetic energy, and epsilon; the rate of kinetic energy dissipation [4]. Wall functions are used in this model, so the flow in the buffer region is simulated using analytical function. The k-epsilon model was chosen due to its good convergence rate and reliable performance around sharp geometries.

The obtained velocity field is shown in Figure 2 (the warmer the color, the higher the velocity, unit is m/s). The full-length inflow pressure is 3 atm. As one can see on the zoom part of the figure, the simulated velocity profile shows how the nebulizer gas could drag the flow from the separation channel suggesting avoidance or sharp edges.

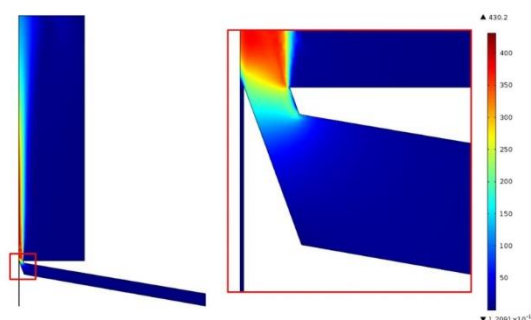


Figure 2. Simulated velocity profile in the microfabricated nebulizer device using $k-\epsilon$ turbulent model

CFD modeling and simulation of microfabricated nebulizer device are well developed, but more importantly, in addition to being considered as just a design tool, they can also be used during the interpretation of experimental data. Deeper understanding of various physical phenomena by means of CFD may lead to engineering of more efficient CE-ESI-MS interfaces.

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KN-10

ANALYSIS OF OXIDIZED PHOSPHOLIPIDS AS BIOMARKERS OF OXIDATIVE STRESS

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Oxidized low-density lipoproteins (OxLDL) are involved in pathophysiological processes such as atherosclerosis. They may be formed under conditions of chronic hyperlipidemia which may lead to lipoprotein aggregation within the intima of the blood vessel and subsequent oxidation by reactive oxygen species (ROS). Macrophages target OxLDLs by endocytosis via scavenger receptors, which are distinct from LDL receptors. The oxidized LDL accumulates in the macrophages and other phagocytes forming so-called foam cells. They may represent the origin of plaque build-up in blood vessels. In this study, we are interested to analyze oxidized phosphatidylcholines (OxPCs) in OxLDL in the plasma. They are regarded as potential biomarkers for oxidative stress, being indicators for a mismatch of pro-oxidative and anti-oxidative processes in the body.

In our study, we used the specific affinity of anti-OxLDL-antibodies (Abs) conjugated to gold nanoparticles (GNPs) [1] for extraction and enrichment of OxPCs *via* selective trapping of OxLDL from plasma combined with the sensitive detection by liquid chromatography / tandem-mass spectrometry (LC-MS/MS). In this presentation the development of the GNP-antibody conjugate will be discussed in detail. Successful bioconjugation chemistry of Abs was accomplished *via* bifunctional polyethylene glycol (PEG) spacer [2] and subsequent antibody coupling. The effect of structural parameters and the surface chemistry on the effectiveness of the immunotrapping step will be presented. The antiOxLDL@GNP bioconjugate was also characterized by determination of the dissociation constant K_d of OxLDL *via* static binding capacity measurements. The new bioconjugated immunoaffinity nanomaterial was utilized for selective extraction of OxLDL from plasma and subsequent LC-MS/MS analysis [3]. In summary, the application of GNP-based bioanalysis for selective targeting of OxLDL and the fast and sensitive detection by LC-MS/MS offers new possibilities for targeted lipidomics in lipoproteins as well as for oxidative stress lipid biomarker screening.

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L-10-1**DETECTION OF GLUTARALDEHYDE CROSS-LINKED GELATIN NANOPARTICLES IN A LIQUID FOOD MATRIX VIA
FLUORESCENCE LABELING, IMMUNOPRECIPITATION AND CHIP
ELECTROPHORESIS**

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Lately, the application of nanoparticles, i.e. particles in the range of up to few hundred nm diameter is gaining popularity in various fields like e.g. pharma- or nutraceuticals. Especially the ability to interact with and pass through cellular membranes of organisms together with the possibility to act as carrier for smaller compounds creates interest in nanoparticle material. If biodegradable, even a sustained, targeted delivery of compounds is feasible with clearance of nanoparticles at the same time from the system by digestion [1, 2]. However, toxicological concerns related to engineered nanomaterials have been raised only recently (see e.g. [3]).

Capillary electrophoretic separations in the chip format (chip CE) on a commercially available instrument by changing the standard, company suggested instrumental setup has already been successfully demonstrated for a couple of fluorescently labeled analytes in the nanometer size range, i.e. a human pathogenic virus and liposomes [4]. Additionally, chip CE was further adapted to allow also the analysis of a biodegradable nanoparticle carrier material (glutaraldehyde cross-linked gelatin) possibly employed for nutrient, vitamin or flavor additive supplementation to food [5]. It was now our aim to demonstrate the isolation of gelatin nanoparticles from a spiked liquid food matrix followed by chip electrophoresis in combination with immunoprecipitation (i.e. the attachment of analytes to specific, magnetic bead immobilized antibodies in order to allow matrix removal and enrichment). We suppose that such an assay will gain importance in the future to allow for nanoparticle detection even in complex matrices. Spiking of the liquid food matrix is preceded by characterization of nanoparticles in terms of (i) diameter, (ii) amount of nanoparticle building blocks present and (iii) amount of nanoparticle cross-linking (indirect by the degree of analyte modification via a fluorophore targeting the same reactive groups as the employed cross-linker).

The proof of principle for the described workflow could be impressively demonstrated but several further questions like the application in different matrices or of nominally identical analyte batches (however varying in nanoparticle size and cross-linking) as well as the increase of the dynamic range of the assay still need to be targeted in future work.

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L-10-2

DETERMINATION OF POLYCATION LOG D DISTRIBUTIONS BY MICELLAR AND MICROEMULSION ELECTROKINETIC CHROMATOGRAPHY

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The characterization of the hydrophobicity of polymer compounds in solution remains a challenging issue of importance, especially for biomedical or pharmaceutical applications. In this work, for the first time, the log *D* distributions of statistical cationic polypeptides in the (-1; +3) range were characterized using micellar or microemulsion electrokinetic chromatography at physiological pH (7.4) [1]. The log *D* distributions of the polymer samples were obtained from the electrophoretic/chromatographic retardation of the polymer derivatives in presence of neutral micelles (or neutral microemulsion), using small cationic molecules of β -blockers for calibration. Separating electrolytes were based on a TRIS-chloride buffer containing a neutral surfactant (BRIJ35: polyoxyethyleneglycol dodecyl ether) for the formation of micelles (in water) or microemulsion (in water/n-pentanol mixture). The log *D* distributions obtained at pH 7.4 using this method were in good agreement with the chemical structures of cationic polypeptides: poly(lys, phe) 1:1 > poly(lys, tyr) 1:1 > poly(lys, trp) 4:1 > poly(lys, ser) 3:1 > poly(L-lysine), where x:y represents the molar ratio of each amino acid in the copolymer. Weight average octanol-water log *D* values and the dispersion of the log *D* distribution were also defined and determined for each polymer sample. Knowing the importance of the distributions in chemical composition and in molar mass on the properties of application of polymer samples, the opportunity to characterize the distribution in hydrophobicity seems very attractive. For example, in the pharmaceutical / biomedical field, it is known that the introduction of hydrophobic groups in polycations generally improves the transfection efficiency of gene delivery polyplexes into cells.

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L-10-3**OPTIMIZING MEKC SEPARATIONS: SURFACTANT SELECTIVITY REVISITED**

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In the optimization of MEKC separations partition equilibria of solutes between the aqueous phase (background electrolyte, BGE) and the dispersed phase (micelle) is usually invoked. Solute retention can therefore be manipulated by altering either the bulk solution properties or the micelle structural backbone. In order to alter the solution properties, a myriad of electrolyte modifiers has been proposed as BGE additives. Organic solvents especially those classified in the Snyder triangle and classically used in chromatographic separations (methanol, acetonitrile and tetrahydrofuran) have been shown to exert important selectivity changes in MEKC separations. Improved solubility of solutes in the BGE, distinct solute-solvent intermolecular interactions, differences in cavitation work as well as measurable changes in micelle structure (cmc, aggregation number, etc) have all been accounted as solvent effects in MEKC. Interestingly, selectivity changes imparted by surfactant selection are not as straightforward. Many LSER/QSRR studies aiming at establishing phase selectivity differences using Abraham descriptors have failed. In these studies, a variety of surfactants even with different headgroups, chain lengths and counterions all seem to behave alike [1].

In this work we propose that surfactant selectivity in MEKC can be rationalized by considering the details of the micellar phase and the manner by which solutes solubilize into the micelle compartments. A SDS micelle is viewed as an entity composed of numerous compartments of distinct hydrophobicities; at least three can be readily defined: the inner core, the interface and the surface. By knowing the solute set major locus into the micelle it is possible to promote changes in that specific compartment and therefore manipulate resolution. Moreover, selectivity changes due to the micellar phase can only be visualized if a particular set of solutes that assesses the modified compartment is used. From LSER/QSRR studies it is well known that the solute volume is the most prominent variable in multiple linear regressions of $\log k$ versus solute descriptors. Indeed the solute volume governs partition due to the remarkable differences in cavitation energy between aqueous bulk and micelle. In fact, retention coefficients (as $\log k$) of neutral compounds as a function of solutes McGowan volumes for homologues series depict linear relationships. The same behavior is observed for multifunctional series such as flavonoids, anthocyanins, amines, terpenes, and steroids, objects of our studies. The slope of $\log k$ versus volume can be interpreted as the solute penetration degree into the micelle and the intercept is related to the effective phase volume the solute assesses. Our results revealed that the solubilization loci in SDS for terpenes is the inner core, for steroids the interface and flavonoids, anthocyanins and amines anchor at the micelle surface. Therefore, if surfactant selectivity is to be explored to optimize separations of flavonoids, the basicity of the sulfate heads must be changed by introducing micelle spacers, such as co-surfactants and/or anchored solvents (e.g. pentanol). To optimize the separation of terpenes, the inner core must be altered which can be achieved by selecting surfactants of differing tail chain lengths. And finally, to optimize separation of steroids, the use of mixed micelles constituted by SDS/non ionic surfactants can be attempted.

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KN-11**HYPERCROSSLINKING: A NEW ROUTE TO POROUS POLYMER MONOLITHS IN CAPILLARIES AND THIN LAYERS WITH ENHANCED SURFACE AREA, REACTIVITY, AND CHROMATOGRAPHIC PERFORMANCE**

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The first generation of porous polymer monoliths emerged in the early 1990s. Their well known advantages included ease of the preparation, robustness, high permeability to flow, and mass transfer via convection. These features made them useful for the chromatographic separation of large molecules and as supports for immobilized enzymes. However, these monoliths lacked mesopores, and therefore, exhibited only a small surface area. This then prohibited the polymer-based monolith from use for the highly efficient separations of small molecules. To avoid this weakness, we have developed and demonstrated the second generation monoliths prepared using hypercrosslinking of poly(chloromethylstyrene-co-styrene-co divinylbenzene) polymers and obtained materials with a surface area as large as 600 m²/g that enabled the desired separation of small molecules. We also used two approaches to modulate the chemistry of these porous monolithic polymers: (i) chemical reactions of the hypercrosslinked polymers [1, 2] and (ii) hypercrosslinking of copolymers containing functional styrene derivatives such as 4-acetoxystyrene and 4-methylstyrene [3]. Recently, we used “plain” poly(styrene-divinylbenzene) monoliths and hypercrosslinked them with external crosslinkers including 4,4'-bis(chloromethyl)-1,1'-biphenyl, 1,4-bis(chloro-methyl)benzene, and dimethoxymethane [4]. Polymers with extremely large surface areas reaching up to 900 m²/g were obtained via hypercrosslinking precursor monolith polymerized for only 2.5 h. The increase in chromatographic performance of monoliths modified using this new procedure was comparable to the performance obtained with earlier monolithic polymers containing chloromethylstyrene. However, the preparation of the binary copolymer poly(styrene-divinylbenzene) precursor is simpler than that of ternary copolymer containing chloromethylstyrene. Experiments were carried out using capillary format [5] and thin layers [6].

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L-11-1

**DEVELOPMENT OF FEMTOLITER SCALE LC USING EXTENDED-NANO CHANNEL
TOWARD SEPARATION OF PROTEINS WITH MILLION PLATE NUMBERS**

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A femtoliter scale liquid chromatography system was developed using 100 nm scale nanofluidic channel (extended-nano channel) toward protein separation with million theoretical plate numbers. In this paper, the performance of the extended-nano chromatography system was verified by separation of two fluorescent dyes, which showed a plate number of 36,000, that is well accorded with a theoretically estimated value, with only ~10fL injection volume.

Higher efficiency with smaller volume sample has been targeted in separation science for a long time. Especially, in chromatography, development of packed column with smaller particle and monolith column, utilizing higher pressure and smaller capillary column have been intensively researched. Contrary, our group has developed fundamental fluidic techniques in extended-nano scale to investigate unique liquid properties of water confined in fused silica nanochannels. Furthermore, our group applied the extended-nano fluidic control techniques to analytical devices including chromatography and verified a separation efficiency of 440,000 plates/m using an open extended-nano channel as a separation column. However, the total plate number was only 450 due to the short length of nanochannel (1.2 mm). Herein, we designed and developed a new high pressure nanofluidic control system and longer nanochannel for higher separation efficiency and verified its performance.

Because the extended-nano channel is diffusion dominant even for molecules with small diffusion constants, it has a potential of ultrahigh efficient separation of proteins. Here, we designed the size and length of nanochannels and theoretical plate number using a theoretical equation of open tubular chromatography [1], diffusion constant of bovine serum albumin [2] and a withstanding pressure of a fused silica chip (20 MPa). As a result, a nanochannel of 300 nm wide and deep and 89 mm long could realize theoretical plate number of 1,000,000.

In an experimental setup using a high pressure control system and a chip with nanofluidic channels two fluorescent dyes, Pyrromethene 597 (P597) and Coumarine 460 (C460) were used as samples. The mobile phase and stationary phase were hexane with 10% 2-propanol and bare silica surface. The sample of ~10fL was cut and injected into the 89 mm long nanochannel by high-speed pressure switching using the solenoid valves under 8 MPa, followed by detection using fluorescent microscope.

Results of separation obtained by conventional HPLC and extended-nano chromatography were compared. As a result, the theoretical plate numbers were 3,700 for HPLC and 36,000 for extended-nano, respectively. The theoretical plate number of extended-nano was well accorded with a theoretically-estimated value (39,000). The degree of separation was also improved from 4.9 (HPLC) to 7.5 (extended-nano). From these results, performance of the new system was verified and protein separation with million plates would be expected. Moreover, the sample volume was decreased 8 orders of magnitude (μ L to 10 fL), which promise application to single cell proteomic analysis because the volume is much smaller than a single cell (pL).

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L-11-2**NEW DETECTION APPROACHES FOR MICROSCALE SEPARATIONS BASED ON LASER DESORPTION MASS SPECTROMETRY**

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Fast, sensitive and specific detection is mandatory for modern separations. Laser desorption mass spectrometry (MS) is characterized by all these attributes and, in addition, it offers advantages of off-line coupling such as archiving and independent optimization of the separation and MS steps. We demonstrate two novel approaches with applications in today's omics research, namely metallomics and proteomics.

The first approach is an alternative to microcolumn separations coupled to MS with electrospray and inductively coupled plasma (ICP) ionization techniques for metalloproteomic research. Unlike these on-line interfaces, it does not require effluent splitting or repeating the separation for each of the detection modes. Our method is based on an off-line coupling of a single capillary electrophoresis (CE) run to both substrate-assisted laser desorption (SALD) ICP MS and matrix-assisted laser desorption/ionization (MALDI) MS [1]. The effluent fractions are collected on a custom-designed Au-coated polyethylene terephthalate glycol (PETG) sample target that is compatible with the both MS detection methods. The approach is demonstrated on successive analysis of rabbit-liver metallothionein (MT) isoform mixture. MT isoforms are separated by CE coupled to a sub-atmospheric fraction collector via a liquid junction interface. The separation record is then covered with MALDI matrix and analyzed consecutively by MALDI MS and SALD ICP MS providing information about both molecular mass of present proteins and metal distribution and quantity, respectively. Furthermore, two different MALDI matrices at acidic and neutral pH may be applied alternately at the deposited fractions. Thus, masses of MT apoforms are revealed under acidic condition and metal-protein complexes at neutral pH using MALDI MS.

The second approach aims at simplicity and low cost. It employs a newly developed technique for determination of trace elements in submicroliter sample volumes, diode laser thermal vaporization (DLTV) ICP MS [2]. Here, samples are deposited on paper with preprinted rectangles or a line, dried and vaporized with a diode laser, which induces pyrolysis of the paper. The generated aerosol is then carried into the ICP MS. The technique can also be used for separation of simple mixtures using thin layer chromatography (TLC). In this case, the diode laser beam scans across the thin layer with separated zones rather than across discrete sample spots on paper. Here, we present determination of cobalamins using TLC – DLTV ICP MS to prove this concept. Ascending TLC separation of cobalamins is performed on cellulose/aluminum sheets. The TLC chromatogram is scanned in a simple laboratory-built chamber with the diode laser. The chamber is made of a glass tube; the laser is attached to a syringe pump providing the translation over the sample in the chamber. The simplest possible chamber construction provides the minimal dead volume and reduces the turbulent flow as well as wash-out time. This approach is sufficient for elemental speciation and presents a simple and cost-effective alternative to HPLC coupled to nebulizer ICP MS.

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L-11-3**NOVEL APPROACHES FOR THE STUDY OF ULTRASMALL SAMPLES BY FAST CAPILLARY ELECTROPHORESIS-MASS SPECTROMETRY**

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The hyphenation of capillary electrophoresis (CE) with mass spectrometry (MS) is an attractive tool of instrumental analysis. However, the combination of conventional CE systems with MS using sheath-liquid ESI sprayers is usually associated with the implementation of rather long capillaries (longer than or equal to 60 cm). Consequently, the window of migration times is typically in the range of 5-10 min. In order to speed up CE-MS separations much shorter capillaries and rather high separation voltages should be applied.

We present a novel instrumental approach for fast CE-MS measurements in the time scale of seconds; in addition ultrasmall samples in the nanolitre range can be studied.

Samples are handled by means of a microprocessor controlled injection system with an integrated capillary. The injection capillary is moved out of the injection cell for sample take-up from a microenvironment, and after repositioning it is facing the fixed inlet of a short separation capillary (15 cm in length), then a small sample plug is injected onto the inlet of the separation capillary. In this way separations of catecholamines could be carried out in less than 12 s.

The capability of handling samples in the nanolitre range allows for a rapid pre-concentration protocol based on the simple volume reduction by evaporation from μL to nL sample volumes. The efficient usage of available sample is of key importance in many bioanalytical questions for which CE-MS is an attractive method. The main advantage of separations in short capillaries is the dramatical decrease in migration times which is an important requirement for high-throughput analysis in the life sciences.

L-11-4

EVALUATION OF A “HOME- MADE” MINI STIR BAR FOR FLUOXETINE DETERMINATION IN HUMAN PLASMA BY PDMS SBSE/HPLC-UV APPLYING EXPERIMENTAL DESIGN

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Depression is a complex psychiatric syndrome that affects a considerable part of the world population. Therefore therapeutic drug monitoring of antidepressants is necessary. However, sample preparation usually is laborious. Recently, stir bar sorptive extraction (SBSE) highlights as a promising microtechnique for sample preparation, allowing the extraction and preconcentration of a wide variety of matrices [1, 2]. The use of experimental design ally with this technique enables to verify the best analytical conditions. A “home-made” polydimethylsiloxane mini stir bar sorptive (PDMS SBSE, 10 mm length, 1 mm thickness) has been developed, covering direct the metal with commercial polymer (Sylgard 184/curing agent) cured using silicon mold in oven for 1 h at 60°C, followed by increasing temperature at 10°C min⁻¹ rate until 200°C maintaining then for 1 hour. Sample preparation using the PDMS SBSE was carried out adding 250 µL of human plasma, 25 µL of fluoxetine solution (500 µg mL⁻¹) and 975 µL of phosphate buffer (10.0 mmol L⁻¹) proceeding extraction for 1 h at 60°C under magnetic stirring. Desorption modes (magnetic and ultrasonic stirring) and kinetics were studied, showing that the magnetic stirring was the most appropriate desorption mode, requiring 40 minutes to equilibrium establishment. An approach using simplex centroid design of three components was applied to assess the best composition solvent phase among methanol, acetonitrile and water for fluoxetine (FLU) desorption from plasma sample. Fluoxetine's analysis was performed in a high performance liquid chromatographic system (HPLC). The HPLC an Alliance e-2695 (Waters) consisted in a quaternary solvent delivery pump using isocratic elution of acetonitrile: phosphate buffer acidified with HCl (pH 3.07) (70:30, v/v) at flow rate of 1.0 mL min⁻¹ with 20.0 µL injected by autosampler in a Microsorb® C8 column (5 µm, Varian) at 30°C, coupled with photodiode array detector (2998 PDA) scanning wavelength from 200 to 300 nm, fixed at 225 nm. The best results for simplex centroid design were obtained for methanol: acetonitrile (75:25, v/v) as desorption phase. The influence of temperature and pH as variables on PDMS SBSE FLU extraction in plasma sample was studied associated by a factorial experimental design for the first time in literature, so far as our knowledge, showing significant contribution of these factors. So, a central composite design was performed and the optimal extraction conditions were obtained for temperature between 75 and 82°C and pH = 9.00, showing synergism of those variables on the PDMS SBSE extraction. It presents high recovery rates of 100.3% even after 50 analyses. Some of the advantages are pointed out - the excellent cost/benefit ratio, easy confection, with the dimension of the bar and the thickness of the coating may be selected and even the selectivity of the polymer can be changed. Enables also preparation of many samples at the same time in a multipoint stirrer and desorption can be taken directly in sample vial of the autosampler. All of these factors demonstrate that our developed mini bar of PDMS SBSE represent a powerful analytical tool that will contribute for drugs extraction in biopharmaceutical analyses.

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KN-12**MICROSCALE PROTEIN ISOLATION/ENRICHMENT USING RP-HPLC**

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Microscale protein isolation and/or enrichment is becoming a major direction in proteomics and in many protein related biochemical research. There are several major avenues, among these RP-HPLC. Novel, large pore size stationary phases made HPLC analysis of macromolecules feasible, and a real alternative to the mainstream 2D gel based protein separation methods.

In proteomics one major direction is the study of protein families; mostly diverse post-translational modifications of the same protein. While these “families” are typically separated in 2D gels, in RP-HPLC the protein families are expected to elute in one band. While superficially it is a disadvantage, this allows fractionation the whole family, which may be studied in detail in a subsequent set of proteomics methodologies.

This avenue will be explored in the presentation. Following method optimization we show that protein families (various glycoforms) indeed elute at the same time (can be collected in a given fraction). The separation method is capable of handling complex mixtures, like blood plasma, using only simple sample preparation. Fractionation is easy to perform on standard 4 mm wide columns, injecting ca. 10 µL blood plasma onto the column. The proteins amounts in the isolated fractions are sufficient to perform detailed proteomic analysis, including sample handling and preparation (enzymatic digests), followed by nano-HPLC-MS/MS analysis.

The results enable us to study native protein families. Our studies focus on determining N-glycosylation patterns in various plasma proteins. Several such examples will be discussed.

Acknowledgement

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L-12-1

A NEXT GENERATION MASS SPECTROMETRY PLATFORM FOR THE RAPID IDENTIFICATION OF (MULTI-)DRUG RESISTANT *GRAM-NEGATIVE* BACTERIA

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The ever increasing spread of multi-drug resistant micro-organisms demands for fast and accurate analysis of suspect organisms to supplement existing techniques in the clinical laboratory. Targeted proteomics may offer a number of advantages over more traditional approaches of screening for suspect organisms with times scale of the assay being the most significant one.

A practical implementation of targeted proteomics is based on the identification of β -lactamases, the class of enzymes which confer resistance to penicillin like antibiotics. To ensure a high sensitivity for such an analysis, an analytical platform combining capillary electrophoresis coupled to high resolution ToF mass spectrometry (CE-MS) via a sheathless interface (a porous sprayer) was developed. Bacterial cell pellets were lysed and protein extracts were digested with trypsin. The resulting peptide mixtures were analysed by CE-MS and proteins were identified after searching of the spectral data against bacterial protein databases. The method was validated using recombinant β -lactamase and lab strains and was subsequently tested using a set of well characterised OXA-48 and KPC β -lactamase containing clinical isolates.

The CE-MS peptide analysis was optimized with regards to separation window and number of identifications within a one hour analysis. The performance of the system was first evaluated using a recombinant TEM-1 β -lactamase, resulting in 67% of the amino acid sequence being covered by 20 different unique peptides. Subsequently, a resistant and susceptible *Escherichia coli* lab strain were analysed and based on the observed peptides, the two strains could easily be discriminated.

Finally, the method was tested on a collection of well characterised OXA-48 (n=17) and KPC (n=10) clinical isolates. The minimal inhibitory concentration (MIC) for meropenem in this set ranged from 0.5 mg L⁻¹ to more than 30 mg L⁻¹, indicating a varying degree of antimicrobial resistance. A modified Hodge test was applied to confirm the functional resistance. The developed CE-MS was able to identify the presence of OXA-48 and KPC in all of the samples, independent of species and degree of susceptibility. Furthermore, a number of extended-spectrum β -lactamases (ESBL) were identified in the same analysis, confirming the multi-resistant character of the clinical isolates.

In conclusion, a method has been developed which allows for accurate determination of the presence of OXA-48 and KPC. The method performed reliably for a collection of clinical isolates. Due to the unbiased nature of the method, a number of ESBLs were also revealed for which the isolates had not been previously tested. The same method is currently developed for direct phenotypic resistance monitoring, by measuring the antibiotic and its β -lactamase-mediated breakdown products after short time co-culturing of bacteria with the individual antibiotic compounds.

L-12-2**IMPROVING PREDICTION OF IVF SUCCESS: LOOKING FOR PUTATIVE BIOMARKERS IN IVF-MEDIA UPON EMBRYO CULTIVATION**

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L-12-3**USE OF ALCOHOLS AS MOBILE PHASES FOR ENHANCED PEPTIDE SEPARATION AND IMPROVED SEQUENCE COVERAGE**

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For separation of tryptic peptides by nano RP-HPLC mobile phase systems containing acetonitrile as modifier organic are commonly used. Now, a new approach using HPLC eluents containing combination of different alcohols such as methanol and 2-propanol instead of and in addition to acetonitrile is described. The percentage of alcohol in the mobile phase was varied and the results were compared to separation of the same tryptic peptides under (otherwise) default separation conditions (organic mobile phase containing acetonitrile) [1, 2].

The increase of the alcohol part in mobile phase led to significant selective changes in peptide retention times on the reversed-phase system and in intensity of the MS/MS spectra. These changes are the consequence of different physicochemical conditions for retention such as the change of the overall charge distribution and density, polarity and relative hydrophobicity of peptides dissolved in a solvent of different properties. The peptides eluted uniformly over the complete gradient window without grouping in certain areas, thus enabling generally better separation and enhanced MS detection.

As a further consequence of using new mobile phases, a changed selectivity for the separation column was observed.

The use of the new "alcoholic" mobile phase enabled detection of higher amounts of peptides and proteins.

A significant increase in identifications of unique peptides and improved sequence coverage for identified proteins were observed with the "alcoholic" mobile phases.

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Acknowledgment

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L-12-4**RATIONAL DESIGN OF PEPTIDES AGAINST ALZHEIMER'S DISEASE: A PROTEOMIC APPROACH**

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Alzheimer's disease (AD) is the most prevalent form of neurodegenerative disorders. Recently, it is widely accepted that oligomeric amyloid-beta peptide (A β) toxicity may be responsible for the initiation of AD. Interaction of A β with proteins is the key event in the pathology of Alzheimer's disease. Mapping the A β interaction partners could help to discover novel pathways in disease pathogenesis and to find targets for drug development.

The aim of our research was to discover small peptides inhibiting the interaction of these proteins with A β .

Our proteomic studies have revealed that the fibrillar A β (1-42) peptide binds to a huge number of proteins in rat synaptosomal membrane fractions. Majority of these identified proteins have already been associated with Alzheimer's disease.

The protein array technology is a novel method to investigate peptide-protein, protein-protein interactions in a high-throughput manner. A protein array with 8163 recombinant human proteins was incubated with oligomeric A β and we identified 324 proteins as potential interaction partners. These extra and intracellularly localized proteins have diverse functions. The most highly impacted cellular system was the protein translation machinery. Based on the protein sequences identified in the above mentioned experiments we wanted to extract such peptide subsequences that could inhibit binding of A β to these proteins. Such peptides could be a base set in the design of new, putative neuroprotective compounds. Using a mathematical algorithm we devised a peptide chip containing 4000 hexapeptides with sequences from the previously identified proteins and performed a binding experiment with oligomeric A β (1-42). We have demonstrated interaction of several peptides with A β . Some of these peptide sequences were prepared by solid phase peptide synthesis and those of them which showed inhibitory effect on the toxicity of A β in MTT test were used further experiments. Penetration of our most promising peptides into neuronal cells was investigated, too.

These peptides will be used as lead compounds in rational drug design against AD.

Acknowledgement

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KN-13**ASSESSMENT OF THE EFFECT OF FOOD SUPPLEMENTS IN ACUTE-ON-CHRONIC DISEASES BY MONITORING BIOMARKERS OF INFLAMMATION USING IMMUNOAFFINITY CAPILLARY ELECTROPHORESIS**

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The term microbiota represents an ensemble of microorganisms that resides in a previously established environment. Humans live in close association with communities of microorganisms (the human microbiota) that inhabit every exposed surface and cavity in the body. The adult human gastrointestinal tract is home to trillions of bacteria, archaea, and eukarya cells (commonly known as the gut microbiota). The collective genetic information of the human microbiota represents a second genome (the human microbiome) that outnumbers human genes by several orders of magnitude.

Recent studies have demonstrated that bacterial community composition is dramatically altered in diseases such as obesity; with healthy subjects typically exhibiting distinct, diverse and temporally stable bacterial consortia when compared with patients displaying disease symptoms. The shift in microbiota elicited by diet and other factors (e.g., hygiene, medical therapies, etc.) are key to host health, particularly because the structure of the gastrointestinal microbial population has been associated with protection against pathogens. The approximately one-hundred trillion microbes that live in our gastrointestinal tract represent an anaerobic bioreactor programmed to synthesize several molecules that direct the human immune system locally and systematically. The biosynthetic variability of our gut microbiota, which remains largely unexplored, could shape the human physiological phenotypes and is viewed as modifier of the host epigenome.

Studies on drug polypharmacology indicate that many of the known drugs have more than one target and highlight the need to undertake a systems level approach to understand drug efficacy and adverse drug reactions considering the effect of a molecule in a global physiological environment, including the gut microbiota.

Owing to the increased prevalence of diseases and disorders associated with gut microbiota imbalances and the fact that traditional treatments such as antibiotic administration appear to have the potential for long-term disruption, microbial manipulation of the host microbiome to treat chronic diseases has become the focus of recent renewed interest. Manipulation may be elicited through probiotics, prebiotics, or synbiotics (supplements composed of a combination of both probiotics and prebiotics), and fecal transplant. The goal is to develop novel therapies, including products of fermented food, to restore or promote beneficial microbial communities to help combat a myriad of chronic diseases.

Determination of selective protein biomarkers by immunoaffinity capillary electrophoresis was carried out in serum and stool samples of lean and obese animals before and after feeding them with a diet containing fermented food.

Current information support the fact that the complexity of the host stool proteome mirrors the complexity of microbiota composition. However, the application of metaproteomic approaches to complex human samples still faces considerable challenges.

Immunoaffinity capillary electrophoresis can provide a new avenue for non-invasive monitoring of host-microbiota dynamics via host-produced proteins in stool. In particular, affinity capture of selected proteins and/or peptides that can become specific biomarker molecules associated with detecting and diagnosing gastrointestinal diseases and other inflammatory diseases.

L-13-1

DIRECT COUPLING OF AFFINITY CHROMATOGRAPHY AND CAPILLARY ISOELECTRIC FOCUSING IN A SINGLE CAPILLARY

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One of the most selective separation method, affinity chromatography, and a high-resolution separation method, capillary isoelectric focusing (CIEF), were directly coupled in a single capillary. To our knowledge, this is the first report for this type of coupling. The inner wall of the capillary was separately coated with two different polymers. The inlet part (20 cm) of the capillary (50 μm i.d., 50 cm long) was covalently coated with polyglycidylmethacrylate (PGMA) and the rest of the capillary was covalently coated with polydimethylacrylamide (PDMA). The epoxy groups of the PGMA coating were reacted with iminodiacetate, and the resultant chelating polymer coating was loaded with nickel ions (2+). In this way, the nickel-chelate adsorbent and the PDMA-coated capillary for CIEF were directly connected as a single entity. The proof of principle experiment was carried out using fluorescence-labeled recombinant Fab (rFab) having a hexahistidine tag as a sample and Beckman P/ACE MDQ instrument. After loading a sample mixture of the rFab and fluorescence-labeled bovine serum albumin (BSA), the capillary was washed to remove the labeled BSA and, then, filled with a carrier ampholyte solution. Bound rFab was eluted by injecting an anolyte solution, 100 mM phosphoric acid, until its front end reached the boundary of the two coatings. CIEF separation was initiated by applying the voltage of 25 kV with the anolyte and 100 mM NaOH as an catholyte. To block the electroosmotic flow that was produced in the affinity column towards the anode, a pressure of 0.2 psi was applied at the anode for 2 min, and the pressure was reduced to 0.1 psi thereafter. As the decrease of the electroosmotic flow with the progress of focusing, the focused protein in the pH gradient was conveyed to the cathode and detected with LIF at 10 cm from the cathodic end. The electroosmosis produced in the neighboring affinity column can be practically suppressed by using the anolyte solution having a relatively high conductivity in comparison with that of the carrier ampholyte solution, and/or by applying the pressure balancing the electroosmosis. Under such conditions, CIEF in the PDMA-coated capillary can be successfully achieved without any compromise in resolution. The affinity capture in front of the CIEF capillary is also quite effective to remove salts that may affect the CIEF separation. The pressure-osmosis balance is very important for successful separation. The results of a detailed investigation on such balancing will be reported.

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L-13-2**USING NON-EQUILIBRIUM CAPILLARY ELECTROPHORESIS OF EQUILIBRIUM MIXTURES (NECEEM) FOR SIMULTANEOUS DETERMINATION OF CONCENTRATION AND EQUILIBRIUM CONSTANT**

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Non-equilibrium capillary electrophoresis of equilibrium mixtures (NECEEM) is a versatile tool for studying affinity binding. Here we describe a simple procedure that facilitates simultaneous determination of both the equilibrium constant, K_d , and binder concentration by NECEEM. In essence, NECEEM is used to measure the equilibrium fraction of unbound binder for a binder with known concentration. The measurements are performed at varying concentrations of this binder but at a constant concentration of the second binder, for which the concentration is unknown. The experimental dependence of the fraction of unbound binder on its concentration is fitted with a function describing their theoretical dependence. Both K_d and the concentration of the second binder are used as fitting parameters and their sought values are determined as the ones that generate the best fit. This procedure was applied to study the interaction between ABH2 protein, a human oxoglutarate-dependent oxygenase, and its DNA aptamers. Enzymes of this class catalyse the removal of N-methyl groups from the damaged DNA to prevent mutation and carcinogenesis. Silencing the demethylating enzymes may be beneficial for enhancement of chemotherapeutic treatments and can reduce their cytotoxic effects through reducing therapeutic doses. We used our NECEEM-based procedure to find K_d and an unknown concentration of ABH2. The aptamers were fluorescently-labeled and their concentrations were known. The protein concentration found was within 10% of the one measured by an independent method. The values of K_d were determined for the interaction of ABH2 and its aptamers for the first time.

L-13-3**AFFINITY CAPILLARY ELECTROPHORESIS-MASS SPECTROMETRY:
ISOFORM-SELECTIVE ASSESSMENT OF PROTEIN-PROTEIN INTERACTIONS**

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Protein-protein interactions play a major role in biological processes and pharmacological activity of biopharmaceuticals. Affinity capillary electrophoresis (ACE) represents a powerful technique for the assessment of protein-protein interactions by measuring ligand electrophoretic mobilities as function of receptor concentration. Employing the efficient separation of intact proteins as provided by CE, ACE offers the unique possibility to simultaneously study the interaction of multiple proteins with a target receptor under homogeneous and near-physiological conditions.

Here, we present the on-line combination of ACE with mass spectrometry (MS) as a novel and powerful tool for the determination of protein-protein affinity constants. It will be demonstrated that ACE-MS allows (i) label-free detection of protein ligands, (ii) assignment of molecular weight and composition of unknown ligands in mixtures, and (iii) simultaneous detection of ligand and affinity complex in one peak. MS-compatible multilayered coatings consisting of Polybrene, dextran sulfate, and Polybrene were employed to allow efficient and reproducible CE of protein ligands (also in presence of receptor) and ensure precise determination of affinity-induced changes of ligand mobility. Due attention was paid to the CE-MS interfacing conditions to ensure proper transfer of the complex from the liquid into the gas phase. A strategy to negotiate possible ionization suppression by the receptor present in the BGE was evaluated as well. The binding of the protease inhibitor aprotinin to trypsinogen was used as protein-protein affinity model; the trypsinogen sample comprised several isoforms. ACE-MS analysis of the trypsinogen in the presence of different concentrations of aprotinin yielded isoforms-selective dissociation constants, and at the same time allowed identification of the isoforms, which were mainly deamidated variants of trypsinogen. The affinity data showed that these modifications did not significantly alter the affinity, with dissociation constants all in the μM range. In order to appreciate the utility and added value of the data obtained with ACE-MS, comparisons were made with affinity measurements by direct infusion MS and ACE with UV detection.

KN-14

SAW-MALDI MS: OPEN CHIP FOR BIOMOLECULE SAMPLE HANDLING - PAINFUL CELL EXPERIMENTS?

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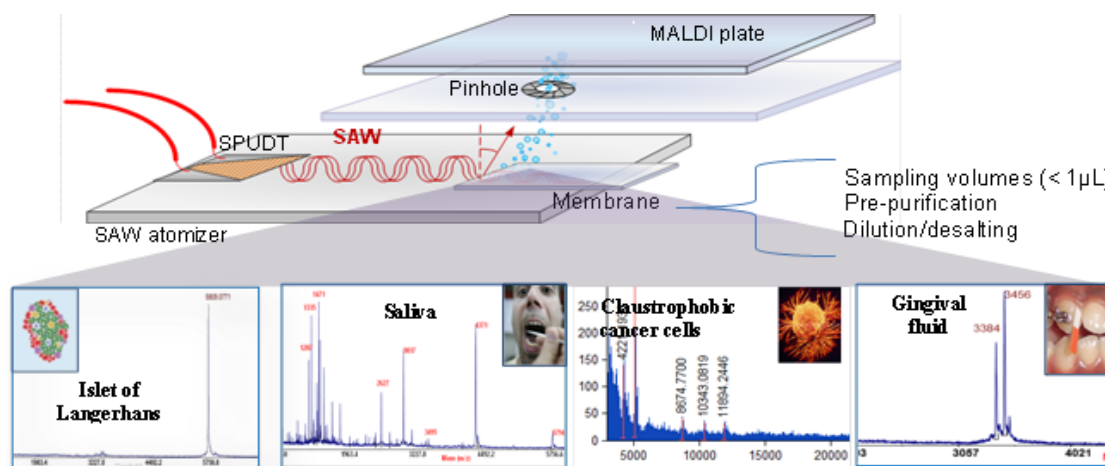
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SAW atomizer has been interfaced with MALDI MS for fast analysis of small volumes (< 1 µL) sampled in a membrane [1]. SAW propagate through and underneath the membrane and atomize the liquid bound sample [2] into 2-10 µm diameter aerosol which is subsequently deposited on a MALDI plate for further analysis. Fast peptide profiling of living islets of Langerhans and bio fluid (saliva, gingival fluid) has been achieved and facilitates further analysis utilizing airborne chemistry [3] leading to a wall-less analysis with low contamination and high sensitivity [4] at single-cell level.

Scaffold-entrapped, claustrophobic, cancer cells are monitored during their growth phase using SAW-MALDI-MS, on the releasate. Peptides will be identified as potential growth inhibitors in the treatment of cancer.



SAW-MALDI-MS based cell releasate analysis.

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L-14-1**PNEUMATIC MICROVALVE-BASED HYDRODYNAMIC SAMPLE INJECTION FOR HIGH THROUGHPUT, QUANTITATIVE ZONE ELECTROPHORESIS IN MICROCHIPS AND CAPILLARIES**

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Sample injection plays a major role in determining the sensitivity, resolution, throughput and quantitative power of both capillary-based and microfluidic capillary zone electrophoresis (CZE) separations. For capillary-based CZE, the capillary inlet is moved from the run buffer to a sample reservoir and sample is injected electrokinetically or hydrodynamically. For microchip CZE, gated or pinched electrokinetic injection schemes are commonly employed. For all of these injection techniques, the electric field required for separation is interrupted during sample injection, and as such a separation must generally be completed before a subsequent injection can occur. For gated microfluidic injection, the sample volume can be varied based on injection time and voltage, but a mobility-based quantitative bias results. Pinched microfluidic injection has less of a quantitative bias, but the injection volume is fixed by the geometry of the injection region of the microchip. Here we present a pressure-based injection method utilizing a pneumatic microfluidic valve created by multilayer soft lithography. A separation voltage is applied continuously across the column and a computer-controlled valve is opened to allow sample to be loaded onto the column. The injection method has been used with both microfluidic and capillary-based separations and with both laser-induced fluorescence and electrospray ionization mass spectrometry detection. Importantly, the technique enables variable sample volumes (picoliters to nanoliters), no quantitative bias, and repeated, programmed overlapping separations for optimization and high measurement throughput. The technology is expected to facilitate the rapid analysis of large numbers of samples as well as multiplexed and multidimensional separations.

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L-14-2**PACKED MULTI-CHANNELS FOR PARALLEL CHROMATOGRAPHIC SEPARATIONS IN MICROCHIP**Nagy, Andrea; Gáspár, Attila*University of Debrecen, Dept. of Inorganic and Analytical Chemistry*

Only relatively few chip-based chromatographic systems [1-2] compared to chip-based capillary electrophoretic devices are known. Very recently, microfluidic solid-phase chromatography columns formed by multilayer soft lithography and an application of special bypass channel systems along the length of the chromatographic packing were described [3].

Here we report on a simple method to fabricate microfluidic chip incorporating multi-channel systems packed by conventional chromatographic particles without the use of frits [4]. The retaining effectivities of different bottlenecks created in the channels were studied. For the parallel multi-channel chromatographic separations several channel patterns were designed. The obtained multipackings were applied for parallel separations of dyes. The highest separation efficiency for a dye component was $0.75 \mu\text{m}$ (plate number value was 1,330,000/m), which was obtained with 0.0176 mm/s (4.2 nL/min) flow rate. The flow rates in the different parts of the channel network were calculated by using EPANET software. The calculated values were in good agreement with the flow rate values given from the microchip experiments.

We also studied the possibility of interfacing microfluidic chip-based chromatography to flame atomic absorption spectrometry (FAAS) for a simple and fast separation or preconcentration and subsequent determination of chromium(VI).

The implementation of the several chromatographic separation units in microscopic size makes possible faster and high throughput separations. The simplicity of the replication of the polydimethylsiloxane (PDMS) chips and the minimal consumption of the conventional packing particles (some tens of nanograms for a 10 mm length of packing) makes the chips inexpensive and disposable.

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L-14-3**APPROACHES TO CHIRAL SEPARATION PREDICTION ON IMMOBILIZED POLYSACCHARIDE CHIRAL STATIONARY PHASES**

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It is well known that drug enantiomers exert different pharmacological actions, sometimes toxic or antagonistic. [1] In order to analyze and improve the pharmacological profile, enantioseparation is mandatory. Chiral chromatography is the most used tool in separating enantiomers on analytical or preparative scale. Therefore, predicting the chromatographic behavior of certain compounds is becoming more and more interesting, as it is useful in reducing method development time and solvent consumption.

The present study focuses on generating predictive models for enantioseparations on immobilized polysaccharide CSPs using sixteen beta-blockers as model drug substances. A normal phase screening methodology was developed using a mobile phase gradient consisting of n-Hexane/2-propanol and several basic additives (ethylenediamine, diethylamine and ethanolamine). [2] The obtained chromatographic parameters (k' , t_r , N and R_s) were correlated using decision tree analysis and multivariate analysis with two different classes of easily available molecular descriptors - fixed length [3, 4] and MOSES molecular descriptors.

Fourteen out of sixteen beta-blockers were baseline separated using four commercially available CSPs. A significant degree of complementarity was observed in the case of the studied stationary phases, while the scale of influence of the employed additive is stationary phase dependent.

The used data mining procedures in the case of fixed length molecular descriptors offered prediction models with high percentage of correct classifications (>75%, correct classification threshold $R_s = 1.5$). Multivariate data analysis (PLS and O2PLS methods) offered better predictive models, given that for decision tree analysis a much larger database should be considered.

In case of MOSES molecular descriptors multivariate data analysis was the method of choice for predicting enantiomer separations. Once again the obtained models showed a good predictive ability (66.6% of all the models with correct classifications >80%; the remainder of models - 60-80% correct classifications).

The developed gradient elution screening method turned out to be an efficient protocol to obtain the necessary training data set in the construction of prediction models applicable in the early stages of chromatographic method development. Moreover, the highly predictive models were created using widely available molecular descriptors without the need of sophisticated computational chemistry tools and highly skilled personnel.

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POSTERS

ALPHABETICAL ORDER OF THE FIRST AUTHOR

P-01

INCREASING THE SPEED OF CAPILLARY GEL ELECTROPHORESIS OF PROTEINS

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In protein analysis, sodium dodecyl sulfate-capillary gel electrophoresis (SDS-CGE) with on-line UV detection has developed into a strong alternative for traditional slab-gel electrophoresis, offering automation, enhanced resolution and improved quantitation as well as low buffer and sample volume consumption. Presently, SDS-CGE is used in particular for profiling of biopharmaceuticals, e.g. for monitoring of the presence of related compounds, such as the non-glycosylated antibody and heavy chain (HC) or light chain (LC) fragments in monoclonal antibodies (mAbs). SDS-CGE analysis is generally performed with conventional CE instrumentation using prefabricated kits comprising rinsing solutions for capillary conditioning and sieving gel solutions as background electrolyte (BGE). Following standard procedures, SDS-CGE separation times are relatively long, taking typically at least 30 min to cover a protein size region of 10-225 kDa.

In this study, we have investigated possibilities to increase the speed of SDS-CGE. The study focused on the separation voltage and the effective capillary length. The effect of increasing the separation voltage from 15 kV to 30 kV on the total analysis time and protein resolution was evaluated using a mix of seven protein size markers ranging from 10-225 kDa. Reduction of the effective capillary length from 20 to 10 cm was achieved by applying the short-end injection procedure (SEIP). During this modification, the sample injection zone relative to the migration distance was kept constant. By using both higher voltage and SEIP, separation of the proteins in the test mixture was achieved within approximately 7 min. This is a four-fold decrease of separation time. RSD values (n=3) of migration times, peak widths, and corrected peak areas were generally within 5.0%, which is similar to the standard SDS-CGE method. Plate numbers were decreased by 2.0-8.8 for the 10-225 kDa range. Resolution was decreased by 1.9-3.2 with respect to the standard method, however, all test proteins were well baseline separated with the high-speed method. The applicability of the developed high-speed SDS-CGE method was demonstrated by the analysis of non-reduced and reduced samples of pharmaceutical mAbs. The high-speed method allows the detection of LC and HC fragments in mAbs.

P-02

DETERMINATION OF BINDING CONSTANTS AND THE INFLUENCE OF THE CYCLODEXTRIN CAVITY SIZE FOR THE EMO ON THE SEPARATION OF AMINO COMPOUNDS BY CAPILLARY ELECTROPHORESIS

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The search for new chiral ligands which can be efficiently applied in biology and asymmetric catalysis is currently a field of great interest in organic chemistry. Amino compounds and their derivatives are important intermediates and chiral catalysts in organic syntheses. Among them, Jørgensen's catalysts play important roles in the synthesis of various enantiomerically pure compounds in organic and medicinal chemistry. The amino acid amides serve as building blocks for different peptides and peptide analogs. A number of antibacterial β -lactam agonists, which contain an amine-substituent at position 2, display Gram-positive and Gram-negative antibacterial activity.

The development of methods for the separation of enantiomers has attracted great interest, since it became evident that the potential biological or pharmacological applications are mostly restricted to one of the enantiomers. The important analytical task of the separation of isomers is achieved mainly by chromatographic and electrophoretic methods. Capillary electrophoresis is one of the most efficient separation techniques available for the separation of both large and small molecules. One of the important issues is the migration order in CE especially for the determination of the enantiomeric purity of a compound. Different factors lead to the migration order of enantiomers: 1, the binding strength of the analytes by the selector; 2, the direction and magnitude of the mobility of the free analyte; 3, the direction and magnitude of the mobility of the chiral selector; 4, the direction and magnitude of the mobility of the selector-analyte-complex; 5, the direction and magnitude of the EOF; 6, the concentration of the chiral selector.

The present study was described in order to develop a capillary electrophoresis method for the separation of amino compounds containing one chiral center. Experiments were performed at 25 °C employing 50 μ m I.D. fused-silica capillary with an effective length of 50 cm and total length of 60.2 cm. Using a background electrolyte composed of 20 mM sodium phosphate-buffer adjusted to pH 2.5 by addition of phosphoric acid and a separation voltage of 15 kV, the separation of all amino compound enantiomers could be achieved. Several CDs including α -CD, β -CD, γ -CD and their derivatives were investigated as a chiral selector. Elution order changes were achieved by using different cavity size sulphated- β -cyclodextrins.

P-03

HPLC-MS BASED MONITORING OF STEROID CONTAMINANTS OF LAKE BALATON

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Worldwide, one of the most studied research fields of environmental effects is the contamination of anthropogenic origin due to the continuously growing population and urbanisation, which affects primarily the fresh water ecosystems. The occurrence of highly persistent chemical pollutants in different compartments of the aquatic environment have become a major threat to the healthy state of the aquatic ecosystems.

Among chemical pollutants, hormones represent a significant source of danger. Oral contraceptives and endogenous estrogenic and androgenic steroid hormones are eliminated and removed from the body mainly through renal systems and entering in ecosystems they can accumulate. Instead of steroid hormones being chemically very stable (ring structure), highly lipophilic and poorly soluble in water their biological properties have a strong impact on the endocrine regulation of aquatic species. Changes in the reproductive system may also initiate disturbances of the population dynamics of different species.

Considering that there is limited quantitative information about steroid hormone contaminations of Lake Balaton and its drainage basin our aim was to chart the area with novel liquid chromatographic methods. At first we focused on estradiol (E2), estriol (E3), testosterone and progesterone levels. We pre-concentrated our samples using solid phase extraction (SPE). A Dionex LC system with a 2.6 µm C18 100Å column and a quadrupole-orbitrap mass spectrometer (MS) as detector was used for measuring. As a second aim of our investigation we compared the performance of the Q-Exactive (orbitrap-MS-microflow-HPLC) system with an Agilent iFunnel Q-TOF by which separation is carried out on a HPLC chip. Our early experiments show a slight advantage of the Chip Cube based on its excellent sensitivity, though it has less good reproducibility what can make the orbitrap system the first choice for routine analyses.

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P-04

COMPARISON OF PORE SIZE DISTRIBUTION OF RP-HPLC STATIONARY PHASES WITH THE MOLECULAR THEORY OF SIZE-EXCLUSION CHROMATOGRAPHY

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Important factors of high column performance and efficiency are the physico-chemical properties of liquid chromatography stationary phases [1]. To characterize the pore structure, the pore size distribution is relevant, because the mass transfer across the particles is a very important component of the band broadening [2-4].

Inverse Size-Exclusion Chromatography is a very comfortable and preferred method for estimate the porosity of HPLC columns, because it needs only a HPLC instrument with minor changes and the researchers are going to able to characterize their own columns. This method based on the relationship between the retention volumes of polymer fraction with known mass and narrow mass distribution and its size, namely the mass and diameter respectively [5].

The stochastic models depict the chromatographic process at a molecular level via the random migration of the individual molecules. The stochastic theory of size-exclusion chromatography (SEC) based on the characteristic function, developed by Dondi et al. [6] allows taking into account the kinetics of the pore ingress and egress processes, the heterogeneity of the pore size and polymer polydispersion.

In this study we will show, how to estimate the pore volume, the pore size, and the PSD of particles on the basis of experimental data using a chromatographic model extended to wide PSD in case of cylindrical and conical pore shape [7]. For the validation we used the column having different structure HPLC columns.

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P-05**PREDICTION OF PEPTIDE AND GLYCOPEPTIDE SEPARATION FROM GLYCOPROTEIN DIGESTS IN CAPILLARY ELECTROPHORESIS-MASS SPECTROMETRY**

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P-06

TEMPERATURE DEPENDENCE OF THE RETENTION FACTORS OF RESORCINARENE BASED CAVITANDS ON C8 AND C18 REVERSED STATIONARY PHASES

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Cavitands (e.g.: calixarenes, resorcinarenes, cyclodextrins and their derivatives) are cavity-shaped cyclic oligomers and they can create host-guest interactions. Cavitand-bonded silica particles may have use as selectors in high performance liquid chromatography (HPLC). Many of them are prepared for the separation of aromatic positional isomers [1, 2], geometric isomers [3], enantiomers of chiral compounds [1] and polycyclic aromatic hydrocarbons [2].

We have investigated the chromatographic behavior of three resorcinarene based cavitands as analytes in HPLC. This is important to know before their covalent binding to the stationary phase. The experiments were performed at four different temperature values (15 °C, 25 °C, 35 °C, 45°C) on two types of reversed stationary phases (C8 and C18) from two different manufacturers (Waters Xterra® and Thermo BDS Hypersil). To our knowledge, there is only one reference that demonstrates the chromatographic separation of resorcinarene based cavitands [4]. The retention mechanisms were studied with the help of thermodynamic parameters calculated from the van't Hoff equation:

$$\ln k' = -\frac{\Delta H^\circ}{RT} + \frac{\Delta S^\circ}{R} + \ln \Phi$$

where $\ln k'$ is the natural logarithm of the retention factor, R is the gas constant and Φ is the phase ratio of the column (the volume of the stationary phase divided by the volume of the mobile phase). The slopes and the intercepts were used to calculate the enthalpy (ΔH°) and entropy (ΔS°) for the transfer of the solute from the mobile phase to the stationary phase. Gibbs free energy (ΔG°) was also calculated.

We have found different retention behaviors between resorcinarenes containing free hydroxyl groups on the upper rim and cavitand, which has methylene bridges between the hydroxyl groups.

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P-07

EFFECTS OF CLARY SAGE OIL AND ITS MAIN COMPONENTS LINALOOL AND LINALYL ACETATE ON THE PLASMA MEMBRANE OF *CANDIDA ALBICANS*: AN *IN VIVO* EPR STUDY

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Effects of clary sage (Cs, *Salvia sclarea* L.) oil and its two main components, linalool (Lol) and linalyl acetate (La) on the eukaryotic human pathogen yeast *Candida albicans* cells were studied. Dynamic and thermodynamic properties of plasma membrane were investigated by electron paramagnetic resonance spectroscopy, using 5-doxylstearic acid (5-SASL) and 16-doxylstearic acid (16-SASL) spin labels. The phase transition break point temperature of plasma membrane of cells labelled with 5-SASL treated with 0.125 $\mu\text{L mL}^{-1}$ for 15 min have been identified at 13.15 °C and 10.35 °C, 12.70 °C, 9.55 °C of untreated and Cs oil, Lol, La, respectively, showing significant increase in fluidity via interactions with plasma membrane of cells. Results spin label proved that Cs oil and La induced increased level of fluidization in comparison to Lol treatment had two opposite consequences namely it induced a less (1%) ordered bilayer organization at superficial regions simultaneously with an increased (10%) order of the membrane leaflet at deeper layers. Acute toxicity tests and results of EPR proved that composition- and chemical structure-dependence of cytotoxicity and plasma membrane disturbing effects of examined oils. In comparison to control, Cs oil, Lol and La treatment induced loss of 13.0%, 12.3% and 26.4% of metabolites absorbing at 260 nm, respectively, as biological consequence of the plasma membrane fluidizing effects.

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P-08

**PREPARATIVE CAPILLARY ISOTACHOPHORESIS USED IN MULTIDIMENSIONAL SEPARATIONS
OF PROTEINS**

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Capillary isotachopheresis (CITP) is mainly used as an on-line pretreatment technique prior to the other electroseparation methods, e.g. capillary zone electrophoresis (CZE). Moreover, CITP is also used for micropreparative purposes. In this case, CITP is employed as (1) sample purification technique for isolation of selected components from the sample matrix into fraction(s) or (2) sample fractionation technique for splitting the sample into several fractions with limited number of constituents which reduces the risk of peak overlapping in final separation.

The aim of this work was to study the effect of simplification of multicomponent proteinous samples by preparative CITP on the resolution of the compounds obtained in CITP-CZE and planar SDS-PAGE separations. As a model sample intracellular fluid (cytosol) obtained from bacteria *Mycobacterium smegmatis* (*M. Smeg.*) was chosen.

Preparative process was carried out on a column – coupling separation unit in discontinuous fractionation mode (at interrupted separation current). The apparatus consisted of two joined wide-bore capillaries (800 µm I.D., 90 mm length) and micropreparative valve (6 µL) placed at the end of second column. A cytosol sample contained relatively high concentration of low molecular weight cations (sodium, potassium, magnesium) originated from the *M. Smeg.* cells and also from the buffer. An optimal condition for isolation of cell organelles was achieved by proper selection of buffer. An adequate concentration of protein in isolated fractions for further analysis by CITP-CZE and/or SDS-PAGE required a high injection volume of cytosol (up to 100 µL) into the preparative CITP device, which unfortunately resulted in the long separation time (about 1.3 h). Sample desalting by ultrafiltration (MILLIPORE YM10) reduced the separation time in half.

Fractions were analyzed by on-line combination of CITP-CZE, where the CITP served as a concentration technique prior to CZE with UV photometric detection at 254 nm. The splitting of desalted cytosol compounds into six fractions enabled to improve the peak resolution in CZE stage of CITP-CZE. Good repeatability of the fractionation procedure was confirmed by CITP-CZE analysis. SDS-PAGE separation using precast gradient gel (ExcelGel XL SDS 12–14) was used to consider the molecular weight of the separated substances. SDS-PAGE analysis of fractions obtained by preparative CITP showed the presence of proteins of various molecular weights in all fractions. This fact indicates no relation between the effective mobilities of the proteins separated under employed CITP conditions and their molecular weights and consequently, the orthogonality of the separation mechanisms in both CITP and SDS-PAGE techniques.

Acknowledgements

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P-09

CE WITH LED INDUCED FLUORESCENCE DETECTION A LOW COST TOOL FOR THE ANALYSIS IN FOOD CHEMISTRY

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For several years, many articles described the use of LED instead of Laser wavelengths. In fact, LED technology presents a lot advantages than lasers: cheaper, less energy consuming, lifetime higher and a large range of different wavelengths. This last point is important because LED is able to be used for the excitation of many different fluorophores. In this poster we will show that LEDIF results in more stable low frequency noise, that the sensitivity depending on the fluorophore and is better than using lasers, and offer a larger variety of LED wavelengths.

We will present analysis of amino acids and biogenic amines labeled with FITC in wine and african drinks. For that, depending of the suitable excitation wavelength of this dye, 480 nm LED has been used.

In the African drinks, we have determined that most essential amino acids for the growth of children were present except lysine. We are also interested to the detection of serotonin. The ESI-MS and ESI-MS/MS experiments confirm that serotonin was correctly labeled with FITC. We have analyzed red wine and serotonin was not found in this sample.

P-10

ADVANCED LIQUID CHROMATOGRAPHY TECHNIQUES FOR PROTEOMIC ANALYSIS

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One of the biggest challenges for analytical scientists, involved in the analysis of proteomic samples, is their enormous complexity due to the presence of thousand of compounds occurring in a wide concentration range. Additionally, proteins can undergo several post-translational modifications (PTMs) e.g. deamidation, oxidation, glycosilation and phosphorylation, thus leading to remarkable structural alterations. In particular, phosphorylation plays an important role in determining nutritional and functional properties of food products.

As a consequence, a high demand is placed both on the separation power and on the sensitivity of detection methods employed. Two dimensional polyacrylamide gel electrophoresis (2D-PAGE), where protein separation occurs according to pI and molecular weight, has been the method of choice for proteome analysis for a long time. Despite the high separation power and the orthogonality theoretically affordable, 2D-PAGE suffers of some limitations such as difficulty of both automation and detection of low abundant proteins as well as proteins with high pI, MW, or strong hydrophobic nature.

Liquid chromatography-mass spectrometry (LC-MS) has recently drawn a considerable attention allowing to address many of the limitations affecting traditional gel-based methods. From an analytical view-point, enhanced chromatographic resolution can be achieved either by extending the stationary phase length or by the employment of orthogonal separation modes (in multidimensional or “comprehensive” LC systems) prior to MS or MS/MS analysis.

An overview of such different chromatographic approaches, employing also capillary stationary phases is here provided, for selected protein isoforms tryptic mapping, followed by ESI-MS characterization and database search for sequence coverage.

P-11

EVALUATION OF THE INTERACTION OF MAGNETIC NANOPARTICLES WITH SMALL MOLECULES

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Capillary electrophoresis has proven to be a useful technique for the characterization of nanoparticles (NPs), as it can be used to estimate the size and charge of the particle under the appropriate experimental setup [1, 2].

In the present work, we apply dynamic equilibration affinity electrophoresis for the estimation of the interactions between magnetic COOH-Fe₃O₄ nanoparticles and small molecules and surfactants (e.g. TRIS, DMAB, CTAB, SDS...)

Different BGE solutions based on 5 mM borate/NaOH pH 9.5 and concentrations ranging of the ligands up to 1 mM were used for screening of the interaction between the nanoparticles and the different compounds. Suspension of nanoparticles was prepared on 5 mM borate/NaOH pH 9.5 in all cases. The variation of the electrophoretic mobility of the nanoparticle – molecule adduct is correlated with the concentration of ligand added into the background electrolyte.

The obtained results indicate that dynamic equilibration affinity electrophoresis can therefore be applied for the evaluation of the interactions of nanoparticles with small molecules and to estimate their binding constants in a similar manner to that carried out for the complexation of small molecules.

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P-12

IMMOBILIZED MONOLITHIC ENZYMATIC REACTORS FOR ONLINE DIGESTION OF PROTEINS SECRETED BY DEVELOPING HUMAN-EMBRYOS

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P-13

APPLICATION OF LC- MS/MS IN β -LACTAMS DEGRADATION STUDIES

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β -lactam antibiotics are susceptible to degradation in conditions of acid-base hydrolysis, oxidation and in the solid state. Degradation products are pharmacologically inactive and their structures are similar to parenteral substance. The aim of the study was to develop the selective analytical methods and identification of degradation products in selected conditions of degradation. For three β -lactam analogues (cefoselis sulfate, ceftazidime hydrochloride and ceftiofur sodium) were developed HPLC MS/MS method. The method allow to separation of parenteral substance from degradation products formed under the influence of stress conditions. The cefoselis was separated on Lichrospher RP-18 column, 5 μ m particle size, 250 mm x 4 mm, using mobile phase acetonitrile 12 mM ammonium acetate (95:5 V/V), flow rate 1.0 mL min⁻¹. The wavelength of the DAD detector was set at 260 nm. Separation was performed at 30°C.

The ceftazidime was separated on Lichrospher RP-18 column, 5 μ m particle size, 250 mm x 4 mm, using mobile phase acetonitrile 12 mM ammonium acetate (92:8 V/V), flow rate 1.0 mL min⁻¹. The wavelength of the DAD detector was set at 260 nm. Separation was performed at 30°C.

The ceftiofur sodium was separated on Lichrospher RP-18 column, 5 μ m particle size, 125 mm x 4 mm, using mobile phase acetonitrile 12 mM ammonium acetate (90:10 V/V) flow rate 1.0 mL min⁻¹. The wavelength of the DAD detector was set at 270 nm. Separation was performed at 30°C. For all compounds chemical structures of degradation products was proposed and degradation pathways were established. The LC-MS/MS was validated with respect to selectivity linearity accuracy and precision.

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P-14

GREENER APPROACH TO THE STABILITY STUDIES BASED ON HPLC-DAD METHOD IN DETERMINATION OF CEFEPIME

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Cefepime is a fourth-generation semi-synthetic cephem analogue. It is very susceptible to degradation in body fluids and in pharmaceutical matrix. The development of fast and variable analytical method for determination of cefepime is important due to the formation of numerous degradation products as well as the possibility of its degradation during analysis.

The aim of studies is development of fast stability-indicating LC-DAD method for determination of cefepime.

For a chromatographic separation of cefepime, a Kinetex C-18 (100 mm x 2.1 mm, 5 μ m) as stationary phase and mixture of 12 mmol L⁻¹ ammonium acetate solution: acetonitrile (95:5 V/V) as mobile phase were used. A quantitative determination of cefepime was carried out by using DAD detector at 254 nm, with a flow rate of 1.0 mL min⁻¹. Volume of injection was 5 μ L.

The selectivity of method was established in the presence of degradation products which are formed under the influence of conditions of acid-base hydrolysis, thermolysis and oxidation in bulk substance and MAXIPIME preparation. The method has been validated with respect to linearity range, limit of detection (LOD), limit of quantification (LOQ), precision, accuracy. The robustness of the procedure was evaluated by changing the following parameters: the composition and pH of the mobile phase, the mobile phase flow rate in the range of 0.8–1.2 mL/min, and the temperature in the range 25–35°C ($\pm$ 1 °C). With a change of each parameter, its effect on retention time, peak resolution, peak shape and peak area (height and width) was evaluated.

Under applied chromatographic conditions, determination of cefepime was possible in short time ~ 1.20 min. Typical retention time of cefepime was about 0.79 min, while the peaks originating from degradation products and related products were eluted faster (< 0.55 min). Separation of degradation products was also possible.

The application of HPLC-DAD for determination of cefepime may be considered as a greening solution for liquid chromatography with potential to replace the less environment-friendly analytical techniques. An application of selective and fast analytical method consuming lower volumes of organic solvents is significant during stability studies when the required number of assays is especially high.

P-15

A NEW MOLECULARLY IMPRINTED POLYMER FOR THE EXTRACTION OF GLYPHOSATE IN WATER

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Glyphosate [(N-phosphonomethyl)glycine] is a non-selective broad-spectrum herbicide which is extensively used in agriculture. This molecule inhibits the plant enzymatic mechanism which enables the plant to produce amino acids that are needed for its growth. The biodegradation of glyphosate (GLY) in the environment leads to aminomethylphosphonic acid (AMPA) and both molecules are included in the European guidelines about the quality of surface and underground waters (DCE 2006/18 circular – appendix 1).

GLY and AMPA monitoring at low concentrations (about 1 µg/L) requires efficient pre-concentration tools before any analytical method. However, none of the commercial sorbents usually used as solid phase extraction (SPE) materials is appropriate to retain very polar and hydrophilic analytes such as GLY and AMPA. For this reason, a molecular imprinted polymer (MIP) has been synthesized to rebind the target molecules in water.

The selected monomer is 1-allyl-2-thiourea which was used by Kugimiya for the synthesis of a MIP dedicated to the capture of phosphate ions in water [1]. The template is phosphonic acid, the cross-linker is ethylene glycol dimethacrylate and the porogenic solvent is acetonitrile.

The assessment of the MIP were firstly carried out by comparison of the recoveries obtained with MIP and NIP (Non Imprinted Polymer, synthesized with the same reagents as MIP, but without template) with a very straightforward SPE protocol (conditioning of 250 mg of MIP/NIP packed in 3 mL polypropylene cartridges with 3 mL ultra pure water, loading of GLY and AMPA (5 mg/L) in ultra pure water (15 mL) and elution by 3 mL NH₄OH (10 mM). For all the experiments, the samples, MIP and NIP extractions were analyzed by capillary electrophoresis that is a high throughput analytical method. Thus, MIP recoveries of GLY and AMPA (105% and 80%, respectively) were higher than NIP recoveries (68% and 19%, respectively) which proved MIP selectivity, that means the presence of cavities able to specifically rebind GLY and AMPA. Moreover, the substitution of ultra pure water by buffers of pH contained between 5.5 and 9 showed that GLY and AMPA were most retained in a narrow pH range from 5.5 to 6. Then, a capacity study showed that one gram of MIP totally retained 0.017 µmol of GLY and 0.005 µg of AMPA. However, the substitution of ultra pure water by mineral water caused the decrease of MIP recoveries (about 30 % for GLY and 5 % for AMPA), for that, a pretreatment of the sample by ionic exchange resins was set up and succeeded in improving recoveries (about 60% for GLY and 25% for AMPA). The MIP was also successfully applied for the extraction of GLY (0.5 µg/L) in mineral water without any resin pre-treatment. Indeed, the elution recovery of GLY was 92%. The low level of GLY concentration required UPLC-MS/MS apparatus.

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P-16**LIF DETECTION METHOD ON PDMS CHIPS**

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The lab-on-chip methods gained wide popularity during the last decades. They offer today fast and reliable separation methods for a relative low price and equipment needs. One of the main goals of its use is to replace the conventional methods with chip-based ones. As a result of the efforts all the significant chromatographic and electrophoretic methods were already “transferred” to chips.

However, after a successful separation on an in-lab developed chip, the detection still can be difficult and tricky several times, even with optical, electrochemical or mass-spectrometric detection methods.

Our poster tries to summarize some ways of the implementation of detection methods on plastic chips, showing its pros and cons from the practical point of view. We mainly focused to develop laser induced fluorescence (LIF) on PDMS chips using integrated optical components. The set-up was built for low-energy laser power source excitation and fiber optic detection of the emission of simple fluorescent components.

Acknowledgement

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P-17

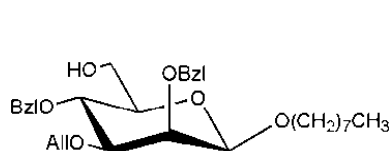
APPLICATION OF CONVENTIONAL AND COMBINATORIAL CARBOHYDRATE CHEMISTRY FOR GLYCOMICS APPLICATIONS

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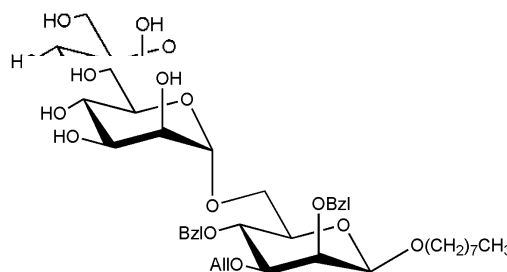
N-glycosylation of proteins is ubiquitous on eukaryotic cell surfaces and in body fluids. The emerging field of glycobiology is devoted to defining structural and functional roles of glycans in numerous biological recognition processes, including, for example, viral and bacterial infection, tumor metastasis, immune response and many other receptor-mediated signaling processes [1]. Chemical synthesis provides valuable means to produce glycans of biomedical interest, which may serve as model compounds to gain insight into the structure of *N*-glycosylation of proteins and function, and offers additional tools to study biomolecular interactions [2]. However, due to the large number of possible configurations, chemical synthesis and the following analysis of oligosaccharides is extremely challenging. Capillary electrophoresis is suitable method for analyzing oligosaccharides with high sensitivity.

Based on our vast experience during the past 30 years [3-5], our research program focuses on the preparation of large number of closely related structural determinants of high mannose antennae by means of the combination of conventional and combinatorial carbohydrate chemistry. After the removal of the necessary protecting groups the generated mixture of random oligosaccharides will be evaluated for their interactions with such important proteins as the HIV gp120.

The key compound of our synthetic route was octyl 3-*O*-allyl-2,4-di-*O*-benzyl- β -D-mannopyranoside **1**, which can be considered as a selectively protected pseudo core trisaccharide, containing β -mannosidic linkage and the chitobiose part is replaced by a simple octyl aglycone.



Compound 1



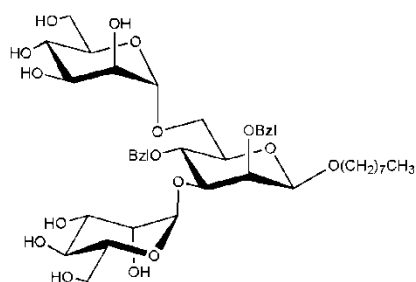
Compound 2

Compound **1** was prepared by conventional carbohydrate chemistry involving nine synthetic steps. The β -mannosidic linkage was created by C-2 epimerization of the initially introduced β -D-*gluco* unit *via* oxidation followed by stereoselective reduction. Condensation of **1** and acetylated mannose imidate followed by deacetylation gave pseudo tetrasaccharide acceptor **2** bearing 4 free OH-groups.

Removal of the allyl function of compound **1** resulted in a diol which was glycosylated with acetylated mannose imidate to yield a pseudo pentasaccharide. Deacetylation of this latter derivative resulted in acceptor **3** with 8 free OH-groups.

Random glycosylations of the pseudo tetra- (**2**) and pentasaccharide (**3**) acceptors with various mannosyl donors yielded the combinatorial mixtures of predominantly pseudo penta- and hexasaccharides, respectively.

The generated structures are characterized by capillary electrophoresis with laser induced fluorescent detection (CE-LIF). After the removal of the protecting groups from the synthetic products the screening experiments are carried out by means of interrogation with HIV gp120.



Compound 3

Random glycosylations of the pseudo tetra- (**2**) and pentasaccharide (**3**) acceptors with various mannosyl donors yielded the combinatorial mixtures of predominantly pseudo penta- and hexasaccharides, respectively.

The generated structures are characterized by capillary electrophoresis with laser induced fluorescent detection (CE-LIF). After the removal of the protecting groups from the synthetic products the screening experiments are carried out by means of interrogation with HIV gp120.

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P-18

MATRIX-COMATRIX SYSTEMS FOR MALDI-TOF MS ANALYSIS OF BACTERIAL ENDOTOXIN PROFILES

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Matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) has been already used for the classification and identification of bacteria based on their protein mass patterns (or protein profiles) [1]. Nowadays several companies offer user-friendly software packages containing different algorithms for the identification and classification of bacterial protein mass patterns, as well as databases with several thousand bacterial reference entries [1].

Since most Gram-negative bacteria have endotoxins, *i.e.* lipopolysaccharides or lipooligosaccharides (LPSs or LOSs) on their outer membrane, the question arises whether these molecules and/or their building blocks could also represent a class of biomarkers for bacteria determination. In general, an LPS molecule consists of a phosphoglycolipid unit (called lipid A), and two variable saccharide regions: (i) the core oligosaccharide (core OS) and (ii) the O-polysaccharide. A LOS molecule lacks the polysaccharide chain and sometimes portions of the core OS too. Micro- and macroheterogeneities describe the native LPS/LOS samples. The detection of the complete LPS profile is still a great challenge with mass spectrometry. Meanwhile, several MALDI-TOF MS methods have been published on structural characterization of intact LOS and isolated lipid A samples [2]. In case of LOS samples, besides the complete LOS quasimolecular ions, well-defined fragments appear in the MALDI mass spectra, as the acid-labile glycosidic bond between the core OS and the lipid A units is readily cleaved in the mass spectrometer [2, 3].

To get a comparable and reproducible endotoxin profile with MALDI-TOF MS applicable in bacterial determination, the information content variation of the mass spectra due to different experimental conditions and energetics should be understood. In this study, LPS and LOS samples extracted from *Shigella sonnei* phase I and II bacteria, respectively, and their lipid A units isolated from the appropriate LPS/LOS samples were analyzed as model systems. Negative ion MALDI-TOF MS experiments were performed in reflectron and linear modes, using an Autoflex II (Bruker) mass spectrometer. The matrix-comatrix systems, the ionization parameters were varied and their effects on the in source and post source decays, and thus, on the acquired mass spectra were compared.

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P-19

FIRST INSIGHTS OF PTERIDINES ANALYSIS BY CE-MS

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Pteridines belong to a broad family of compounds known for their implication in various biological systems such as the synthesis of tyrosine, serotonin, L-Dopa, and nitrite oxide. Tetrahydro-biopterin (BH4) is a precursor of numerous pathways, including important inflammatory processes. Recently, neopterin (NEO) appeared as an efficient biomarker for human African trypanosomiasis and subarachnoid hemorrhage where its concentration was significantly increased in cerebrospinal fluid and plasma, respectively. Because BH4, NEO and analogous molecules are highly polar (logP between -1.1 and -2.4) and ionizable, the on-line combination of capillary electrophoresis (CE) with mass spectrometry (MS) appears as an appropriate method for trace analysis of pteridines in biological fluids.

In this study, 11 compounds involved in the biosynthesis of BH4 and NEO were selected. The main objective of the study was to develop CE-MS compatible conditions for the simultaneous detection of all selected compounds. It has to be noted that these analytes experienced a significant stability issue: NEO is the oxidized form of dihydro-neopterin (NH2) while biopterin (BIO) is the oxidized end-form of dihydro- (BH2) and tetrahydro-biopterin (BH4). To ensure a sufficient stability of stock solutions, the latter have to be prepared in an aprotic solvent (acetonitrile) and diluted in water.

Because pteridines are amphoteric compounds, two modes of separation were investigated, i.e., capillary zone electrophoresis (CZE) under acidic and basic conditions where a complete separation of the 11 molecules was obtained with a buffer made of 160 mM borate adjusted with ammonia at pH 10.3. This separation was achieved thanks to the specific interactions between borate and the vicinal diols of the pteridines. In order to enable MS hyphenation while maintaining effective interaction, borate was then substituted by a volatile electrolyte (heptafluoroisopropanol, HFIP) mixed with 60 mM of borate adjusted to pH 10.3 with ammonia.

Another MS-compatible method was also investigated with an acidic BGE. Given their low basic pK_a values, a highly concentrated formate BGE was used (formic acid 2%, i.e., 525 mM). All compounds were ionized and separated, except isoxanthopterin and guanosine triphosphate, which migrated as neutral and negatively-charged compounds, respectively. The method was transferred to ESI-MS/MS in SRM mode with a sheath liquid interface. The molecules' stability was also an issue since in-source reduction reactions occurred. Three peaks were detected for BH4 transitions, whereas two of them corresponded to BH2 and BIO peaks. The same result was observed for other oxidized compounds such as NEO that was detected with the NH2 SRM transition. This work has not been published or presented yet.

P-20

PHOSPHOPEPTIDE ENRICHMENT ON A MONOLITHIC COLUMN CONTAINING SUPERFICIALLY IMMOBILIZED NANO TiO₂

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Protein phosphorylation plays an important role in regulating a broad spectrum of key physiological processes like cell signaling, cell growth and cell differentiation [1]. Prior to LC/MS analysis, enrichment of phosphopeptides is a crucial step because of their low stoichiometry in biological sample, longer retention on reversed phase columns, and lower ionization efficiency compared to non-phosphorylated peptides [2].

The use of metal oxides, most prominently of TiO₂ enabled efficient and relatively simple phosphopeptide-enrichment. In this study a new monolithic column from BIA Separations packed with immobilized TiO₂-nanoparticles was tested for its ability to enrich phosphopeptides. The TiO₂-column was also tested for possible carryover originating from biological samples. The enrichment procedure was conducted according to an altered and optimized protocol described by Mazanek et al. [3], sample was loaded onto the column using a syringe pump and five fractions were collected. Briefly: the flow-through fraction, two wash and two elution fractions. The same sample treatment was performed using the "blank" monolithic column, i.e. without immobilized TiO₂ nanoparticles.

Resulting fractions were re-injected onto the reversed phase nano separation column and detected with MS.

Results show that the "blank" column was unable to discriminate between the phosphorylated and unphosphorylated peptides.

In conclusion, tested monolithic TiO₂ columns show significant binding ability for phosphopeptides and are considered as suitable for phosphopeptide enrichment. Additional optimization steps will be applied for better prevention of carryover and for more efficient peptide trapping by using different loading buffers.

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P-21

CHARACTERIZATION OF ORGANIC SULFUR COMPOUNDS IN FOSSIL FUELS USING FLOW MODULATED COMPREHENSIVE TWO-DIMENSIONAL GAS CHROMATOGRAPHY AND TANDEM MASS SPECTROMETRY DETECTOR

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The main forms of organic sulfur compounds (OSC) in fossil fuels such as coal and petroleum, are thiophenes linked to aromatic rings and called polycyclic aromatic sulfur heterocycles (PASH), which consist primarily of alkylated benzothiophenes and dibenzothiophenes. The presence of OSC in these matrices is undesirable, due mainly to the release of sulfur oxides into the atmosphere during burning processes. In general, identification of these compounds requires various steps of isolation and fractionation, mainly due to co-elution of these compounds with polyaromatic hydrocarbons (PAH). From the analytical point of view, there is no known method that is fully satisfactory for the separation of PASH and PAH in these matrices. Furthermore, there is the problem of the large number of isomers of alkylated PASH, which makes the separation of these compounds even more than an analytical challenge. Overcome this challenge is important so the monitoring of recalcitrant OSC in final products can be possible.

The objective of this study was evaluate the potential of GC×GC added to the selectivity of tandem mass spectrometry (MS/MS) for direct injection of fossil fuel samples for qualitative characterization and PASH target determination. Specifically, a flow modulation (FM) system and a triple-quadrupole mass spectrometer were employed.

The use of FM GC×GC-MS/MS, applied to fossil fuel OSC analysis, has shown that the determination of PASH is possible without the pre-fractionation step, and it can represent an analytical advance in the petrochemical area.

P-22

THEORETICAL PRINCIPLES OF CAPILLARY ISOTACHOPHORESIS IN MOVING-BOUNDARY SYSTEMS

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Capillary isotachophoresis (ITP) is a well established electrophoretic technique with high potential of on-line analyte enrichment [1]. Its high sample stacking capabilities are nowadays employed predominantly by its combination with other separation techniques and/or various universal (e.g. contactless conductivity) or selective (e.g. mass spectrometry) detection principles [2]. This often requires advanced manipulation of selectivity and tuning of the system's stacking window. We have recently shown [3,4] that a promising solution is the application of a generalized concept of ITP performed in more complex electrolyte setups based on moving boundary systems. This contribution provides the fundamental theoretical background of ITP in moving-boundary systems. It is based on the approximation of capillary ITP as a 1D process and the general condition of ITP migration, i.e., the existence of a self-sharpening boundary capable to form a stack of zones moving with equal velocities. Using a quite simple system with binary co-ion as an example, the basic relationships describing the properties of the system are derived from the appropriate set of moving-boundary equations. It is demonstrated that such moving-boundary systems behave in the same way as regular ITP systems. This includes also the principle of ITP concentration adjustment except the fact that additional parameters apply, namely the concentration ratio(s) of the multiple co-ions. Most telling are equations derived for the simplified case of strong electrolytes that allow insight into the nature of the system and reveal surprising properties. Based on the theoretical description, conditions for ITP stacking of analytes are defined. The simplicity of the presented relationships employs the recently introduced [3, 4] concept of zone related mobility of an ITP boundary, defining a pair of mobility values related to the pair of zones forming the given boundary. The stacking conditions further allow to define the range of analytes that provide ITP zones (i.e. are stacked at the ITP boundary) of a given moving-boundary ITP system, from which its analytical potential can be evaluated. Various possible ITP setups are shown and possibilities of their selectivity tuning are demonstrated using computer simulations and calculated stacking-window diagrams.

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P-23

MICRO-BORE SIZE-EXCLUSION CHROMATOGRAPHY OF HUMIC SUBSTANCES ISOLATED FROM ENVIRONMENTAL SOURCES

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The aims of the presented work are design and development of novel micro-bore Size-Exclusion Chromatography (SEC) method for the analysis and characterization of some distinct environmental macromolecules, e.g. humic substances (HSs) and their fractions obtained by the methods working on independent separation principles, for example RP-HPLC and capillary isotachopheresis (CITP). From the point-of-view of chemical analysis, characteristic feature of HSs as analytes is diffuse non-distinct analytical signal produced by many detection techniques. This signal does not usually result in an exact numerical physical-chemical data, but is described also by their distribution function or range of validity. This dictates the necessity of development of automated complex separation procedures with minimal sample pre-treatment, and the use of on-line (off-line) multidimensional chromatographic techniques as a logical solution to these requirements. Until now SEC is the most utilized separation method of HSs for relative molar mass determination [1, 2] and is used also for purpose of HSs macromolecules size based fractionation in spite of the fact, that the effective size of HS macromolecule or supramolecule is influenced by many variables related to their quality, concentration and the composition of mobile phase (e.g., pH and ionic strength). SEC is the outstanding technique for measuring molar mass distributions (MMDs) of natural and synthetic polymers and macromolecules. Micro-bore SEC (columns are usually 1 – 3 mm inner diameter [3]) has a number of important advantages, such as the use of greatly reduced amounts of samples and solvent, the use of reduced-bore columns instead saves up to 70% of eluent. Authors of the contribution got ample experimental evidence that narrow (1 mm I.D.) and micro-bore columns can give excellent SEC separations advantageous for combined techniques (e.g., with CITP or RP-HPLC) and a more flat MW calibration curve than analytical columns.

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P-24

APPLICATION OF NOVEL CORE-SHELL TYPE POLYSACCHARIDE-BASED CHIRAL STATIONARY PHASE FOR SEPARATION OF ENANTIOMERS IN NANO LIQUID CHROMATOGRAPHY

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Capillary electrochromatography (CEC) is a relatively new separation technique governed by both chromatographic separation principles and electrokinetic migration of analytes. If properly operated, CEC provides higher separation efficiency compared to chromatographic separation techniques under otherwise similar experimental conditions. This has been systematically proven in achiral, as-well-as chiral separations using CEC. Faster intraparticle mass transfer due to flow through particles has been identified as one of the major contributions to the peak efficiency enhancement in CEC experiments compared with nano-liquid chromatographic (nano-LC) experiments performed on the same capillary column. The fact that intraparticle mass transfer in CEC experiments is faster compared to nano-LC is unquestionable as it has been clearly illustrated experimentally by a lower C-term in the van Deemter equation, as well as based on the effects of silica pore size, ionic strength of the background electrolyte and other factors on peak efficiency in CEC. Core-shell silica particles which became commercially available recently have a nonporous core potentially prohibiting (or at least impeding) flow through particles. Thus, a comparative evaluation of the performance of such materials in nano-LC and CEC should provide further direct experimental evidence of the important role played by intra-particle flow in enhancing peak performance in the latter technique.

In addition, the current opinion is that core-shell particles do not perform as well in smaller bore (2.1 mm I.D.) columns as in standard columns of 4.6 mm I.D. The advantages of chiral stationary phases (CSP) based on core-shell silica in standard diameter HPLC columns have been recently demonstrated. It seems interesting to evaluate the performance of core-shell particle based chiral capillary columns as such columns should prove even less efficient given their even lower aspect ratio (i.e. column internal diameter to particle diameter ratio) compared to 2.1 mm I.D. columns.

In order to answer the above questions, six polysaccharide-based CSPs were prepared by coating the same polysaccharide derivative on core-shell and totally porous silica particles. These materials were evaluated for the separation of enantiomers in nano-LC and in CEC.

Three important conclusions can be drawn based on the results reported in this preliminary study: 1) core-shell materials which have been found to underperform in narrow bore columns may be quite useful under CEC conditions in spite of the much larger aspect ratio of capillary columns; 2) Flow through-particle (assumed to be affected by the presence of the core in core-shell particles) cannot be considered a major factor in producing lower HETP values for the same capillary column when operated in CEC mode versus nano-LC mode, and 3) the subtle mechanisms leading to higher observed plate numbers in CEC compared to nano-HPLC on the same capillary column need further investigation, especially for columns made with core-shell materials.

P-25

COPPER(II) AND PHENOL BIOSORPTION BY CELL SURFACE TREATED *CANDIDA TROPICALIS* IN AQUEOUS SUSPENSION

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An experimental study was performed to determine the feasibility of using physically treated *Candida tropicalis* cells for sorption of Cu(II) and phenol, the role of competition between phenol molecules and Cu(II) ions. The yeast cells were lyophilized (LC), heat treated at 65°C for 24 hours (HT1), at 90°C for 24 hours (HT2) and 72 hours (HT3), inactivated at 120°C and 104 kPa for 20 min (PC). The biosorption isotherms were determined in batch system. Experimental equilibrium data were evaluated using Langmuir, Freundlich and Dubinin-Radushkevich isotherm models by linear and non-linear regression. The adsorbed Cu(II) and phenol amounts by cells were decreased due to the physically treatment of cells. The Cu(II) biosorption capacity was also determined in the presence of phenol at various concentrations in aqueous suspension and in these systems phenol biosorption isotherms were determined. The results were compared with experimental data obtained by using activated carbon as sorbent.

Keywords: *C. tropicalis*, treatment, copper, phenol, biosorption, equilibrium

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P-26

MODELING OF SEPARATION EFFICIENCIES OF LARGE BIOMOLECULES IN LIQUID CHROMATOGRAPHY

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Retention factor of large biomolecules affected by pressure significantly due to the relatively large change of molecular volume of these compounds during adsorption. Since there is a pressure drop in chromatographic columns, the migration velocities of chromatographic bands of large biomolecules vary during the elution [1]. The change of velocity affects the widths of peaks and efficiency of separation through the peak expansion and solute release processes. In this work, the separation efficiencies of large biomolecules are analyzed. The effect of pressure on the retention factor of solutes are integrated in the equilibrium dispersive model of chromatography [2] that is solved by the Martin-Synge algorithm [3] for different biomolecules studied previously [1]. The calculated band profiles are analyzed. The effects of peak expansion and solute release processes on the efficiency are studied independently. Practical considerations for the separation of large biomolecules are also discussed.

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P-27

EVALUATING THE INTEGRATION OF CE, ESI AND MASS SPECTROMETRY FOR THE QUANTITATIVE ANALYSIS OF AMINO ACIDS IN THE CATIONIC METABOLOME

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Objectives: The integration of capillary electrophoresis and electrospray ionization into a single dynamic process (CESI) combined with mass spectrometry (MS) has been previously demonstrated. The aims of the study were to investigate the quantitative capability of CESI-MS in metabolomics using commonly available sample preparation methods for bio-fluids including plasma, urine and saliva.

Methods: A prototype CE separation capillary with integrated ESI emitter was developed by etching the terminal portion of the capillary with Hydrofluoric Acid (HF) until porous. This prototype CESI setup was interfaced with commercial mass spectrometry and evaluated using a performance standard mixture of 17 underivatized amino acids to assess sensitivity and linearity. We used this calibration mixture to determine the concentration of amino acids within a plasma sample characterized by the National Institute of Standards and Technology (NIST). Sample preparation of calibrators and unknowns included a 3kD protein filtration followed by simple dilution and pressure injection on the CESI-MS.

Results: Quantitative evaluation using CESI-MS analysis of plasma, urine and oral fluid for each underivatized component in the amino acids mixture was demonstrated and showed excellent sensitivity and linearity ($R^2 > 0.98$) over the range 2.5 to 2500 μM . Quantitative analysis of a NIST characterized Human Plasma sample gave results which were within expected experimental range.

Conclusion: The results of the study demonstrate that CESI-MS may be used for the quantitative analysis of amino acids from bio-fluids. This has the potential to be an effective tool to study endogenous polar compounds in untargeted and targeted analyses of the cationic metabolome.

P-28

CAPILLARY ELECTROPHORETIC ENANTIOSEPARATION OF β^2 -AMINO ACIDS USING MODIFIED CYCLODEXTRINES AS CHIRAL SELECTORS

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In recent years β -amino acids were found to be key components of numerous bioactive molecules, developmental pharmaceuticals, β -peptides and peptidomimetics, that is the reason why they have come under increased scrutiny by the scientific community.

Monosubstituted β -amino acids can be subdivided into β^2 - and β^3 -amino acids, depending upon the position of the side-chain on the 3-aminoalkanoic acid skeleton. In general, β^3 -amino acids are commercially available, whereas most of the β^2 -amino acids are not. This is a consequence of the ease of synthesis of β^3 -amino acids from the corresponding α -amino acids by homologation. On the other hand, the syntheses of β^2 -amino acids in chirally pure form have proved much more challenging and these amino acids must be prepared through the use of multistep procedures. The synthesis of β^2 -amino acids in racemic form and their subsequent enantioseparation is therefore another possibility for the preparation of chirally pure β^2 -amino acids.

The separation and identification of β -amino acid enantiomers have mainly been performed by indirect and direct high-performance liquid chromatographic (HPLC) methods. Among the numerous possibilities for resolution, capillary electrophoresis (CE) is a relatively new technique, which complements the most frequently applied HPLC methods.

The present work describes direct capillary zone electrophoretic method for the enantioseparation of racemic β^2 -amino acids with application of different sulfated cyclodextrins (CDs) and CD sulfates (CDSs). All the investigated β^2 -amino acids possess differently substituted aromatic ring(s) on their α carbon atom.

All experiments were performed using a HP^{3D}CE full automated instrument (Agilent Technologies, Palo Alto, CA, USA) equipped with a diode array detector and Chemstation software for data handling. The applied bare fused silica capillaries (50 μ m I.D.) were preconditioned prior to all runs by flushing with Milli Q water (0.5 min), 0.1 M NaOH (1.0 min), Milli-Q water (1.0 min) and BGE (10.0 min). Detection was accomplished via measurement of the UV absorption at 210 and 225 nm. The capillary was thermostated at 25 °C. Samples were injected hydrodynamically (50 mbar $\cdot$ 4 s).

The optimum background electrolyte (BGE) composition, pH and applied voltage were determined with respect to the migration, selection and resolution. Based on the obtained results it can be stated that the enantioselectivity strongly depends on the substitution degree and cavity size of the selector molecules, on the structural characteristics of the amino acids and on the applied conditions (i.e. pH and BGE composition). The developed CE methods proved to be quite successful for the direct enantioseparation of various β^2 -amino acids.

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P-29

ANALYSIS OF NMDA MODULATORS WITH CE-LIF IN DIFFERENT BIOLOGICAL SAMPLES

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Before the few previous decades it was believed, that only the L-amino acids were present in the biological systems. Recent results suggest that several D-amino acids occur in many living organisms, including the human body. D-serine and D-aspartate can be detected in different tissues, such as neuronal tissue, where they have neuromodulator functions. While the effect of D-serine is rather established, the function of D-aspartate is less well understood. It is probably involved in modulation of neurogenesis, neuronal plasticity and memory formation. Analyzing D-amino acids is challenging, because of their small amount in the samples compared to the L-enantiomers. Moreover microdialysis sample volume is only a few μL . To overcome these difficulties an appropriate analytical instrument, capillary electrophoresis has been applied. This method has the known advantages of low sample volume requirement and high separation efficiency. The amino acids lack of easily detectable moiety. Since the LIF offers a much better sensitivity, compared to the conventional UV absorbtion, fluorescent labeling was chosen for detection. A fluorogenic agent (7-fluoro-4-nitro-2,1,3-benzoxadiazole, N-BDF) was used for the derivatization procedure. NBD-F offers some advantages such as fewer hydrolysis products and the relatively fast derivatization reaction. Distinguishing between the enantiomers can be problematic, as they share common physical and chemical properties. An amino-modified β -cyclodextrin (6-monodeoxy-6-mono (3-hydroxy) propylamino- β -CD) was tested for chiral analysis of different amino acids. The effect of the amino modified cyclodextrin concentration on the separation has been studied. At 3 mM concentration one of the hydrolysis products of NBD-F co-migrates with the D-serine, while at 7 mM there is no further beneficial effect on the resolution. At 5 mM concentration of this chiral and chemical selector the baseline separation of D-aspartate, L-aspartate, L-glutamate, D-serine and L-serine was achieved. The effect of the buffer pH has also been tested. 100 mM borate buffer, pH 8 resulted in baseline separation for all the analytes of interest in the microdialysis samples. Nevertheless the high amount of glycine present in the brain tissue samples tended to co-migrate with the D-serine. Decreasing the pH of the separation buffer to 7.5 showed a better peak shape for glycine and chemical selectivity. As further decrease of the pH made the resolution worse, pH 7.5 was chosen for analyzing brain tissue samples. All the determinations were accomplished in a polyacrylamide coated capillary and reverse polarity was used for the analysis of the negatively charged analytes. The developed method has been validated. The LOQ of the amino acids was 0.1 μM and the LOD was in the low nM range. Both intra and inter day accuracy and precision has been determined. The methods were tested on microdialysis and chicken brain tissue samples to determine the concentrations of the NMDA modulator amino acids.

P-30

POTENTIAL UTILIZATION OF VIOLET LASER FOR CE-LIF DETERMINATION OF FLAVINS – QUALITY ASPECTS AND APPLICATION FOR FOODSTUFF TESTING

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In order to obtain a high sensitivity of mass-limited analytes CE often utilizes laser-induced fluorescence (LIF) detection. Since its introduction the limit of detection has been greatly improved and a growing number of excitation sources is available.

Flavins are group of chemical compounds occurring natural fluorescence. This feature makes them often subjected for fluorometric assays. Some relevant CE-LIF methods for flavin analyses have been reported in the literature with application of range of 442 nm – 488 nm laser excitation wavelengths. The aim of the study was to verify the possibility of using a 405 nm laser (violet) for testing of flavin derivatives – riboflavin (Rib), riboflavin phosphate (FMN) and flavin adenine dinucleotide (FAD) in term of sensitivity and reproducibility comparing to referenced 488 nm blue laser. The new excitation source was used for quality control of pharmaceutical ingredients and flavin determination in foodstuffs.

Capillary electrophoresis was performed on a PA800 plus instrument equipped with build-in LIF detector, solid-state laser 488 nm (3 mW) and laser beam splitter module (Beckman Instruments, USA). Second semi-conductive violet laser (GaN) 405 nm, 30 mW (Spectra-Laser, Opole, Poland) was connected as an external excitation source. The laser has an input modulation signal 0-10kHz TTL and adjusted output 0-100% using a potentiometer. Emission signal was filtered by 510 nm long pass filter (Omega Optical, VT, USA).

Separations based CZE mode were performed in 60/50 cm 50 µm fused silica capillary using 10+100 mM Tris borate buffer pH 9.0 applying 25 kV and cooling to 20°C. Samples were introduced to the capillary by pressure (0.5 psi) for 10 s that corresponds to approx. 8 nl of injection volume.

The highest signal (expressed as S/N) for 405 nm laser was obtained at 90% of output power. The background electrolyte was optimized to increase the separation of secondary peaks in FMN. Validation data proved comparable repeatability (precision and intermediate precision) between both excitation sources.

The highest signal (expressed as S/N) for 405 nm laser was obtained at 90% of output power. The background electrolyte was optimized to increase the separation of secondary peaks in FMN. Validation data proved comparable repeatability (precision and intermediate precision) between both excitation sources. Sensitivity with 405 nm laser was found at the same level as with 488 nm: for Rib LOD attained 0,5 ng/ml and 0,7 ng/ml respectively. The only difference occurred at level of noise that was about 5-8 times lower for 405 nm laser.

The method was used for related substance assay in Rib and FMN pharmaceutical ingredients. Based on comparison of peak profile obtained with current CE method and compendial Pharmacopoeial HPLC method some of impurities could be probably identified (Fig.1).

Method applicability was also verified by testing of selected foodstuffs. Different profiles of Rib / FMN / FAD were found in fermented milk products as a result of activity of various types of microorganisms. It was also found that fresh milk offered in a glass or plastic bottles are prone to flavins degradation comparing to carton-boxed drinks.

The two lasers 405 and 488 nm are comparable concerning sensitivity and applicability for flavins determination.

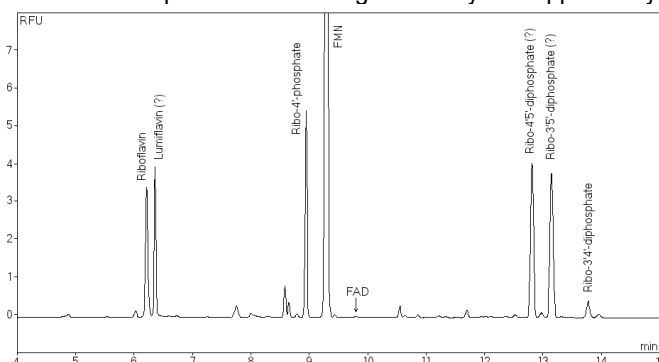


Fig. 1. Electrophoregram of riboflavin 5'-phosphate (FMN) – active pharmaceutical ingredient according to Ph.Eur., conc. 9 µg/ml revealing a profile of related substances with probable identification of some of peaks.

P-31

CONTROL SYNTHESIS OF GO/Fe₃O₄/Au/PEG COMPOSITES FOR THE HIGHLY SELECTIVE ENRICHMENT OF N-LINKED GLYCOPEPTIDES FROM BIOLOGICAL SAMPLES

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Enrichment of glycopeptides from complex samples was indispensable for MS analysis and the subsequent evaluation of glycosylation sites, but still remained a great challenge [1]. Several methods including lectin affinity, hydrazine oxidation, boronic acid affinity and hydrophilic interaction chromatography (HILIC), [2-5] have been developed for the isolation and identification of glycopeptides, among which HILIC displays the advantages of high selectivity, excellent reproducibility and good compatibility with MS analysis, thus making it a promising strategy for glycoproteins analysis. However, the co-elution of non-glycopeptides with glycopeptides might affect the recognition specificity for glycopeptides. Beside to increase the hydrophobicity of non-glycopeptides by adding ion-pair reagents, a more effective solution is to develop new materials with improved hydrophilicity, to increase the retention of glycopeptides.

In this work, GO/Fe₃O₄/Au/PEG composites were synthesized and applied as the hydrophilic interaction chromatography adsorbent for selective enrichment of glycopeptides. GO/Fe₃O₄/Au/PEG composites showed remarkable selectivity toward glycopeptides at a low ratio of glycopeptides/non-glycopeptides (m/m, 1:10) because of its excellent hydrophilicity. The entire enrichment procedure was controlled within 5 min due to the special structure of GO/Fe₃O₄/Au/PEG composites. Furthermore, GO/Fe₃O₄/Au/PEG composites showed excellent stability and good reusability. 270 unique glycopeptides mapped to 131 different glycoproteins was identified from 20 µg tryptic human serum low abundant proteins by this approach. The results clearly demonstrated that the GO/Fe₃O₄/Au/PEG composites have great potential for the selective enrichment of the low-abundant glycopeptides in complex biological samples.

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P-32

NEW ADVANCES IN CAPILLARY ELECTROPHORESIS ANALYSIS OF CARBOHYDRATES: PSEUDOSTATIONARY AND MONOLITHIC PHASES

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The significant impact of high performance analytical separation techniques during the past several decades in life sciences and medicine suggests the continued need for new methods with high and/or special resolving power. Capillary electrophoresis and high performance liquid chromatography are critical separation tools, which are widely used in the bioanalytical field, especially for the analysis of biologically important polymers, such as nucleic acids, proteins and carbohydrates. Carbohydrates play a major role in protein bioactivity, bioavailability and antigenicity and therefore the understanding of the glycosylation of protein molecules is important in the development of effective glycoprotein therapeutics. In recent years there has been considerable activity in the development of simple, rapid and reliable separation methods for the analysis of complex carbohydrates released from a variety of glycoconjugates including glycoproteins, glycolipids, proteoglycans and glycosaminoglycans.

In the first part of this work, we compared the separation capabilities of polymeric additives (pseudostationary phases) of polyethylene oxide and linear polyacrylamide in respect to polymer concentration, separation temperature and organic background electrolyte additives. Thermodynamic treatment by means of Arrhenius plots was given to demonstrate the activation energy differences for the various polymers and its effect on separation performance. The goal of this part of the study was to establish a solid theoretical understanding of the physicochemical underpinning of the migration process involved.

We also describe the preparation of special monolithic in fused silica capillaries to elaborate the monolithic stationary phase mode. Performance of the monolithic –and pseudostationary phase modes was demonstrated with the use N-glycans, released from model glycoproteins of IgG, fetuin and ribonuclease B. We envision these methods to provide a new separation approach with broad application possibilities in the –omics field, especially in glycomics.

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P-33

MASS SPECTROMETRY OF ENDOTOXINS FROM WHOLE-CELL LYSATES

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Endotoxins, chemically lipopolysaccharides (LPSs), make up approximately 75 % of the cell surface of almost all Gram-negative bacteria and are invariably associated with the Gram-negative bacterial outer membrane whether the organisms are pathogenic or not. Much interest is at present focused on endotoxins as modulators of the immune response, and particularly on the relation between the structure of LPS and its role in the development of sepsis that is the leading cause of death in intensive care units worldwide. LPS from all bacteria share a common architecture, consisting of a lipid portion (called Lipid-A) which anchors the molecule in the outer membrane and which is linked to a more or less complex carbohydrate chain. This chain consists of a Core oligosaccharide connected to an O-polysaccharide in case of S-type LPS. A second type of LPS, called R-form LPS or lipooligosaccharide (LOS), consists only of Lipid-A and Core region.

Mass spectrometry (MS), either alone or in combination with other techniques, is a powerful technique used for the analysis of purified LPS or LOS obtained from bacteria by the long lasting (5-10 days) extraction procedures, namely the hot phenol-water [1] or the phenol-chloroform-petroleum ether [2] methods, respectively. Our study provides, for the first time, the mass spectrometric analysis of LOS obtained directly from bacterial cells by the relatively short (ca. 40 hr), small-scale method of Hitchcock and Brown [3]. This method involves the enzymatic digestion of whole-cells with lysosyme and proteinase K, leading to a whole-cell extract that contains LPS approximately free from protoplasmic carbohydrates, peptides and lipids. Such whole-cell lysate solutions of *Shigella sonnei* R-type bacteria [4] were, after a rapid desalting step, directly subjected to structural analysis with matrix assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) in negative ionization and linear mode, using THAP (2,4,6-trihydroxyacetophenone) as matrix. The mass spectra of the partially purified LOSs (whole-cell lysate samples) closely resembled to the spectra of the purified LOS analogs. For the proper detection of signals, densely inoculated liquid bacterial cultures were needed. The individual LOS components present in the whole-cell extracts could be sensitively detected with MS in each sample. Hence, we conclude that LOS profiling by MS of whole-cell lysates might be used to provide preliminary data concerning the LOS content of bacteria. This could be important in the structure-function analyses of endotoxins or in epidemiological investigations to trace cross-infections between patients.

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P-34

OPTIMIZATION OF THE EXTRACTION PROCEDURE OF STEROID HORMONES FROM HUMAN URINE SAMPLES BEFORE MEKC SEPARATION IN THE CONTEXT OF ANTI-DOPING RESEARCH

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Many steroid hormones can be considered as potential biomarkers and their determination in body fluids can create opportunities for the rapid diagnosis of many diseases and disorders of the human body. Most existing methods used for the determination of steroids are usually time- and labor-consuming and quite costly indeed. Therefore, the aim of analytical laboratories is to develop a new, relatively low-cost and rapid implementation methodology for their determination in biological samples. Due to the fact that there is little literature data on concentrations of steroid hormones in urine samples, we have made attempts at the electrophoretic determination of these compounds. For this purpose, an extraction procedure for the optimized separation and simultaneous determination of seven steroid hormones in urine samples has been investigated. The isolation of analytes from biological samples was performed by liquid-liquid extraction (LLE) with dichloromethane and compared to solid phase extraction (SPE) with C₁₈ and hydrophilic-lipophilic balance (HLB) columns. To separate all the analytes a micellar electrokinetic capillary chromatography (MECK) technique was employed. For full separation of all the analytes a running buffer (pH 9.2), composed with 10 mM sodium tetraborate decahydrate (borax), 50 mM sodium dodecyl sulfate (SDS), and 10% methanol was selected.

Therefore, the aim of this study was to develop electrophoretic methods for the identification and determination of steroid hormones in urine samples of volunteers and amateur weight-lifters by the micellar electrokinetic capillary chromatography (MEKC) technique. This new methodology meets all the requirements of analytical methods. We provided a new strategy for solving the problem of sample treatment when endo- and exogenous steroid hormones are simultaneously determined in biological samples. Moreover, the goal of the study was also to demonstrate the use of the application of the elaborated method for routine doping control and the quantification of steroid hormones in human urine samples. Generally, the manual sample preparation steps (e.g. multiple extraction) have been eliminated and the time required for sample processing has been greatly shortened compared to other laboratory techniques.

Applicability of the method was confirmed for the analysis of urine samples collected from volunteers - both men and women (students, amateur bodybuilding using and not using steroid doping). Concentrations of endogenous steroid hormones, which have been obtained in some urine samples power sports enthusiasts origin, well above the physiological level

The data obtained during this work can be successfully used for further research on the determination of steroid hormones in urine samples.

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P-35

PREPARATION AND ANALYSIS OF PAHS IN OILS, BY GC-MS

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Polycyclic aromatic hydrocarbon (PAH) molecules are the largest group of compounds, causing pollution. Generated, wherever organic materials are burned imperfectly.

Seven PAHs in particular are known for their carcinogenic properties: benzo[a]pyrene, benzo [b]fluoranthene, benzo[k]fluoranthene, chrysene, dibenz[a,h]anthracene, and indeno[1,2,3-cd]pyrene. As such, they are monitored in oils.

Benzo[a]pyrene is found in a number of edible oils due to the production process of such oils. The occurrence of PAHs in food is due to environmental contamination, technological processing or home cooking (i.e. grilling and smoking). [1, 2]

The aim of this study, the preparation and analysis of PAHs -before and after cooking potatoes - from olive oil, cooking oil, coconut oil and rapeseed oil. The sample preparation will focus on benzo[a]pyrene and other PAHs. The analysis, were performed by GC-MS after solid phase extraction (SPE).

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P-36

DYNAMIC SORPTION STUDIES: IMMOBILIZATION OF *SPIRULINA PLATENSIS* CELLS

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Nowadays biotechnological processes involving biosorption and bioaccumulation have attracted the attention for environmental decontamination. Study of biosorption using a variety of biomasses (bacteria, algae, yeast, and fungi cells) has already given some promising results for removal of inorganic and organic pollutants from aqueous medium, however, information on interaction of biomass cells and pollutant compounds for technological application under dynamic condition is still limited. These biosorbents are an ideal candidate for the treatment of high volume and low concentration (1-100 mg/L) complex wastewaters contaminated with heavy metal ions.

Heavy metal biosorption by immobilized *Spirulina platensis* cells were studied. The composition of column biomaterial, initial ion concentration and the flow rate were varied during flow condition. Based on breakthrough curves the composition of immobilized beads were optimised. The beads contain 2 m/w % Na-alginate and 4 m/w % chitosan suspension. In both cases the amount of algae cells were 0.1 m/w % in terms of dry weight content. Immobilized cells may be re-used as column packing biomaterial, immobilization reduces the cell wastes, minimizes the loss of product and facilitates product recovery, in particular when in situ product removal is performed.

Acknowledgement

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P-37

MONOLITHIC MACROPOROUS CRYOGELS WITH IMMOBILIZED LECTINS FOR GLYCOPROTEIN ANALYSIS

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The biocompatibility of chromatographic stationary phases as well as supports for affinity ligands immobilization plays a crucial role in analyses of proteins and peptides. Macroporous cryogels have been introduced as a unique hydrophilic monolithic material for a wide range of applications including separation, cell cultivation and tissue engineering [1].

In this work we have developed a hydrophilic poly(hydroxyethyl methacrylate-co-poly(ethylene glycol) diacrylate) cryogel placed in the centrifugal filter device. The composition of the polymerization mixture as well as the polymerization conditions were optimized in order to prepare a mechanically stable, highly porous material suitable for spin column lectin chromatography. The surface of the monolithic scaffold was activated by epichlorohydrin and used for multi-step immobilization of various lectins utilized in affinity extraction of glycoproteins. In one example, the hydrophilic cryogel served as a matrix for immobilization of concanavalin A to provide the affinity support for selective isolation of glycoproteins containing high-mannose glycans. Fucosylated glycan containing glycoproteins were extracted using the immobilized fucose specific lectin support. Efficiency and selectivity of the developed lectin modified macroporous cryogels were evaluated by analyses of protein mixtures and/or released glycans followed by mass spectrometry detection.

Macroporous cryogels with immobilized affinity ligands placed in the centrifugal filter device represent a simple tool enabling rapid as well as efficient isolation of targeted analytes in glycoproteomic analysis.

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P-38

EVALUATION OF THE MASS-TRANSFER PROPERTIES IN CHROMATOGRAPHIC COLUMNS PACKED WITH SUB-2 - μm PARTICLES

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In the previous years, various sub-2- μm packing materials with different silica structures have been introduced. Next to the HSS (High Silica Strength) and BEH (Ethylene Bridged Hybrid) packings, partially-porous packing materials dedicated for UHPLC measurement are available on the market.

The mass-transfer properties of superficially-porous packing materials, with 1.6 μm and 1.3 μm particle diameter, and of fully porous packing materials with 1.7 μm and 1.8 μm particle diameter were investigated and compared. The first and the second central moments of the peaks of the homologous series of alkyl benzenes, over a wide range of mobile phase velocities were measured, and used for the calculation of the mass-transfer coefficients.

Theoretically, high efficiency and the simultaneous reduction of analysis time can be achieved by decreasing the particle diameter [1 2]. The use of smaller particles requires elevated operation pressure and optimum mobile phase velocity, which leads to thermal dissimilarities in the column, through the frictional heat of the mobile phase [3]. For the evaluation of the band broadening caused by the thermal dissimilarities, the measurements were also repeated with the adiabatic insulation of the columns.

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P-39

MICROSOMES AND DRUGS ON-LINE INCUBATION MONITORED BY CAPILLARY ELECTROPHORESIS COUPLED TO MASS SPECTROMETRY

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Capillary electrophoresis (CE) combined with quadrupole - time of flight mass spectrometer (QTOF-MS) represents a universal tool for microscale enzyme assay, because capillary can be used not only as separation tool but also as reaction chamber. Recently published method, which is based on transverse diffusion of laminar flow profiles [1], provides reaction mixture with uniform concentration of reactants and fulfills requirements on generic mixing approach [2]. Detection by mass spectrometry greatly enhances the utility of CE with information about structure and identity of separated analytes.

On-line CE-QTOF-MS method for study of diclofenac (DC) metabolism mediated by cytochrome P450 2C9 isoenzyme (CYP2C9) or human liver microsomes (HLM) is presented. Injection procedure of published method was adapted and separation conditions were changed to be compatible with MS. Volatile background electrolyte was chosen and conditions for electrospray ionization were optimized.

Analyses were performed using Agilent 7100 CE System in bare fused-silica capillary (75 cm length, 75 μ m i.d.). Solutions of CYP2C9 or HLM (E) and mixture of substrate and cofactors (S) dissolved in incubation buffer (IB) were introduced into capillary by hydrodynamic pressure 15 mbar as consecutive plugs - solution S for 3, 3, 3 and 6 s and solution E three times for 3 s between S plugs. Reaction mixture containing 17.5 nM CYP2C9 or 0.25 mg/mL HLM, 1 mM NADPH and required concentration of DC was incubated for 10 minutes in capillary and enzymatic reaction was terminated by voltage application. 30 mM ammonium acetate (pH 8.7) was used as BGE and separations were accomplished by application of voltage -22 kV at 37°C. Positive pressure 100 mbar after 6.5 min of analysis was applied. Under these conditions current 48-50 μ A was observed. CE was coupled with Bruker maXis impact QTOF-MS by sheath liquid co-axial interface from Agilent and sheath liquid, which contained isopropanol-water (1:1, v/v) consisting 0.5% ammonia, was delivered by isocratic pump by flow 2.5 μ L/min. MS conditions for the analysis were set as follow: dry gas 5 L/min at a drying temperature 180°C. ESI field was 5000V and nebulization gas 0.2 bar. Spectra were acquired in the mass range from 50 to 1600 m/z in spectra rate 1 Hz in positive mode.

Michaelis constant for system DC – CYP2C9 was determined. This method was also used for reaction of DC with HLM, which presents a more complex enzymatic system than CYP2C9 itself. Transverse diffusion of laminar flow profiles based mixing can be used for creation of reaction mixtures with changeable compositions and MS allows detection of broad compounds spectrum, therefore this approach is generic and robust and it can be used for the reaction of other drugs with HLM as well.

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P-40**ANALYSIS OF LYSOZYME-STABILIZED COPPER NANOCLUSTERS BY CAPILLARY ELECTROPHORESIS**

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This study presents a simple and efficient capillary electrophoresis (CE) method for the analysis of copper nanoclusters. The ones examined are water-soluble, polydisperse, negatively-charged lysozyme-stabilized copper nanoclusters (Lys-CuNCs). Due to their biocompatibility and highly luminescent properties, these nanoclusters exhibit potential applications in medical research. CE coupled with a UV detector was well established for the separation of small molecules or nanomaterials in solution due to their different charge-to-size ratio. Thus, CE was utilized for characterizing the sizes of Lys-CuNCs. Under the optimal conditions, the migration order was found to be from 10 to 40 mg/mL Lys-CuNCs. The UV spectrum also showed that the Lys-CuNCs have an absorbance maximum at 404 to 391 nm. The blue shift in UV spectrum with the concentration of lysozyme increased from 10 to 40 mg/mL indicates a reduction of the Lys-CuNCs core sizes, which could be attributed to the effect of charge-to-size ratio. In addition, the surface charges of Lys-CuNCs were measured by the zeta potential. The results indicated zeta potential increased from 12 to 35 mV with an increasing concentration of lysozyme in the preparation of Cu nanoclusters. Our results also showed a significant charge-to-size ratio difference between Lys-CuNCs. The present study demonstrated that capillary electrophoresis could not only enhance the separation of copper nanoclusters but also provide an alternative tool for characterizing the sizes of copper nanoclusters.

P-41

HYDROPHILIC BORONIC ACID FUNCTIONAL CORE-SHELL PARTICLES FOR PH-RESPONSIVE RECOGNITION OF GLYCOPROTEINS BASED ON 'THIOL-ENE' CHEMISTRY

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Glycosylation is one of the most common post-translational modifications of proteins, and plays an important role in a variety of biological activities, such as molecular recognition, immune response and inter-and intracellular signaling. To investigate the biological functions of glycoproteins, the global profiling of N-linked glycosylation sites is required. Therefore, exploring a selective glycoprotein enriching strategy followed by MS techniques becomes more imperative. So far, boronic acid functionalized materials for the solid-phase extraction of glycopeptides (SPEG) have been increasing attention in recent years due to their capability. 1, 2

Herein, we report a new and efficient approach to obtain the hydrophilic core-shell polymer particle. This novel strategy could high efficiently modify the functional boronic acid on the surface of inorganic silica particles and the simultaneous polymerize the water-compatible monomer, which reducing the nonspecific adsorption of proteins in the biological samples, as shown in Figure 1. Therefore, more recognition sites of the boronic acid were exposed on the particles due to the larger boronic acid loading yield and stronger hydrophilicity of the matrices through this novel strategy. The obtained boronate affinity micro particles, Sil@P(MBA-co-APBA), showed well stimuli-responsive towards cis-diol containing molecules. When 5000-fold weight nonglycoprotein (bovine serum albumin (BSA)) was existed, the glycoprotein (horseradish peroxidase (HRP)) was also could be isolated. The maximum adsorption capacity of glycoproteins (HRP) could reach 81 mg/g. With such particles, 2 glycoproteins (ovalbumin and conalbumin) were captured from the egg white, as shown in the Figure 2. The results demonstrated that the synthetic Sil@P(MBA-co-APBA) can be a powerful glycoprotein enriching materials, and hold much promise for profiling the glycosylation site occupancy and corresponding glycan heterogeneity.

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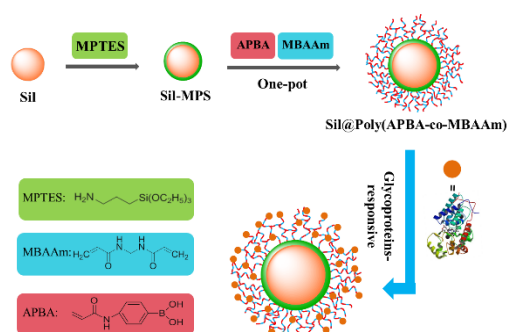


Figure 1. Fabrication of Sil@Poly(APBA-co-MBAAm) core-shell-structured particles

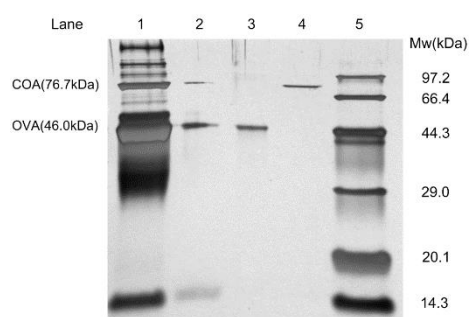


Figure 2. SDS-PAGE analysis of egg white samples with and without treatment by Sil@Poly(APBA-co-MBAAm) particles. Lane (1) egg white without treatment (1.5 µg); (2) eluate from nanoparticles; (3) COA (50 ng); (4) OVA (50 ng) (5) marker.

P-42**CAN LC-MS-MS BE USED IN HORSE MEAT DETECTION?**

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Following the UK Food Standards Agency (FSA)'s announcement in January that horse and pig DNA had been identified in beef products sold by several supermarket chains, further testing across Europe and beyond has revealed widespread incidences of such food contamination. However, most testing methods are based on detection of species-specific DNA in meat, using the polymerase chain reaction (PCR) – which does not detect or identify proteins. This is a concern because DNA can be easily disrupted or removed during standard meat processing and food manufacturing. As a result, horse tissue or other contaminants remain undetected in food samples, despite strong presence of the contaminating proteins. An alternative protein-based method, ELISA (enzyme-linked immunosorbent assay), can be used to complement DNA testing, but this method has limitations, including that it detects only one part of the protein and not multiple protein markers. The LC-MS/MS-based method presented offers a more accurate and reliable approach to meat speciation than PCR or ELISA-based techniques or other indirect methods, and also allows for the detection of veterinary drug residues in the same analysis, which is not possible by ELISA or PCR. The method is developed using an Eksigent micro LC system coupled with an AB SCIEX QTRAP® 5500 LC/MS/MS system and uses multiple reaction monitoring (MRM) to detect peptide markers for horse and is capable of providing sequence information by acquiring a product ion scan for each triggered MRM which can be used to further confirm the peptide's / proteins and therefore the species identity. Using the same extraction and LCMSMS method it is also capable of simultaneously of detecting veterinary drug residues by adding additional MRM experiments. The method has been shown to be capable of simultaneously detected phenylbutazone below 10 µg/kg as well as a 1% contamination of horse meat in beef. This approach offers food analysts the ability to detect multiple species and veterinary drug residues in a single approach which is not possible by any other technique to date.

P-43

EXPLORING THE SENSITIVITY DIFFERENCES FOR TARGETED PEPTIDE QUANTIFICATION IN THE LOW FLOW RATE REGIME

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A common view of the protein/biomarker research pipeline is that one starts with high sensitivity global discovery and narrows the list of protein targets using high throughput targeted quantitation. While much focus has been on mass spectrometry innovations, the importance of the separation component for getting high quality data cannot be underestimated. Sensitivity, robustness and throughput of the LC strategy must also evolve as research progresses from discovery to targeted workflows. The nanoflow regime is used extensively for high sensitivity discovery experiments but more recently researchers are exploring the use of microflow for increased throughput and robustness for targeted quantification. In this study, sensitivity & throughput differences between nano and micro flow rates for targeted quantitation are explored.

Separation of protein digests was performed on an Eksigent ekspert™ nanoLC 425 system and cHiPLC® system (Eksigent, USA) using four different flow regimes. Column diameters and flow rates used were: 75 µm cHiPLC® column at 300 nL/min, 200 µm cHiPLC® column at 1 µL/min, 300 µm microflow LC column at 4 µL/min, 500 µm microflow LC column at 10 µL/min. Gradient lengths were optimized for each flow rate, with faster run times utilized for the larger diameter columns run at higher flow rates. Concentration curves on a set of ten tryptic peptides were analyzed and the lower limits of quantification (LLOQ) for each peptide at each column size was measured using MRM acquisition on the QTRAP® 5500 System. Data was processed using MultiQuant™ Software.

The LLOQs for a set of ten tryptic peptides were measured using the 4 different columns and flow rates. A nanoflow source with glass capillary tips was used for the 75 and 200 µm I.D. column experiments and the high flow source with a low dead volume 25 µm I.D. hybrid electrode was used for the 300 and 500 µm I.D. column experiments. The average increase in LLOQ (or the decrease in sensitivity) relative to running a 75 µm ID column at 300 nL/min was measured for each peptide at each column diameter. For the 200 µm I.D. column running at 1 µL/min, a 2.5 fold decrease in sensitivity or increase in LLOQ was observed. Moving to the 300 µm I.D. column at 4 µL/min, a 3x difference in sensitivity was seen. Finally, 4x difference in sensitivity was observed when the 500 µm I.D. column at 10 µL/min was compared to the same experiment on a 75 µm ID column. However the separation was performed in less than half the time with the 500 µm I.D. column compared to the 75 µm I.D. column (7 minutes @ 10 µL/min vs. 18 minutes @ 300 nL/min). Therefore, as flow rate increases, increased robustness and throughput can be obtained with just a small decrease in sensitivity. In addition, with the larger column diameters, more sample can often be loaded (if available) to offset this sensitivity difference.

This difference in throughput, while maintaining high sensitivity, is key to the continued advancement in the use of mass spectrometry in biomarker research applications, and offers an important alternative to antibody based assays, that typically take much longer to develop when a new antibody must be generated, or when antibody cross-reactivity is a concern.

P-44

ALLERGEN DETECTION IN WINE BY LC/MS/MS

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In wine production fining a wine eliminates any appearance of cloudiness by removing sediment. In this process fining agents, such as casein, are stirred into barrels of wine where they act as magnets by picking up the sediment in the wine and depositing it at the bottom of the wine barrel. Once the wine has been clarified, racking of the wine is done to separate the wine from the sediment residue.

In 2011 EFSA concluded that wines fined with casein/caseinate/milk products may trigger adverse reactions in susceptible individuals following a survey of wine where the detection of casein was reported in trace amounts [<2 mg/L (2 parts per million)] in two (out of 32) experimental wines without bentonite treatment and in three (out of 61) commercial wines with unknown treatment] [1]. This fact together with new European Union legislation (that states that wine after 30 June 2012 wine must disclose on the label if fining reagents such as casein / egg ovalbumin have been use in processing) [2] has driven the need for methods which are capable of detecting casein products in wine at low levels.

Here we present new data using microflow LC in combination with a LC-MS/MS method developed on an AB SCIEX QTRAP® 5500 system utilizing the *Scheduled* MRM™ algorithm which detects casein in wine at sub part per million levels. The method utilizes a simple digestion of the protein in situ in the wine followed by dilution and injection and has been designed to limit extensive sample preparation and perform all protein modification in the same Eppendorf tube. In the presentation we will discuss the benefits of MicroLC over higher flow rate separations.

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P-45

METHOD DEVELOPMENT FOR THE ANALYSIS OF CARBOXYLIC ACIDS AS A PART OF A METABOLOMIC TOOLBOX FOR A HUMAN EMBRYO VIABILITY ASSESSMENT

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Human embryo viability assessment is an important task carried out prior to an embryo implantation in the assisted reproduction. Light-microscopy based techniques are routinely used, however they are limited to an embryo appearance. Metabolomics can provide information at the level beyond the bounds of possibility of a light-microscopy. Monitoring of the composition of an embryo culture medium can be done noninvasively and the connection between metabolism of an embryo and its viability has been already revealed [1]. The new information about the embryo metabolism can improve accuracy of the embryo viability assessment thus a single embryo transfer could be feasible with an adequate pregnancy outcome.

The embryo culture medium always contains salts, buffers, some amino acids, and pyruvic and lactic acids. Pyruvate and lactate are involved in the embryo energy metabolism. Uptake of pyruvate and production of lactate are proportional to a cellular energy demands and correlates with the embryo viability. Capillary electrophoresis in combination with contactless conductivity detection (CE-C⁴D) is suitable analytical tool to quantify pyruvate and lactate in the culture medium. CE-C⁴D is a beneficial combination because CE uses only a few nanoliters of a sample for an analysis and C⁴D can directly detect ionic compounds. Simply speaking, there is no need for a sample treatment.

Method development for the analysis of pyruvate and lactate in the embryo culture medium based on CE-C⁴D is presented and the in-house-assembled C⁴D detector properties are shown. Presented method follows the articles dealing with carboxylic acid analysis [2-4] which agreed on electroosmotic flow (EOF) reversal thus the study of the effect of the addition of the most common dynamic EOF modifiers on C⁴D signal is shared as well.

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P-46

STRUCTURE OF COLITOSE-CONTAINING O-POLYSACCHARIDES FROM THE LIPOPOLYSACCHARIDES OF *PROTEUS MORGANII* O34 (8662/64), *ESCHERICHIA COLI* O111 AND *S. ENTERICA* SV. ADELAIDE O35

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Identification and classification of somatic O-antigens are among the most commonly used methods for typing *Gram-negative* bacteria. It is based on the serological and immunological detection of differences in the antigenic composition of the cell envelope. The immunodominant molecule is the lipopolysaccharide (LPS), specifically the O-polysaccharide. It is made of oligosaccharide repeating units and their structures varying according to the different serogroups.

The serological cross-reactivity might be due to the lipopolysaccharide components of bacterial cell wall, but the protein components can also play a role in this phenomenon [1].

Péterfi *et al.* have determined the outer membrane protein antigens, which play role in the serological cross-reactivity when analyzing these three bacteria [2].

In 1967 Rauss, Ralovich and Vörös described the antigenic relationship between *Proteus (Morganella) morganii* O34 (8662/64) and *Escherichia coli* O111 based on tube agglutination [3].

The O111 O-specific polysaccharide is composed of a pentasaccharide repeating unit with two colitoses bound to the C-3 and C-6 of glucose in a trisaccharide backbone; this structure is identical to that of *Salmonella enterica* sv. *Adelaide* (O35), another enteric pathogen [4].

In a previous study we have determined and found three different electrophoretic profiles of these LPS components by microchip electrophoresis [5].

In this work the O-antigen monosaccharide constituents of the LPSs were characterized by GC-MS techniques, and NMR. We demonstrate that all three pathogens contain similar LPS monosaccharide structures.

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P-47

**FUUL-FORMAT ISOTACHOPHORESIS – A TECHNIQUE FOR HIGHLY VERSATILE,
SENSITIVE AND SELECTIVE CE-ESI MS ANALYSES**

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Capillary electrophoresis with mass-spectrometric (MS) detection is generally less sensitive than other techniques such as liquid chromatography. This can be overcome by using various sample preconcentration and/or stacking techniques. One of them frequently used is transient isotachopheresis (ITP) capable to stack sample analytes very effectively although the following zone electrophoretic step brings major additional dispersion again [1]. Remedy is performing ITP in permanent format where the analytes remain stacked all the way to the detector. This however brings two other problems: limited flexibility by limited available number of ESI-compatible electrolyte components and limited selectivity when all stacked analytes comigrate within a single stack. We have recently begun to investigate how to overcome both these problems and already shown that there are possible solutions [2-3]. The principle is advanced system architecture of permanent-format ITP which is based on a universal concept of fully functional ITP system represented by the existence of a self-sharpening boundary capable to form a stack of zones moving with equal velocities. Such approach allows a very flexible electrolyte system selection from a much broader spectrum of possibilities, tailored to the needs of an actual analytical problem. This contribution provides a comparison of various ways of successful ITP-ESI MS system setups that can be used for sensitive analysis of ionogenic trace components. Regular ITP performed in the classical way is one possible solution well applicable to some kinds of samples. ITP in moving boundary systems offers more flexibility and selectivity while maintaining the same high level of sensitivity. Both the leading and terminating zone contain the same multiple co-ions and electrolyte tuning is done by varying their concentration ratio. Even more flexibility and selectivity can be reached in systems composed of multiple co-ions in such a way that multiple migrating sharp boundaries are formed. Several ways how to reach this are shown and discussed. By way of examples of functional anionic systems, the principles of each setup are explained. Their application areas are characterized by their calculated stacking window diagrams showing the range of stackable analytes. The performances of each of the systems are demonstrated both by computer simulations and experiments.

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P-48

DETECTION AND CHARACTERIZATION OF DIFFERENT CONFORMERS IN A THERAPEUTIC ANTITHROMBIN BY CE AND CE-ESI-MS

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Antithrombin (AT) is the most important physiological inhibitor of blood coagulation. For clinical use, AT can be produced either from human plasma or by genetic engineering and presents conformational variations combined with a microheterogeneity due to its glycosylation. AT shows both anticoagulant and cytoprotective properties. One strategy to extend the therapeutic indications of AT is to dissociate the two properties allowing for instance its use for the treatment of severe sepsis at higher level without bleeding risk. A new generation of mutated recombinant AT (>300 variants) is currently produced.

The aim of this study is to develop a capillary electrophoresis-electrospray-mass spectrometry (CE-ESI-MS) to analyse the heterogeneity of AT arising from both conformation and glycosylation variations. Indeed, latent form of AT has been described in the past as inactive form [1]. The characterization of variants and the quantification of the ratio of latent to native form aim at selecting the hits with the highest therapeutic potential.

To date, only two works have dealt with CE-ESI-MS of AT [2, 3]. They were performed either under denaturing conditions or without a sufficient resolution circumventing the separation of the different forms. In the present work, the influence of the main CE parameters on the separation performance was assessed. The impact of the interface and ESI parameters on AT ionization was evaluated as well.

First, classical acidic conditions generally used for CE-MS of proteins were tested with a 125 cm and PVA coated capillary. Some separation of the different isoforms was obtained. All the isoforms reported in the literature were identified and additional ones were highlighted. For the first time, we detected the presence of heterodimer of AT. Then, different MS-compatible capillary coatings and background electrolytes at physiological pHs were tested. The developed capillary zone electrophoresis (CZE) method under physiological conditions allowed the separation of more than seven peaks. The native AT could be distinguished from the latent form and its heterodimer. Then, two sheath liquid interfaces (a conventional ESI source and the Agilent Jet Stream (AJS) technology) were compared. Using the conventional ESI source and the PVA capillary that suppress the EOF, a pressure application was mandatory at the CE inlet to ensure the electrical integrity but this was accompanied, to a certain degree, by a loss of resolution. On the other hand, the AJS source induced a strong suction effect that allowed avoiding the use of the inlet pressure. Finally, the composition of the sheath liquid was also studied. A shift towards lower charge states and a spread of the mass spectrum was obtained with a non-denaturing sheath liquid, which induced in turn a decrease of the global sensitivity. However, in these conditions, the signal of heterodimer was much more intense (Figure 3B).

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P-49

CAROTENOID PROFILE DETERMINATION OF DIFFERENT KINDS OF PUMPKINS (CUCURBITACEAE)

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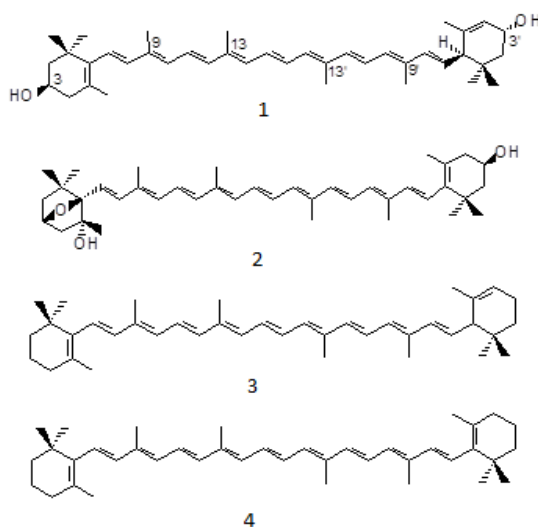
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Epidemiological studies have shown inverse correlations between the consumption of vegetables and fruit rich in carotenoids and the incidence of cancer and cardiovascular diseases. Most vegetables are processed rather than eaten raw. Processing includes household cooking and industrial canning, baking, freezing, dehydration, and pickling. These processes involve thermal treatment and exposure to oxidation, which is enhanced by light or the presence of enzymes.

Carotenoids in edible vegetables may be concentrated in different parts of the plants, such as roots, stems, leaves, flowers, fruits, or seeds.

Earlier we have investigated the changes in carotenoid composition of some vegetables during cooking. We found that lutein can convert to 3'-epilutein in certain conditions.

In the present work we report on the carotenoid analyses of different kinds of raw, cooked and baked pumpkins. In all of the kinds of pumpkin lutein (1), cucurbitaxanthin A (2), α - (3) and β -carotene (4) were detected as main carotenoids. Special attention was paid to the occurrence of minor carotenoids.



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P-50**MULTIPLE SWITCHING DEVICE FOR COMPLEX AND ACCELERATED HPLC SYSTEMS**

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For complex HPLC separation problems as in proteomic analysis the integration of on-line sample preparation and multiplexed chromatography becomes a necessity, especially when optimizing the aspect of mass spectrometric detection efficiency.

A new multiple switching/selection valve module has been constructed to fulfill these tasks: In a first version it is constructed of high pressure (10.000 psi) automated two six-position selection valves and eight six or ten 2-position valves. The actual status of the individual valves is monitored on a small touchscreen on the module which is bi-directionally connected to a PC-system.

Because of the logical complexity when interconnecting the individual valves a PC-based graphical surface has been developed, displaying the actual flow paths between valves. When utilizing multiplexed chromatographic systems the timing and the overview of the (multiple) switching status becomes a problem: As solution the new software tool ChromCommander has been implemented to integrate the multi-valve module into multiple time-bases under the Chromeleon chromatographic software. The new valve module has been tested for a new multidimensional proteomic solution integrating on-line sample preparation with two nano-LC-MS systems in tandem mode.

Acknowledgements

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P-51

μLC-MS FOR QUANTITATIVE ANALYSIS OF ANILINE LABELED N-GLYCANSMichael, Claudia; Rizzi, Andreas*University of Vienna*

Among all post-translational modifications, glycosylation is the most common one and plays crucial roles in various biochemical processes like cell adhesion, protein folding, receptor functioning or signal transduction. In the past decade much effort has been invested in glycan analysis, but the detailed characterization of protein glycosylation remains still challenging due to the diversity of glycan structures (e.g. linkage, branching, length and composition of the oligosaccharide chains). However, a detailed analysis of glycan structure and heterogeneity is required for improving our understanding of disease-related changes in glycan expression levels as well as their context to specific biological functions. HPLC-MS techniques proved as most powerful and versatile technique for structure elucidation of glycans, either still linked to peptides or present as neat glycans.

In this work isotope-coded glycan labeling using $^{12}\text{C}_6$ -/ $^{13}\text{C}_6$ - aniline as labeling reagent is reported aimed at the quantitative *N*-glyco-fingerprinting of selected/treated samples relative to control. The stable isotope coded labeling (*via* reductive amination) allowed the quantitative analysis of combined samples being carried out in one HPLC-MS run. Size- and shape-selective separation of the aniline-labeled oligosaccharides could be attained using porous graphitized carbon (PGC) as stationary phase distinguishing so between branching-, linkage- and anomeric- isobaric isomers.

The main benefit of using isotope-coding in the context of comparative glycomics lies in the improved accuracy and precision of quantitation. Advantages of the specific aniline-based approach proposed lie in the low costs of the used $^{13}\text{C}_6$ -aniline, the mass difference of 6 Da between the heavy/light labeling variants (being sufficient for avoiding overlap of isotopic peaks), suitability for size-, and shape-selective separation of isobaric isomers on PGC-stationary phases.

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P-52

DETERMINATION OF BINDING AND THERMODYNAMIC CONSTANTS OF BOVINE SERUM ALBUMIN-SALICYLIC ACID INTERACTIONS USING CAPILLARY ELECTROPHORESIS

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The binding constant (K_a) is commonly used to describe the strength of binding between protein and ligand such as drug. The strength of the interaction has a significant effect on the biological activity of the drug, and knowledge of the nature and extent of protein-drug binding can help us to understand the pharmacokinetics and pharmacodynamics of a drug. Different modes of capillary electrophoresis can be used to characterize protein-drug interactions for example the capillary electrophoresis-frontal analysis (CE-FA), Hummel Dreyer methods (HD), affinity capillary electrophoresis (ACE), vacancy peak (VP) and vacancy affinity capillary electrophoresis (VACE) [1, 2].

The utilization of the capillary electrophoresis-frontal analysis (CE-FA) for study of binding between bovine serum albumin (BSA) and salicylic acid (SA) was investigated. The optimized method was created and used to determine the binding parameters of BSA-SA. For the experiments, a series of samples containing a constant concentration of BSA (50 μM) and varying concentrations of drug (50 – 900 μM) was prepared. All calibration curves show good linearity in the entire range of concentrations ($R > 0.99$). The binding curves obtained by nonlinear regression analysis were in good fitting of experimental points. Furthermore, the influence of the temperature on the binding constant was examined. Van't Hoff plots showed a correlation between value of the binding constant and the sign and magnitude of various thermodynamic parameters (ΔG , ΔS , and/or ΔH). CE-FA can provide useful and rapid data for the analysis of protein-drug interactions with minimum consumption of sample and other chemicals [3].

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P-53

IN-CAPILLARY ELECTRICAL CELL LYSIS FOR FAST β -GALACTOSIDASE QUANTIFICATION USING CAPILLARY ELECTROPHORESIS

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A novel capillary electrophoresis (CE) based enzymatic assay was developed to evaluate beta-galactosidase (β -Gal) quantity in whole bacterial or human cells. This enzyme catalyzes the hydrolysis of para-nitrophenyl- β -D-galactopyranoside (PNPG) into paranitrophenol (PNP). Its expression in cells is used as a biomarker for assessing replicative senescence in mammalian cells.

The developed CE assay was conducted in five main steps: 1) injection of few whole intact cells in the capillary, 2) injection of the substrate PNPG, 3) in-capillary lysis of these cells by using the electric field to release β -Gal, 4) in-capillary hydrolysis of PNPG by the released enzyme, and 5) on-line detection at 405 nm of the PNP formed and quantification. Consequently, this CE assay is based on the electrophoretically mediated microanalysis (EMMA) approach [1]. The voltage, usually applied during this process to merge reactant plugs, was also used to in-capillary disrupt the cell membrane. The developed method was applied to *Escherichia coli* as well as to human keratinocyte cells at different replicative stages. Results were in excellent agreement with those obtained when analyzing off-line cell lysates.

This work shows for the first time that cell membrane can be in-capillary disrupted by electroporation and that the released enzyme can be subsequently quantified in the same capillary. The EMMA approach has never yet been used for this purpose. The developed CE method is fully automated and simple to conduct. It has attractive applications in studying intracellular material for early disease diagnostic or for highlighting our knowledge of complex biological systems.

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P-54

LIPID OXIDATION LEVELS OBSERVED IN PATIENTS DIAGNOSED WITH ACUTE LYMPHOBLASTIC LEUKEMIA

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Acute lymphoblastic leukemia (ALL), a malignant disorder is the most common cancer, accounting to 30% of diagnosed cases in children's less than fifteen. It is characterized by uncontrolled proliferation and maturation arrest of lymphoid progenitor cells (lymphoblast) in the bone marrow. An increase of reactive species production occurs in ALL with involvement of oxidative stress (OS). Reactive oxygen species particularly free radical (FR) induce tissue damage by lipid peroxidation. The direct determination of FR *in vivo* is difficult. Therefore, methods to assess their reaction products are necessary. Malondialdehyde (MDA) is one of the lipid peroxidative products widely used as biomarker of cellular damage in plasmatic samples due to OS. The objective of this study was to evaluate OS variations in young children diagnosed with ALL compared to healthy patients, determined in plasma samples by modified and validated method, verifying the use of MDA levels as biomarker to distinguish groups, associating with disease's risk. Blood samples (18 healthy, 27 ALL) were collected at Londrina University Hospital (Brazil). Plasma was separate by centrifugation (3000 rpm, 10 min) kept frozen until analysis. Extraction protocol was adapted from Bastos[1] using 250 μL of plasma, mixed to 36 μL of 0.2%BHT and 6.25 μL of 10.0mol L⁻¹ NaOH, vortexed and kept (60°C, 30 min). After, 1500 μL of 7.2%TCA/1%KI solution was added, vortexed and centrifuged (3000 rpm, 10 min). To supernatant (1000 μL) was added 500 μL of 0.6 %TBA incubated by 45 min. MDA stoke standard solution was prepared with 22 μL TEP(1,1,3,3-tetrametoxyp propane) diluted in 10 mL of 1%H₂SO₄, protected from light for 2h, being after 5 μL diluted with 1.5 mL of 1%H₂SO₄, with concentration read at 245 nm ($\epsilon_{245\text{nm}} = 13700 \text{ mol L}^{-1} \text{ cm}^{-1}$). MDA calibration curves (0.05-2.00 $\mu\text{mol L}^{-1}$) were prepared with known MDA concentrations spiked to a plasma pool (6 healthy). Liquid chromatographic system was a Waters Alliance e2695 consisting of quaternary pump with degas module, autosampler, column oven, photodiode array detector (2998 PDA) managed by Empower 2. Run was conducted in isocratic mode at flow rate of 0.7 mL min⁻¹ using CH₃OH : potassium phosphate buffer (10.0 mmol L⁻¹, pH 7.0) (35:65, v/v). The PDA was set at 532 nm for detection adduct of TBA-MDA. Guard column (4.6x12.5 mm, 5 μm) and analytical column was a Zorbax Eclipse XDB-C18 (4.6x250 mm, 5 μm , Agilent Technologies). The method showed to be linear ($r^2=0.9928$) with 0.20 $\mu\text{mol L}^{-1}$ for limit of detection and 0.66 $\mu\text{mol L}^{-1}$ for quantification, repeatability (RSD 3.0%) with recovery rate within 103.0-109.0%. Plasma samples indicated substantially high OS for ALL patients compared to the control group. Mean levels of MDA in the control group was of 0.91 $\pm$ 0.18 mmol L⁻¹ ranged from 0.58-1.20 mmol L⁻¹, while for patients diagnosed with ALL the MDA level was of 2.17 $\pm$ 0.77 mmol L⁻¹ ranged from 0.97-3.67 mmol L⁻¹. MDA levels analyzed by proposed method proved to be an adequate biomarker, allowed by principal component analysis distinction healthy from ALL patients letting by patient's records to associate the risk of disease.

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P-55

MDA IN BALB/C PLASMA INFECTED WITH *LEISHMANIA AMAZONENSIS* TREATED BY TOPICAL AND INTRAMUSCULAR APPLICATIONS OF *TANACETUM PARTHENIUM* PHYTOCHEMICAL

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Leishmaniasis is a neglected disease that affects the poorest regions of the world however; plants have shown great potential in treating tropical diseases. The dichloromethane fraction (DF), obtained from hydroalcoholic extract of *Tanacetum parthenium* (L.) Schultz-Bip, is a phytochemical rich in sesquiterpene lactones which proved to have antileishmania activity. Parthenolide, the most abundant sesquiterpene lactone of DF, causes cell's apoptosis, thereby can justify the raising of oxidative stress. Malondialdehyde (MDA) is one of lipid peroxidation products, widely used as biomarker of oxidative stress in plasmatic samples. Thus, this study aimed to evaluate efficiency of topical and intramuscular treatment, by applying phytochemical DF, through measuring the MDA levels in plasmatic samples of BALB/c mice infected with *Leishmania amazonensis*. Seven groups were formed by treated and untreated animals (each group containing 8 mice). The extraction protocol had employed 200 μ L of mice plasma, mixed to 36 μ L BHT (0.2%) and 6.25 μ L NaOH (10.0 mol L⁻¹), vortexed and kept at 60 °C for 30 min. Then 1500 μ L of solution (7.2%TCA/1% KI) was added, vortexed and centrifuged (3000 rpm for 10 min). Supernatant (1000 μ L) was mixed with 500 μ L of TBA (0.6 %) and incubated for 45 min. MDA standard solution was prepared with 22 μ L TEP (1,1,3,3-tetrametoxyp propane) diluted in 10 mL of H₂SO₄ (1%), protected from light for 2 h, being after, 5 μ L diluted with 1.5 mL of H₂SO₄ (1%), by concentration read at 245 nm (ϵ_{245nm} = 13700 mol L⁻¹ cm⁻¹). MDA calibration's curves (0.05-6.40 μ mol L⁻¹) were prepared with known concentrations, spiked into a plasma pool of 6 healthy animals. Liquid chromatographic system (Waters Alliance e2695) consisted of quaternary pump with degas module, autosampler, column oven, photodiode array detector (2998 PDA) managed by Empower 2. Isocratic chromatographic runs were made using CH₃OH: potassium phosphate buffer (10.0 mmol L⁻¹, pH 7.0) (35:65, v/v) at 0.7 mL min⁻¹ flow rate, with TBA-MDA adduct detected set PDA at 532 nm, applying a guard column and an analytical column Zorbax Eclipse XDB-C18 (4.6x250 mm, 5 μ m, Agilent). Method showed to be linear ($r^2=0.998$) with limit of detection 0.14 μ mol L⁻¹ and limit of quantitation 0.46 μ mol L⁻¹, repeatability (RSD 3.5%) with recovery rates of 97.0-101.0%. Analyses of plasma samples indicated little variation in MDA amounts. However, MDA levels showed an increase of oxidative stress due to treatment. Groups were distinguished by principal component analysis. Hemolyzed samples had to be excluded. Healthy control group had MDA of 1.08 μ mol L⁻¹ and the infected untreated group of 1.25 μ mol L⁻¹. Groups treated by topic use of phytochemical have had MDA of 1.19 μ mol L⁻¹ for placebo while for the DF (5%) was of 1.51 μ mol L⁻¹. Groups treated by intramuscular with phytochemical have had MDA of 1.39 μ mol L⁻¹ for placebo, of 1.48 μ mol L⁻¹ for Glucantime®-reference drug for leishmania, whereas the highest level was of 1.81 μ mol L⁻¹ for DF (30 mg kg⁻¹). Thus, the MDA analysis showed that the treatment using the phytochemical DF, rich in parthenolide was effective, confirming previously activity *in vitro* - *in vivo* determined.

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FABRICATION OF THIN-METAL MICROPARTICLES USING LIFT-OFF PHOTOLITHOGRAPHY

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This poster presents simple method of preparation of thin-metal particles utilizing lift-off photolithography and metal sputtering. Method offers complete control oversize, shape and properties of microparticles with submicrometer thickness. Standard double layer lift-off photolithography was employed, with lift-off resist of high dissolution rate serving as sacrificial layer, which was covered with positive tone photoresist. [1] Several approaches for exposure were considered, of those the most efficient and precise was the use of Heidelberg µPG-101 laser writer. [2] Classical exposure through photomask was also experimented with, using plotter film mask and mercury-vapor lamp. Masks were prepared using Bungard-Filmstar-PLUS raster photoplotter. This method proved to be much faster, but also less precise than exposure in laser pattern generator. Developed structures were coated by metallic layers in sputter coater. Thin-metal particles were released from the glass wafers by the dissolution of resists in N-methyl-2-pyrrolidone. Release was facilitated by the submersion of wafer into the ultrasonic bath. Lift-off approach reduces the need to work with hazardous chemicals in comparison to chemical etching. This is also reflected in wider possibilities in combinations of metals, which are not influenced by the choice and availability of etchants. Chemisorption on the metal surface, e.g. gold, provides a basis for various applications ranging from oligonucleotide arrays to immunoassays. In combination with ferromagnetic properties these particles could be used for separation and movement control using magnetic field. With the aim to verify their usability and versatility the aliquots of Au or Ni metallic particles have been biofunctionalized by covalent bond with PEG-biotin and the quality of biospecific interaction between a planar silanized substrate coated by streptavidin and biotinylated Au (Ni) particles was monitored. Various materials including PTFE, PDMS, UVIBOND UV391 gloss varnish or silicone were applied for bordering the area designed for specific interaction. The level of nonspecific sorption as the indispensable factor for future application was controlled by PEG-ylated microparticles combined with planar area modified by an inert protein, such as bovine serum albumin. The experiments confirmed the potential for wide variety of uses of the thin-metal microparticles in bioanalytical methods based on molecular recognition.

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P-57

**MEKC AND MEEKC AS A TOOLS FOR EVALUATING THE STATUS OF VITAMINS A, C AND E
IN PATIENTS WITH CYSTIC FIBROSIS**

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The aim of this work is the evaluation of nutritional status, based on the level of vitamin C in urine and vitamins A and E in serum, in patients with cystic fibrosis (CF) using capillary electrophoretic methods. A fast, sensitive, selective and fully automated micellar electrokinetic capillary chromatographic (MEKC) and microemulsion electrokinetic capillary chromatographic (MEEKC) methods for determination of mentioned vitamins has been developed and validated. Optimization of parameters affecting the electrophoretic separation provided adequate separation of the analyte of the interest in a short time of 8 min (for vitamin C by MEKC) and 20 min (for vitamins A and E by MEEKC). The study comprised 28 patients with CF and 10 healthy subjects. Based on the mean concentration values, obtained in the two groups, it can be seen that the levels of each vitamins were significantly lower in patients with cystic fibrosis ($0.97 \pm 0.79 \mu\text{g mL}^{-1}$ for vitamin C, $0.83 \pm 0.061 \mu\text{g mL}^{-1}$ of vitamin A and $5.60 \pm 3.38 \mu\text{g mL}^{-1}$ of vitamin E) than in healthy volunteers ($1.185 \pm 0.91 \mu\text{g mL}^{-1}$ for vitamin C, $0.86 \pm 0.076 \mu\text{g mL}^{-1}$ for vitamin A and $9.84 \pm 2.06 \mu\text{g mL}^{-1}$ for vitamin E). In the case of vitamin C and vitamin A concentrations observed differences in both groups were lower ($0.972 \pm 0.79 \mu\text{g mL}^{-1}$ vs. $1.185 \pm 0.91 \mu\text{g mL}^{-1}$ for vitamin C, and $0.83 \pm 0.061 \mu\text{g mL}^{-1}$ vs $0.86 \pm 0.076 \mu\text{g mL}^{-1}$) than in the case of vitamin E ($5.60 \pm 3.38 \mu\text{g mL}^{-1}$ vs $9.84 \pm 2.06 \mu\text{g mL}^{-1}$). When comparing average content of vitamin C in the urine in both studied groups, it can be seen a little lower level in patients suffering from cystic fibrosis.

Many HPLC assays of vitamins have been published, however the determination of physiological vitamin concentration in biological samples by HPLC requires several preliminary steps consisting in the clean-up and preconcentration procedures. Reports on determination of water soluble vitamins in urine were relatively limited, and in most of the relevant studies, vitamins were not simultaneously determined, but individually identified. This is because the therefore developed methods do not possess sufficient sensitivity.

The elaborated in this work method can find practical application in determination of vitamin C in urine and vitamins A and E in serum from children suffer from cystic fibrosis. This method can become a useful tool for future evaluation of vitamins status and in the longer term for evaluation of nutritional status of patients with CF.

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P-58

LOCAL EXPRESSION OF CALGRANULINS (S100 A8/A9) IN HEAD NECK SQUAMOUS CELL CARCINOMA: AN IMAGING MASS SPECTROMETRIC APPROACH

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Despite the earlier theory, that tumors are homogeneous systems, it is well known and accepted that they are high degree of heterogeneity and complexity. That is why the analytical methods, which can provide morphological information and visualization became more in the focus than those based on sample homogenization such as conventional mass spectrometry.

The development of mass spectrometry has recently entered a new phase, an innovation has enabled the combination of mass spectrometry analyses with visualization of the spatial distribution of various analytes. Matrix-assisted laser desorption ionization imaging mass spectrometry (MALDI-IMS) measurements allow the proteomic analysis right on the tissue cryosection. In this study, we determined the spatial distribution of S100 A8 and S100 A9 proteins in HNSCC tissue. The technique has been applied to hypopharynx carcinoma samples, including cancerous tissue, stromal and healthy regions also. Our results showed that the S100 A8/A9 protein complex localized in the tumor and the neostroma regions and not presented in the healthy tissue areas.

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B-MYB, A TRANSCRIPTION FACTOR IN EARLY STAGE HUMAN EMBRYONAL DEVELOPMENT: NON-INVASIVE SCREENING FOR PREDICTION OF EMBRYO VIABILITY

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The quality of the embryo transferred is of a vital importance to IVF success. Nowadays, various technologies, including morphokinetics, genomics, metabolomics, lipidomics and proteomics were introduced for embryo viability prediction.

The molecular profile measured by matrix-assisted laser desorption ionization tandem time-of-flight mass spectrometry after 3rd and 5th days from the micro-droplets of embryo culture as well as in control blank media. The mass spectrometric results were statistically evaluated by ClinProTools (Bruker Daltonics) clustering software.

The molecular fingerprint of the blastocyst secretome can be a predictive technique to distinguish between higher and lower viability of early stage human embryos. Based on our biochemical and statistical analyses the molecular differences of embryos with good and unsatisfactory implantation properties were significantly detectable after 3rd and 5th days as well.

The different patient cohorts can be significantly distinguished by using ClinProTools statistical analysis. Additionally, several molecular biomarkers were identified included B-myb transcription factor, which has critical role in early embryogenesis and successful implantation.

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P-60

FREE-FLOW ISOTACHOPHORESIS ON GLASS CHIP

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Kist-europe

Isotachopheresis is a separation based on two buffers (leading and terminating) with ions of different mobility presence of electric field. Free-flow electrophoresis can be used for continuous separation [1 - 3]. Here we have combined both methods into continuous free-flow isothachopheresis using microfabricated glass chip. The microfluidic device was designed and its pattern was transferred by conventional photolithography and etched by HF/HCl mixture.

The device consists of five inlets (I), a separation (main) chamber with the size of 23×15 mm, two side chambers for connecting the electrodes and five outlets (O). The three middle inlets are split with a binary tree structure for equal distribution of samples and buffers. Two outer two inlets used to guide flow direction into the main chamber. The side chambers are separated from the main chamber by $25 \mu\text{m}$ wide channels to prevent gas bubbles entering the main chamber. The outlets are designed in similar fashion as the inlets.

All buffers were prepared as follows with resulting pH value of 9. The leading buffer was made by 50 mM NH_4OH mixed with 20 mM HCl, the terminating buffer by 50 mM NH_4OH mixed with 50 mM 2-(N-morpholino)ethanesulfonic acid. The sample was prepared by mixing 50 mM NH_4OH with 2.5 mM O-acetylsalicylic acid and fluorescein and rhodamine 110, both with concentration of $50 \mu\text{M}$.

Chip was mounted on an inverted microscope equipped with fluorescent imaging system. Fused silica tubes with internal diameter of $100 \mu\text{m}$ were connected to the chip I/O using Upchurch connectors. Steady flow of buffers and a sample was achieved by syringe pumps with flow rate of $5 \mu\text{L}/\text{min}$. Once the separation voltage with amplitude of 500V was applied the rhodamine was separated from fluorescein which was focused. Figure 1A show the 3 mm wide sample inlet (pointed by an arrow). Figure 1B shows the separated stream of a fluorescein concentrated 500 fold compare to the original concentration. The concentrated fluorescein flow had width of $60 \mu\text{m}$. This focused stream was driven by positive pressure applied to two outer outlets. This configuration enables steering the focused stream to any output of our choice.

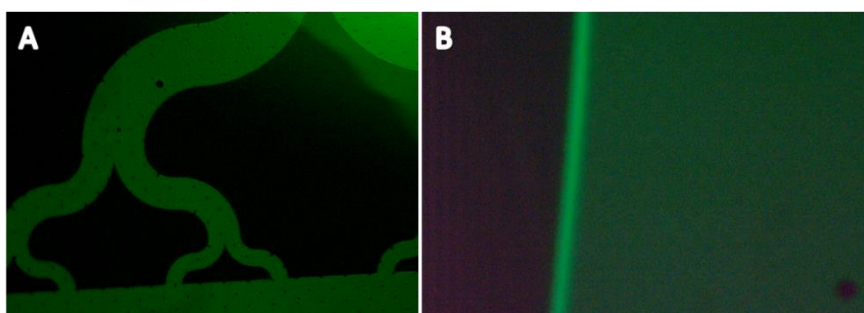


Figure 1: (A) inlet filled with rhodamine and fluorescein, (B) the separation of fluorescein and its focusing. The bright line is fluorescein with 50 fold concentration compare to the input sample ones.

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P-61

**URINARY STEROID HORMONE LEVELS AS POTENTIAL BIOMARKERS
OF NEUROENDOCRINE TUMORS**

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Neuroendocrine tumors (NETs) are relatively rare tumors that arise from the diffuse neuroendocrine system, and are able to produce and to secrete a variety of metabolically active substances like amines, peptides and hormones. Despite of their relative rarity, NETs can cause substantial morbidity in the community because many of the symptoms are innocuous and often non-specific. Additionally, most NET markers are also not specific for a given tumor making difficult the diagnosis of this cancer disease.

The aim of the work was to evaluate urinary steroid hormones as potential new biomarkers of NETs. Thus, the high-performance liquid chromatographic (HPLC) method for the determination of cortisol, cortisone, corticosterone, testosterone, epitestosterone and progesterone in human urine was developed and validated. For each steroid hormone, the limits of detection (LOD) and quantification (LOQ) were 0.5 and 1 ng/mL, respectively. The assay was linear over the concentration range of 1-300 ng/mL ($R^2 = 0.9995$). The intra- and inter-assay precision does not exceed 8.4 and 9.8%, respectively. Next, the developed HPLC method has been successfully applied for the determination of six endogenous steroid levels in urine samples from 20 healthy volunteers and 19 patients with NETs. Finally, a comparison of steroid hormone profiles from both the analyzed groups was performed using parametric tests (Student's *t*-test and the separate-variances *t* test), non-parametric Mann-Whitney U and principal component analysis (PCA). The obtained results confirmed that cortisone and cortisol levels in men with NETs were statistically different against healthy men what confirmed that they could be treated as potential biomarkers of NETs. In the case of women, further study should be continued because the results were not consistent [1]. Thus, the steroid profiles may create new possibilities in diagnosis and prognosis of patients with NETs.

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P-62

**ANALYSIS OF CALIX[4]RESORCINARENE CAVITAND DERIVATIVES WITH
TANDEM MASS SPECTROMETRY**

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Cavitands are cavity-shaped cyclic oligomers and they can create host-guest interactions with various analytes. Host-guest interaction represents the affinity of macrocyclic molecules to form reversible complexes with neutral as well as charged molecules.

Cavitands (e.g.: calixarenes, resorcinarenes, cyclodextrins and their derivatives) may have different uses in Reversed Phase Liquid Chromatography (RPLC). Many resorcinarene-based cavitand-bonded silica phases are prepared for the separation of aromatic positional isomers [1,2], geometric isomers [3], enantiomers of chiral compounds [1] and polycyclic aromatic hydrocarbons [2]. Furthermore, calyx[4]resorcinarene derivatives can be applied as mobile phase additives [4]. They are also employed as dynamic coatings on stationary phase for the separation of aromatic positional isomers and nucleobases [5].

To study the physical-chemical properties and structure of cavitands we have investigated the calyx[4]resorcinarene containing different alkyl chains at the lower rim as analytes in High Performance Liquid Chromatography – TOF-Mass Spectrometry (HPLC-TOF-MS). The identification of the suggested quasimolecular ions was performed with tandem mass spectrometric measurements with ion trap mass spectrometer in MSⁿ mode.

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P-63

OPTIMIZED ON-LINE CAPILLARY ELECTROPHORETIC METHOD FOR CYP3A4 MEDIATED ENANTIOSELECTIVE N-DEMETHYLATION OF KETAMINE

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Enantioselective discrimination is a significant phenomenon influencing biological activity of chiral drugs in humans and animals. Rational drug discovery thus requires an early appraisal of this characteristic impacting on the likely success of a drug candidate in the subsequent clinical testing. Capillary electrophoresis (CE) is a promising technique in this field due to simple implementation of chiral separations, high separation efficiency, minuscule sample and reagent consumption, high throughput by automation and possibility of coupling with mass spectrometric detection. Furthermore, a fused-silica capillary can be used not only as a separation column but also as a nanoscale reaction chamber corresponding to the current trend of miniaturization and throughput enhancement of screening assays in early stages of the development of a new drug.

After initial attempts [1], an improved on-line method for determination of enantioselective metabolism of anesthetic ketamine mediated by cytochrome P450 3A4 (CYP3A4) was developed. As mixing inside the capillary based on diffusion is generic, robust and rapid, the principle of transverse diffusion of laminar flow profiles was adopted [2]. Conceptually, solutions of CYP3A4 and mixture of ketamine and NADPH were injected by hydrodynamic pressure as a series of consecutive plugs with parabolic profiles allowing rapid mixing of the reactants by transverse diffusion [3]. Optimized conditions providing the highest yields of the reaction products and method repeatability were elucidated. All experiments were carried out on the ProteomeLab P800 CE system. Three plugs of 400 nM CYP3A4 solution (each plug was injected for 4 s by pressure of 0.5 psi) and four plugs of ketamine at a certain concentration and 4 mM NADPH solution (each plug was injected for 3 s by pressure of -0.5 psi) were alternately inserted into the 50 μ m i.d. capillary of 64 cm total length that was thermostated at 37°C. The reaction was terminated after 10 minutes and reaction products were separated by application of -20 kV. A 50 mM TRIS-phosphate buffer (pH 2.5) containing 3 % (w/v) of highly sulfated γ -cyclodextrin was used as BGE.

Performed validation showed a good intraday and interday repeatability (RSD < 5 % and 8.5 %, respectively) for both norketamine enantiomers and the optimized method is suitable for on-line study of ketamine metabolism by CYP3A4. The mixing of reactants based on diffusion ensures the versatile applicability of the method for the assessment of the stereoselective aspects of kinetic and inhibition studies of cytochrome P450 enzymes.

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CAPILLARY ELECTROPHORESIS WITH ON-LINE UV ABSORPTION AND LIF DETECTION (488 NM) FOR THE DETERMINATION OF BINDING PARAMETERS BETWEEN APTAMERS AND THEIR TARGETS

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Oligo-desoxyribonucleic acid aptamers can bind to a specific target molecule with high affinity and good specificity. They are usually identified from a large random sequence pool of DNA by an in vitro selection procedure named SELEX. Aptamers can be used for many applications mimicking antibodies as well as for basic research or clinical purposes. Since the works of German *et al* (1998) describing the specific interaction of an aptamer with IgE in serum samples by CE, many studies have been performed to select aptamers using CE (see for example Berezovski *et al*, 2006). In most of the works concerning the determination of the binding interaction parameters between aptamers and protein by CE, the protein was not fluorescent and the synthesized aptamer was covalently bound to a highly fluorescent dye. Usually, laser induced fluorescence is used to detect the free unbound aptamer as well as the complex, but the protein cannot be measured in the same experiment. In our approach to identify as well the aptamer, the complex and the protein, we used a double window capillary: one with LIF detection and one with UV. .

Using this system we were able to measure the K_d of the interaction either using the fluorescence or the UV absorbance.

In order to get a better comprehension of the kinetics of the aptamers folding we studied their behavior both by CE and by circular dichroism and a better knowledge of the reaction between aptamers and proteins was obtained. This work was performed using two proteins: the very well-known thrombin and hRAS.

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P-65

NOVEL LC-MS/MS METHOD FOR THE ANALYSIS OF INTACT R-TYPE BACTERIAL ENDOTOXINS

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One of the most pro-inflammatory bacterial compounds is the lipopolysaccharide (LPS), also called endotoxin, consisting of three domains: lipid-A, the core-oligosaccharide and the O-polysaccharide (O-antigen). LPS is the major outer surface membrane component of Gram-negative bacteria. The amphiphilic and heterogeneous LPS macromolecules are anchored in the phospholipid bilayer with their lipid-A part. As a potent activator of the innate immune system, LPS can induce endotoxic shock in patients suffering from septicemia. The endotoxin that lack the O-antigen and sometimes portions of the core is called lipooligosaccharide (LOS) or R-type LPS. LOS molecules can also be pathogenic, due to their unique mimicry of human ganglioside structures, which can potentially lead to the induction of different syndromes such as Guillain-Barré and Miller Fisher syndrome [1]. The structure elucidation of LPS/LOS is a multi-step task, which includes various techniques besides mass spectrometry (MS), e.g. NMR. These techniques require chemical degradation of the sample in order to obtain defined part structures of LPS/LOS. The part structures are more suitable for further analysis. For example, mild acid hydrolysis of LPS/LOS is routinely used to isolate the saccharide part from the lipid-A. However, a disadvantage of this chemical degradation step is the high risk of losing labile, but biologically important functional groups, e.g. fatty acids, saccharides, non-stoichiometric substitution of 2-aminoethyl(pyro)phosphate or 4-amino-4-deoxyarabinose, besides, it, introduces additional micro-heterogeneity, such as partial dephosphorylation. There are only few reports in the literature demonstrating the successful application of liquid chromatography coupled to MS to separate and detect various species of native LOS/LPS. Up to now, the largest LOS separated and identified by HPLC-MS was a heptose-deficient *Escherichia coli* mutant R-type LPS made up of a lipid-A part and two Kdo-s (3-deoxy-D-manno-octulosonic acids) [2]. None of the studies used tandem mass spectrometry (MS/MS) for the unambiguous identification of the separated molecules. In this study we demonstrate a comprehensive RP/UHPLC-Q-TOF MS/MS analysis of intact LOS of *Salmonella minnesota* R595 containing a lipid-A and two Kdo-s, and of *Shigella sonnei* R41 made up of lipid-A, two Kdo-s and two heptoses, as previously presented by Kilár and co-workers with MALDI-TOF/TOF MS [3]. As compared to the earlier works, we obtained significantly shorter retention times of LOS molecules, and detected several, previously unidentified molecules including structural features otherwise often lost due to sample preparation prior to analysis.

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P-66**CESI-MS FOR MONITORING POST-TRANSLATIONAL MODIFICATIONS OF HISTONE H4**

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P-67

A STOCHASTIC APPROACH TO THE POLYDISPERSITY IN SIZE EXCLUSION CHROMATOGRAPHY

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We investigate the impact of the polydispersity of the sample molecules on the separation process and on the efficiency of size exclusion chromatography. The polydispersity was integrated into the molecular (stochastic) model of chromatography; the characteristic function, the band profile and the main moments of the elution profiles were determined by mathematical formulas for several kind of pore structures. We investigated the effects of the parameters affected by the polydispersity on the separation for a number of pore shapes.

Our results demonstrate that even a small distribution in the molecule size (i.e. polydispersity) can contribute substantially to the total width of the chromatographic peak. The pure effect of the polydispersity can only be investigated via a mathematical model, because its contribution to an experimental chromatogram cannot be separated from other band-broadening effects.

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MODELING THE 2D CORRELATION IN CHROMATOGRAPHY WITH GAUSSIAN PEAK SHAPE

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Two-dimensional correlation analysis is a method of long standing in spectroscopy. It has provided countless unique information about a huge variety of chemical phenomena. Due to its achievements in the recent years it was spread to numerous other analytical fields such as microscopy or gel permeation chromatography. Despite its wide success it hasn't gained a foothold in liquid or gas chromatography yet.

The two-dimensional correlation spectroscopy's (2D-COS) theory and the calculations of the synchronous and asynchronous spectra are well defined for spectral intensities, whether it is in discrete data or matrix form. Using the same algorithms in chromatography, the two methods necessarily have both similarities and differences. Chromatographic data are normally digitalized and stored in matrices, thus it is evident to use matrix calculations for producing the correlation maps. Chromatographic peaks can often be modeled by Gaussian curves, thus it is reasonable to build the calculation with them for discrete data. In this case chromatograms are represented by a sum of Gaussian curves, and correlation maps, like two-dimensional chromatograms, are represented by the sum of two-dimensional Gaussian curves. The Gaussian function can be described with three parameters, the peak height, width and location (retention time). In this study the parameters are extracted from chromatograms and correlation maps for further analysis. Comparing these data will lead to a deeper insight of the correlation method.

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P-69

DESIGN AND NUMERICAL SIMULATION OF MICROFLUIDIC CELL CAPTURE DEVICES

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A novel microfluidic cell capture device design is investigated by means of computational fluid dynamics (CFD) simulation. The device features an array of cylindrically shaped microposts. The physical object is simulated in 2D. The model is based on the laminar form of the Navier-Stokes equation assuming one phase flow with same physical characteristics as water at 293.15 K and constant dynamic viscosity. The velocity field distribution and the formed shear stress are calculated by numerical methods. It is concluded that the cell capture capability of this novel device can be significantly improved by altering the positions of the pillars.

Microfabricated cell capture devices (MCCDs) are inspired by the electrical circuits of the semiconductor industry. MCCDs usually comprise channels, pillars, junctions and the combinations of those, with a goal of maximizing functionally useable area. They are used for sorting, processing and analyzing minute amount of biological fluids or other biological samples, such as rare cells [1, 2]. Cell sorting has particular importance in cancer research since it reduces the complexity of the biological sample (blood) for liquid biopsy [3]. Dealing with such very small number of cells in the target makes MCCDs promising tools for detection, capture and enrichment since the geometrical dimensions of the target cells and the working channels are in the same size range. Due to their small dimensions, experimental analysis with such miniature and complex devices is not only difficult but time and cost intensive. Thus, computational modeling can be used to speed up the development. With regard to MCCDs, computational fluid dynamics (CFD) modeling is widely accepted and probably one of the most frequently used tools today. In this presentation, we focus on the investigation of the shear stress occurring in MCCDs. Furthermore, we demonstrate how CFD simulation can help to detect unexpected dead-volume problems inside the microchip.

The obtained velocity field is shown in Figure 1 (the warmer the color, the higher the velocity). The full-length inflow velocity is 0.001 m/s. As one can see, the wall effect is not significant, since the flow field distribution between the pillars is homogenous. The three hot points, where the flow velocity is higher are negligible since we are interested in the bulk characteristic only.

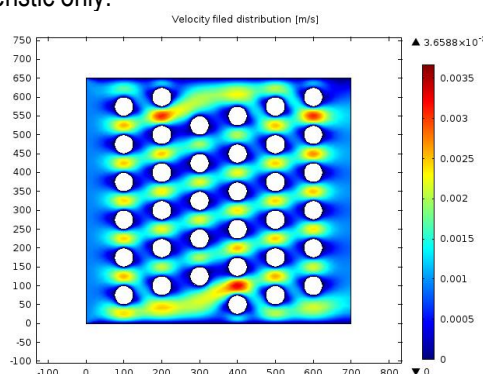


Figure 1. The simulated flow velocity distribution

Based on the flow channel patterns (indicated with turquoise), which are formed between the pillars, it can be concluded that this micropost arrangement is not efficient enough. Cells, which are transported by laminar flow, can move through the chip without any interaction with the functional surfaces, i.e. the surface of the pillars. If one alters the pillar arrangement (shift the columns of pillars in vertical orientation), the pressure drop could be increased. The higher pressure drop could damage the cells; therefore, the shear stress was calculated using the calculated velocity field. The shear stress is the linear function of the velocity field and can be derived as (1):

$$\tau_w = \frac{6\mu Q}{wh^2} \quad (1)$$

where μ is the dynamic viscosity, Q is the flow velocity, w and h are characteristic geometry sizes of the object [4]. The calculated shear stress is around 0.2 Pa, which is much less than the threshold shear stress of 150 Pa, where extensive cell damage could occur. Based on the flow pattern and the calculated shear stress, as a first approximation we can consider that the investigated MCCD can be improved by appropriate alteration of the pillar positions.

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P-70

MODELING OF SAMPLING STRATEGIES FOR CAPILLARY ISOELECTRIC FOCUSING

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Capillary isoelectric focusing (cIEF) is a high-resolution analytical technique for separation and characterization of proteins. cIEF is commonly used to segregate mixtures of compounds on the basis of differences in their isoelectric points (pI).

The sampling strategies, named „sandwich” (ampholyte–sample–ampholyte injection) and „half-sandwich” (ampholyte-sample injection) developed by Kilár et al. [1], offer a unique opportunity for separating and analyzing zwitterionic compounds having pI/s outside the pH range of the carrier ampholytes. These configurations allow a divided introduction of the ampholyte and sample components and have advantages for MS detection.

Seven hypothetical low molecular mass samples (pI: 5.3, 6.4, 6.6, 7.2, 7.9, 8.6, 10.4) were analyzed by computer simulation following the sequential injection protocol in order to reveal their behavior in and outside the pH 7–9 range established by 101 hypothetical amphoteric carrier ampholytes. The first set of simulations in which phosphoric acid and sodium hydroxide were replaced by formic acid and ammonium hydroxide provides a good opportunity to examine the effect of the different electrolytes on the separation. Other types of models including eight extra carrier components (pI: 4.0, 5.5, 6.0, 6.8, 9.5, 9.8, 10.0, 10.6) are imitating edge compounds having pI/s outside the pH gradient.

A one-dimensional computer program, GENTRANS (generalized, transient PC-based electrophoresis model) was used to explore the difference between each consecutive injections to predict separation dynamics of all components, pH gradient formation and stability, and electrophoretic mobilization during and after focusing [2, 3]. Simulation data show that sequential introduction of components into the capillary offers a fast separation of samples, changing in electrode solutions results in different sample and ampholyte behavior and during the focusing all components outside the pH gradient are migrating towards the cathodic or anodic leader forming an isotachophoretic zone.

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P-71

ANALYSIS OF SERUM N-GLYCOME ALTERATIONS IN INFLAMMATORY AND MALIGNANT LUNG DISEASES

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Glycosylation is a complex post-translational modification, which usually influences the biological function of the resulting glycoproteins [1]. Because of to the non-template driven generation of glycans, comprehensive characterization of their structural motifs is helpful to understand their roles in molecular cellular processes [2]. Changes in terminal sialylation, galactosylation and differences in α 1-3/6 fucosylation are well-known markers of several inflammatory and malignant diseases [3]. The scientific interest towards glycan analysis has been significantly growing in recent years, leading to the need of more precise and higher throughput techniques to decipher the complexity of N-glycans, sometimes necessitating combination of different bio-analytical methods [4]. The most frequently used techniques for glycan analysis are high performance liquid chromatography (HPLC), capillary electrophoresis (CE), mass spectrometry (MS) and NMR or the combination of them. Weak-anion-exchange chromatography is a reportedly useful option to prepare and analyze highly sialylated carbohydrate structures. Using the WAX retention times of 2-AB labeled samples, unlabeled glycans can be fractionated and used for further analytical techniques. Capillary electrophoresis with laser induced fluorescence detection (CE-LIF) of APTS-labeled glycans is a robust tool, offering rapid analysis with high resolution and excellent reproducibility. Targeted exoglycosidase digestions in combination with CE-LIF offers a powerful method to reveal both structural and linkage information, thus enables monitoring of highly detailed glycosylation changes. The aim of this study was to analyze the changes of global serum glycosylation in normal, pneumonia, tuberculosis and lung cancer samples focusing on sialylation, fucosylation and branching. Pooled serum samples were PNGase F digested and fractionated by HPLC-WAX. The collected fractions were APTS labeled and first profiled by CE-LIF and further analyzed by sequential exoglycosidase digestion where the major N-glycan structures were identified to specify disease-specific alterations.

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P-72**A STUDY OF TWO SEQUENTIAL MEDIA FOR IVF**

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Culture media for the support of human embryos after in vitro fertilisation (IVF) and before embryo transfer has been a focus of considerable interest over the past decade. These sequential media were formulated according to the changing physiology and metabolic requirements of the human embryo and led to improvements in human IVF outcomes [1]. In this study, we performed proteomic analysis of the two kinds of commercial media (Sydney IVF medium and VitroLife G medium). Human serum albumin is the absolutely abundant protein in the medium sample, so we choose multi-filter cut-off workflow for sample preparation and albumin removal. Firstly, Amicon 30kD cutoff spin column is applied to separate one medium sample into 3 fractions: Filtered, Concentrated, Eluted. Then, every fraction is reduced, alkylated and digested with Lys-C or Glu-C. Secondly, each fraction is filtered with 10kD cutoff spin column like in the first step, and these fractions were collected and labeled: Filtered, Concentrated, Eluted. Altogether nine fractions of each sample are collected, and digested with trypsin. Finally, peptides are analyzed by nanoHPLC-MS/MS. It is interesting that, beside the human serum albumin, other proteins involved in embryonic development are detected in the samples except. For example, there are 30 identified proteins in Sydney Cleavage medium, which are mostly plasma proteins. Because albumin is an important transport protein, the presence of other plasma proteins could be explained with the "contamination" during the HSA production. However, it is still unknown which of these unpredicted plasma proteins have an effect on IVF, but it is an important clue for the next analysis of the embryonal secretome during IVF procedure.

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P-73

CHIRAL ANALYSIS OF ASPARTATE AND GLUTAMATE IN BIOLOGICAL SAMPLES BY CAPILLARY ELECTROPHORESIS WITH LASER-INDUCED FLUORESCENCE (LIF) DETECTION AND APPLICATION IN INVESTIGATING THE RELATIONSHIP WITH ALZHEIMER'S DISEASE

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A simple CD-mediated CZE method equipped with laser-induced fluorescence (LIF) detector was developed for chiral analysis of excitatory amino acids (EAAs), aspartate and glutamate, and to determine the EAAs concentrations in Alzheimer's disease patients' plasma. Before analysis, plasma samples were pretreated with centrifugal filter devices for removing proteins with high molecular weight (molecular weight cut off 3000) and then derivatized with 10 mM 6-carboxyfluorescein N-hydroxysuccinimide ester/ DMSO. The ratio of filtrate to the derivatizing reagent is 5 (v/v= 5 : 1). Mixed samples were sonicated for 2 h at 25°C for chemical derivatization. After the derivatization reaction, reacted samples were diluted 100-fold with water and then hydrodynamically injected into CE instrument (0.5 psi for 5 s). The separation buffer was consisting of borate buffer 50 mM (pH 9.0) with 6 mM γ -CD and 0.1% PVP. The separation voltage was set at 20 kV. This method was applied in determining the EAAs concentrations of 26 patients with Alzheimer's disease. We compared the EAAs concentrations and CDR-SB or MMSE and then discussed the relationship between EAAs concentrations and Alzheimer's disease. From the results, there's a moderately negative correlation between L-Asp concentrations with CDR-SB values.

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P-74

ELECTROPHORESIS IN THE LIQUID AND GAS-PHASE FOR INVESTIGATION OF THE INFLUENCE OF FLUOROPHORE/QUENCHER MODIFICATIONS ON OLIGONUCLEOTIDE CONFORMATIONS

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Data storage in organisms is achieved via nucleotides whose sequence encodes for genetic information. Duplex formation (G-C and A-T) as part of DNA packing enhances the stability of genetic information. This ability of specific base recognition is employed in analytical chemistry e.g. for molecular beacons (MBs), short, single-stranded oligonucleotides modified by a fluorophore/quencher pair at their sequence ends. Under favorable conditions several 3' and 5' proximal bases of a given oligonucleotide form a helical structure (stem) which confines spatial proximity to the fluorophore/quencher pair; hence no fluorescence is recorded (closed hairpin). The nucleotide sequence between stem members (loop) is designed to specifically target other DNA or RNA sequences, e.g. inside cells. Upon presence of such a specific target, the loop region of the MB attaches, the secondary structure of the stem is lost and fluorescence is recorded (open hairpin).

In order to efficiently design MBs and to predict their folding, several freeware computational programs exist, e.g. by Markham and Zuker [1]. However, the impact of modifications (i.e. fluorophore/quencher pairs) on the conformation of oligonucleotides [2] is not regarded for computation. Therefore, we took interest in the impact of an exemplary fluorophore/quencher modification on the conformation of oligonucleotides. Experiments were carried out under conditions not reflecting *in vivo* conditions (Electrolyte solutions were at low ionic strength, divalent cations were absent.). Such conditions are often necessary prerequisites for *in vitro* experimental setups. In order to investigate MB conformations we employed electrophoresis in the liquid phase (standard capillary electrophoresis instrument with UV detection and chip electrophoresis with fluorescence detection [3]) as well as in the gas-phase (gas-phase electrophoretic mobility molecular analysis; GEMMA [4, 5]). Electrophoresis in the liquid phase separates analytes according to their charge and shape. As the analyte charge remains constant, electrophoretic mobility variations can be ascribed to conformational changes. GEMMA on the other hand detects electrophoretic mobility diameter values in the gas-phase and is thus able to discriminate open and closed hairpin conformations.

In the course of our experiments we were able to demonstrate the impact of fluorophore/quencher modifications on the folding of oligonucleotides. Therefore, it has to be kept in mind that upon probe design the choice of a fluorophore/quencher pair influences the oligonucleotide conformational behavior.

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P-75

NEW APPROACH IN EVALUATING THE INERTNESS OF GAS CHROMATOGRAPHIC SYSTEMS

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Over viewing the problem. *Inertness* seems to be an important characteristic of any chromatographic systems, remaining neither out of effective controlling, nor out of commonly acceptable unambiguous definition. Purely intuitive interpretation of this term predominates. In a result of insufficient inertness of chromatographic system, the signals of polar or reactive analytes may be not only strongly distorted on the chromatograms, but even disappear completely. An objective difficulty to determine the inertness is its relative character; it depends not only on equipment, but on the chemical origin of analytes.

Nevertheless, the history of chromatography includes a lot of examples of low inertness of chromatographic systems and ways to overcome it. Most of approaches in its controlling are based on the concept of mixtures of test compounds and evaluating the discrimination effects of relative areas (or heights) of chromatographic peaks of polar compounds relative to those of non-polar constituents. Few dozens test-mixtures were elaborated since beginning of 1970s. One of them (most "popular") has become Grob test mixture containing 12 components (some variations are known).

The over viewing the test-mixtures permits us to conclude the following:

1. All recommendations imply the single analysis of the single mixtures;
2. There is no standard viewpoint of on their composition; two opposite tendencies are observed: the simplification vs. complication;
3. ***Not the composition of the test-mixtures is the main factor determining their using; rather it is the algorithm of data interpretation.***

This report is devoted to the discussion of the new approach in the controlling the inertness of chromatographic systems.

New approach implies:

1. To simplify the composition up to the two constituents: one *n*-alkane and one polar compound (e.g., alkanol);
2. The principal difference from previous recommendations is the refusal from the analysis of single samples. Any test-mixture should be analyzed several times as a series of successively diluted solutions, namely 1:1, 1:10, and so on up to 1:10⁵-1:10⁶. The analysis of the first sample (1:1) is not necessary because of the danger of possible overloading the columns.

The amount of the polar component injected in chromatograph (M_i , g) is calculated using the following relation:

$$M_i \approx 10^{-(i-3)} v_{\text{sample}} [V_1 d_1 / (V_1 + V_2)] / (R + 1), \quad (1)$$

where $V_1 = V_2 = 1$ mL are the initial volumes of mixed liquids; d_1 is the specific gravity of polar component, g/mL = $\mu\text{g}/\mu\text{L}$; v_{sample} is the volume of probe injected into chromatograph (usually 1 μL); R is the split ratio (at using the capillary columns); i is the sequential number of dilution.

The amounts of analytes can be presented in the form of indices, $\text{pM} = -\log M$.

Next step is evaluating the relative peak areas ($S_{i,\text{rel}}$) and their standard deviations, s_i ,

$$S_{i,\text{rel}} = S_i / \sum S_i \quad (2)$$

and calculating the differences of relative peak areas for all diluted solutions and those for the first one in series:

$$D_i = \langle S_{1,\text{rel}} \rangle - \langle S_{i,\text{rel}} \rangle \quad (3)$$

The slightly non-linear dependence of the sums of standard deviations $\sum s_i$ vs. pM_i can be approximated by the linear regression:

$$\sum s_i = a \text{pM}_i + b \quad (4)$$

The dependence $D_i(\text{pM})$ is expressed in larger extent due to the restricted inertness of chromatographic system. It can be approximated by linear regression as well:

$$D_i = a' \text{pM}_i + b' \quad (5)$$

Thus, for relatively concentrated solutions the typical inequality is $D_i < \sum s_i$, while since any stage of their dilution we obtain the inequality, $D_i > \sum s_i$. Graphically it means the crossing of two lines corresponding to regression equations (4) and (5) (see Figure below). The crossing point (pM_{lim}) corresponds to the limiting amount of the analyte when the influence of the insufficient inertness of GC system can be still considered as statistically negligible. The solution of the system (4) and (5) relative to pM_{lim} is:

$$pM_{lim} = (b - b') / (a' - a) \quad (6)$$

So far as the definition of the term “inertness” is unknown; the following proposition for *limit of inertness* can be suggested:

The limit of the inertness of a chromatographic system with respect to the selected analyte is such its quantity in the injected probe [in the form of $pM = -\log(M)$] of the diluted solution of its mixture with conventionally inert component (*n*-alkane), when the differences in relative peak areas of this analyte compared with the more concentrated solutions are beginning to exceed the sums of their standard deviations.

The example of data processing is the following:

Table. The example of data processing for controlling the inertness (quartz WCOT column with HP-5 MS, temperature 80 °C), test-mixture is *n*-octane/1-heptanol

Dilution	$S_{rel}(1-C_7OH)$, %	pM_i	D_i	$\sum s_i$	Parameters of regressions:
1:10	46.9 ± 0.5	5.75	-	-	$\sum s_i = apM_i + b$ (4):
1:10 ²	46.5 ± 0.2	6.75	0.4	0.7	$a = 4.6 \pm 1.1$; $b = -32.2 \pm 9.5$; $r = 0.944$
1:10 ³	44.0 ± 0.5	7.75	2.9	1.0	$D_i = a'pM_i + b'$ (5):
1:10 ⁴	41.2 ± 0.9	8.75	5.7	1.4	$a' = 0.5 \pm 0.1$; $b' = -2.5 \pm 0.5$; $r = 0.981$.
1:10 ⁵	32.0 ± 1.6	9.75	14.9	2.1	Solution: $pM_{lim} = 7.24$, $M_{lim} = 0.06 \mu g$.

Graphical illustration of this mode of controlling the inertness is presented on Figure a) stainless steel packed column, and b) quartz WCOT column. The M_{lim} value in the first case regularly exceeds the second one more than ten times.

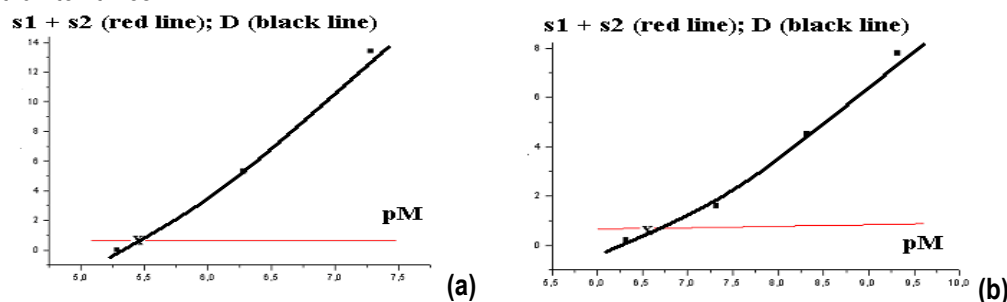


Figure a) The graphical illustration of the revealing the inertness ranges: column's temperature 90 °C. Test-mixture: phenol – *n*-octane, solvent chloroform, dilution of the initial mixture from 1:10 up to 1:10⁴. The limit of inertness for phenol is: $pM \approx 5.4$ ($\sim 4.0 \mu g$ in the probe);

b) The graphical illustration of the revealing the inertness ranges: column's temperature 70 °C. Test mixture 1-hexanol – *n*-octane, solvent 2-propanol, dilutions of initial mixture from 1:10 up to 1:10⁶. The limit of inertness for 1-hexanol is: $pM \approx 6.6$ ($\sim 0.25 \mu g$ in the probe).

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NGS BASED CHARACTERIZATION OF TWO SHIGELLA SONNEI STRAINS WITH DIFFERENT LPS CHARACTERISTICS

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Shigella spp. are gram-negative, rod-shaped, non-motile and non-sporulating intracellular pathogenic bacteria belong to the family *Enterobacteriaceae*. The *Shigella* genus can be classified into four species (i.e.: *S. dysenteriae*, *S. flexneri*, *S. sonnei* and *S. boydii*), which are further divided into multiple serotypes based on the structure of the O-polysaccharide portion of their outer membrane lipopolysaccharide (LPS). More than a century after their discovery, *Shigella* spp. still poses a worldwide major health problem due to the devastating diarrhea that the bacteria may cause. Shigellosis is a severe diarrheal disease associated with high morbidity and mortality rates, particularly in young children. Despite the fact that, most of the currently evolved models of pathogenesis induced by *Shigella* spp. are based *in vitro* and *in vivo* studies using various cell types and different animal models, we still have much to learn on the functions of bacterial virulence factors and the responses of infected cells. Recently cutting edge technologies e.g., whole-genome sequencing, comparative genomics and proteomics are also support the new insight into the genetic basis of *Shigella* virulence.

As part of these efforts, massively parallel next generation sequencing (NGS) technology was applied to characterize two *S. sonnei* strains with different pathogenic abilities. To provide new insights into the structural organization of LPS biosynthetic gene clusters, genome sequencing was performed on Ion Torrent semiconductor sequencing platform. After clonal amplification of library fragments (emPCR), 444.553 Ion Sphere particles were deposited in the chip wells and sequenced. The presented draft genome (assembly comprises 2352 scaffolds) was annotated and genes that play pivotal roles in the LPS biosynthetic processes were identified. Complementary to the structural genomics, to get insight into the gene expression transcriptome profiling has also been performed.

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P-77

ANALYSIS OF MUCIN-TYPE O-GLYCANS BY μ HPLC-ESI-MSSic, Sinisa¹; Rizzi, Andreas¹; Maier, Norbert²¹University of Vienna²University of Helsinki

Glycomics is still an increasing field of research and new tools and methods for the in-detail analysis of protein glycosylation are still required. Qualitative and quantitative analysis of glycan variants attached to proteins (glycan fingerprinting) has gained considerable importance. In this context, the investigation of O-glycosylation is underrepresented, probably due to the lack of enzymes catalyzing their release and, secondly, due to the rather low ionization yield attained for small oligosaccharides.

The focus of this poster is on establishing a sensitive and robust platform for the analysis of mucin-type O-linked glycans. In this approach a chemical one-step reaction is used combining the release of O-glycans from glycoproteins with a derivatisation step. In this paper stable-isotope coded labeling by d0/d5 1-phenyl-3-methyl-5-pyrazolidone (PMP) was introduced as labeling reagent for the relative quantitation of O-glycans. The “heavy“-version of this label, penta-deutero (d5)-PMP, had to be synthesized.

PMP derivatisation of sugars improved the ionization yield by ESI-MS and impacted retention with all types of stationary phases used. On-line HPLC-ESI-MS (using a high resolution Qq-oeTOF instrument) was established for the fast quantitative determination of O-glycan profiles. RP-HPLC offers only a very limited potential for separating isobaric oligosaccharide isomers, however in this case, it is most beneficial for avoiding the presence of sodium clusters in the MS spectrum.

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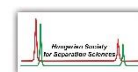
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