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EDITORIAL PREFACE TO SPECIAL ISSUE DEDICATED TO THE 14TH INTERNATIONAL INTERDISCIPLINARY MEETING ON BIOANALYSIS

ANDRÁS GUTTMAN *[1,2] AND HERBERT LINDNER[3]

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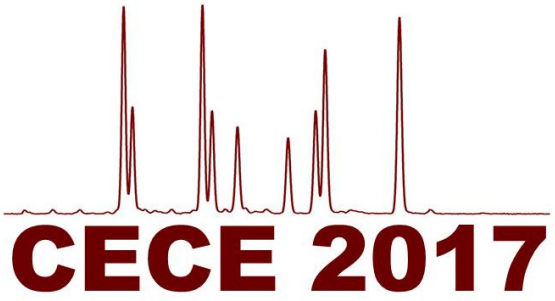
The CECE-2017, 14th International Interdisciplinary Meeting on Bioanalysis was held in Veszprém, Hungary on 8-11 October, 2017. The symposium was organized by the MTA-PE Translational Glycomics Research Group, MUKKI, University of Pannonia. The funding idea of this conference series was "bring together scientists from different disciplines who may not meet at other meetings". Following this initiative, 17 world-class invited speakers delivered stimulating lectures not only about cutting-edge bioanalysis but also about other hot topics, such as aerosol chemistry, etc. The informal atmosphere of the meeting supported direct networking between well-established senior researchers and young scientists at the beginning of their carrier. The first two days of invited lectures was extended with CECE Junior, a dedicated day for PhD students and postdocs, providing them an opportunity to present their results and catch the attention of colleagues both from academia and industry. More than 120 registered participants attended the event during the three days.

The scientific program was preceded by a unique Hungarian social event, a sausage making exercise on Sunday. This national tradition was an exceptional get-together party, where invited speakers, participants, students, sponsors and organizers made spicy and tasty sausage under strict quality control by the father of CECE series, Frantisek Foret.

As an accompanying section, the Horváth Csaba Memorial Exhibition was reopened on the occasion of opening an exhibition for another great Hungarian chromatographer Professor Ervin sz. Kováts. The joint display was dubbed as Hungarian "Chromatography" Mafia Exhibition and these two good friends are together as examples for the next generations.



Overall, the program was of very high scientific level. In this symposium issue, selected contributions are published after normal peer-review process covering very diverse topics of the bioanalytical field.

At this point, the chair András Guttman and co-chair Herbert Lindner would like to thank the hard work of colleagues and students namely Brigitta Mészáros, Beáta Borza, László Hajba, Márton Szigeti, Balázs Reider and Gábor Járvás from the Hungarian side and Bettina Sarg and Klaus Faserl from the Austrian side.

Last but not least, this event would not have happened without the financial support of our sponsors – SCIEX, Unicam Hungary, Thermo-Fischer Scientific, Bio-Science, Agilent, Kromat, Watrex, Waters, University of Pannonia, City of Veszprém and the Hungarian Society of Separation Sciences.

In 2018 the series will return to its home town Brno on 15-17 October, 2018, see you there!

András Guttman and Herbert Lindner

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SEMIQUANTITATIVE ASSESSMENT OF DISHEVELLED-3 PHOSPHORYLATION STATUS BY MASS SPECTROMETRY

KATEŘINA HANÁKOVÁ[1,2], DAVID POTĚŠIL[2], ONDŘEJ BERNATÍK[3], IGOR ČERVENKA[3], MATĚJ RÁDSETOULAL[3], VÍTĚZSLAV BRYJA[3], AND ZBYNĚK ZDRÁHAL *[1,2]

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The focus of this paper is the human Dishevelled 3 protein (hDvl3), an essential component of the Wnt signalling pathway that contributes to their regulation. Mass spectrometry-based analysis of hDvl3 phosphorylations induced by eight associated kinases was performed revealing several dozens of phosphorylation sites. The main outcome of this study was the description of Dvl phosphorylation "patterns" induced by individual kinases.

Keywords: phosphorylation, Dishevelled 3, mass spectrometry, CK1ε, NEK2

1. Introduction

Reversible protein phosphorylation belongs to posttranslational modification which is of high biological significance mainly due to its role in regulating cellular processes such as gene expression, cell division, signal transduction, metabolism, differentiation, apoptosis, etc. [1]

Dishevelled-3 (Dvl3) is a protein involved in the Wnt signalling pathways and gets heavily phosphorylated in response to pathway activation by Wnt ligands [2].

In our study, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) separation of hDvl3 immunoprecipitates was combined with titanium dioxide phosphopeptide enrichment followed by liquid chromatography-tandem mass spectrometry (LC-MS/MS). Data were processed using Skyline software to obtain a semiquantitative assessment with regard to the occupancy of phosphorylation sites induced by individual kinases.

2. Experimental

2.1 Cell culture and transfection

The cell line HEK293 was used for this study. Cells were seeded in dishes 15 cm in diameter and transfected at a confluence of 60%. Two days after transfection, after reaching confluence, they were harvested for immunoprecipitation. A chilled NP-40 lysis buffer with protease inhibitors, dithiothreitol (DTT), phosphatase inhibitors and N-ethylmaleimide (NEM) was used for cell lysis. The lysate was incubated with the antibody Anti FLAG M2.

2.2 Gel electrophoresis, protein digestion and phosphopeptide enrichment

Immunoprecipitates were separated by SDS–PAGE, fixed with acetic acid in methanol, and stained with Coomassie Brilliant Blue for 1 hour then partially destained.

Corresponding 1-D bands were excised. After destaining, the proteins in gel pieces were incubated with 10 mM of DTT at 56 °C for 45 mins. After the removal of DTT excess samples were incubated with 55 mM of iodoacetamide (IAA) at room temperature in darkness for 30 mins, then an alkylation solution was removed and gel pieces were hydrated for 45 mins at 4 °C in a digestion solution (5 ng/µl trypsin, sequencing grade, Promega, Fitchburg, Wisconsin, USA, in 25mM ammonium bicarbonate (AmBic)). The trypsin digestion proceeded for 2 hours at 37 °C on a Thermomixer (at 750 rpm; Eppendorf AG, Hamburg, Germany). Subsequently, the tryptic digests were cleaved by chymotrypsin (5 ng/µl, sequencing grade, Roche, Basel, Switzerland, in 25mM AmBic) for 2 hours at 37 °C. Digested peptides were extracted from gels using 50% acetonitrile (ACN) solution with 2.5% formic acid (FA) and concentrated in a SpeedVac concentrator (Eppendorf AG, Hamburg, Germany). The aliquot (1/10) of the sample was transferred to an autosampler vial and concentrated under a vacuum. Water was used to produce 15 µl of the peptide solution which was directly

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Figure 1: Comparison of phosphorylation sites of hDvl3 induced by CK1ε and NEK2. Experimentally determined phosphorylation sites by CK1ε and NEK2 are indicated by green background color. Phosphorylation sites identified only by NEK2 are indicated by red background color.

analyzed by liquid chromatography coupled with mass spectrometry (LC-MS/MS) for protein identification (ID run).

The rest of the peptide mixture was used for phosphopeptide analysis. MS PhosphoMix 1, 2, 3 Light (Sigma Aldrich) was added to the samples before the phosphopeptide enrichment step in a concentration of 0.1 pmol. Phosphopeptides were enriched using a Pierce Magnetic Titanium Dioxide Phosphopeptide Enrichment Kit (Thermo Fisher Scientific, Waltham, Massachusetts, USA) according to the protocol of the manufacturer and eluted into an autosampler vial. The solution was concentrated under a vacuum to a volume of less than 5 µl, dissolved in water and 0.6 µl of 5% FA was used to obtain 15 µl of the peptide solution before LC-MS/MS analysis.

2.3 Mass spectrometry

LC-MS/MS analyses of the peptide mixture were conducted using a RSLCnano system connected to a Orbitrap Elite hybrid mass spectrometer (Thermo Fisher Scientific) with a ABIRD (Active Background Ion Reduction Device; ESI Source Solutions) and a Digital PicoView DPV550 (New Objective) ion source (tip rinsing by 50% ACN with 0.1% FA) installed. Prior to LC separation, tryptic digests were concentrated online and desalted using a trapping column (100 µm × 30 mm) filled with 3.5-µm of XBridge BEH 130Ĺ C18 sorbent (Waters). After the trapping column was washed with 0.1% FA, the peptides were eluted (flow rate of 300 nl/min) from the trapping column onto a Acclaim PepMap100 C18 column (3 µm particles, 75 µm × 500 mm; Thermo Fisher Scientific) along a 65 min-long gradient. Mobile phase A (0.1% FA in water) and mobile phase B (0.1% FA in 80% ACN) were used in both cases. The gradient elution started at 1% of mobile phase B and increased from 1% to 56% during the first 50 mins (30% in the 35th and 56% in the 50th min), then increased linearly to 80% of mobile phase B over the following 5 mins and remained at this state for the next 10 mins. Equilibration of the trapping column and the anlytical column was conducted prior to injection of the sample into the sample loop. The outlet of the analytical column was directly connected to the Digital PicoView DPV550 ion source.

MS data were acquired in a data-dependent strategy by selecting the top 6 precursors based on precursor abundance in the survey scan (350-2000 m/z). The resolution of the survey scan was 60,000 (at 400 m/z) with a target value of 1×10^6 ions, one microscan and a maximum injection time of 200 ms. High resolution (resolution of 15,000 at 400 m/z) higher energy collisional dissociation (HCD) MS/MS spectra were acquired with a target value of 50,000. The normalized collision energy was 32 % for HCD spectra. The maximum injection time for MS/MS was 500 ms. Dynamic exclusion was enabled for 45 s after the acquisition of one MS/MS spectra and early expiration was disabled. The isolation window for MS/MS fragmentation was set to 2 m/z.

The analysis of the mass spectrometric RAW data files was carried out using the Proteome Discoverer software (Thermo Fisher Scientific; version 1.4) with utilization of the in-house Mascot (Matrixscience; version 2.4.1) search engine. MS/MS ion searches were conducted against an in-house database containing the expected protein of interest with additional sequences from the cRAP (common Repository of Adventitious Proteins) database (downloaded from http://www.thegpm.org/crap/). Mass tolerance for peptides and MS/MS fragments were 7 ppm and 0.03 Da, respectively. Oxidation of methionine, deamidation (N, Q)

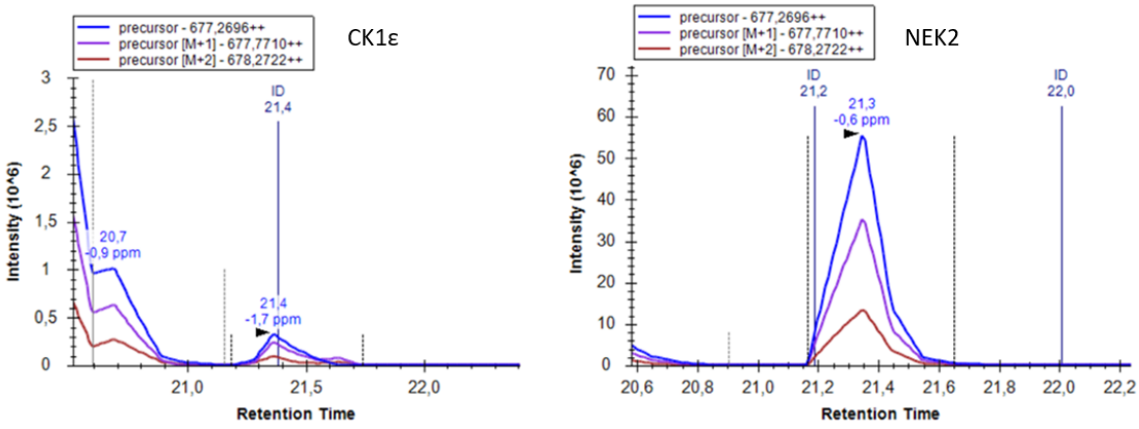

Figure 2: Semiquantitative analysis of phosphorylation site serine 204 (S204) on peptide FSSpSTEQSSASR induced by CK1ε and NEK2. Precursor and selected fragment traces of corresponding hDvl3 phosphopeptides are shown for CK1ε and NEK2 (in Skyline). The highest signal intensity was detected in the case of NEK2.

and phosphorylation (S, T, Y) as optional modifications, carbamidomethylation of C as a fixed modification and three miss cleavages of enzymes were set for all searches. The phosphoRS feature was used for phosphorylation site localization.

Quantitative information was assessed and manually validated in Skyline software (Skyline-daily 3.1.1.8884).

3. Results and Analysis

3.1 Identification of phosphorylation sites

Phosphorylation is important for protein function and regulation. The phosphorylation status of human Dvl3 induced by eight individual Ser/Thr kinases that were previously reported or identified by an unbiased MS screen for Dvl-associated kinases was analysed. Dvl3 contains 131 serines/threonines, which can be potentially phosphorylated. In total, 88 Ser/Thr phosphorylation sites and one tyrosine phosphorylation site in Dvl3 were identified.

3.2 Phosphorylations induced by CK1ε and NEK2

Based on our experiment, a phosphorylation "map" of the Dvl protein was created that described the complex phosphorylation "fingerprint" for each kinase tested. Eight of the kinases used to induce phosphorylation include CK1ε and NEK2. Fig. 1 shows a qualitative comparison of the identified phosphorylation sites using these two kinases. In the case of CK1ε induction, 77 phosphorylation sites were identified, and in the case of NEK2, 87 phosphorylation sites were determined from a total of 131 possible Ser/Thr phosphorylation sites in the Dvl3 protein.

Next in terms of qualitative characterization, a semiquantitative comparison with regard to the occupancy of phosphorylation sites induced by individual kinases was conducted. The Skyline software was used for this evaluation. The individual phosphorylated peptides were compared based on their peak areas. A comparison of a selected peptide phosphorylated in the position of S204 by CK1ε and NEK2 is shown in Fig. 2. The peak area was determined for CK1ε as 7.60e6 and for NEK2 as 1.07e9. Subsequently, double normalization of the data was performed using a set of phosphopeptide standards (added to the sample prior to the phospho-enrichment step) and by unphosphorylated peptides identified in the identification run. The resulting areas (CK1ε: 1.23e7 and NEK2: 1.10e9) were compared with each other.

4. Discussion

Our study focused on the determination of the phosphorylation sites of Dvl3 by MS induced by eight kinases. 88 Ser/Thr phosphorylations from a total of 131 sites and 1 tyrosine phosphorylation were identified which can be potentially phosphorylated.

CK1ε-induced phosphorylation was identified at 77 unique sites and 10 more phosphorylation sites were induced by NEK2. Previous studies in various experimental systems identified several phosphorylation sites spread throughout the structure of the protein Dvl3 [3, 4]. Our data clearly demonstrate that the phosphorylation of the protein Dvl3 is extensive and the number of phosphorylated sites exceeds 60.

5. Conclusion

An approach based on the SDS-PAGE separation of Dvl3 immunoprecipitates, TiO_2 phospho-enrichment followed by LC-MS/MS analysis and data processing using Skyline software was utilized for the evaluation of semiquantitative differences in the phosphorylation level of hDvl3 at particular sites within the set of eight selected kinases.

Differences were observed in terms of the phosphorylation profiles induced by individual kinases, as indicated in Fig. 1. Based on our results, a "comprehensive map" of the phosphorylations of human Dvl3 will be created.

Acknowledgement

This work was carried out with the support of the project CEITEC 2020 (LQ1601) funded by the Ministry of Education, Youth and Sports (MEYS) of the Czech Republic under the National Sustainability Programme II. The Czech Infrastructurefor Integrative Structural Biology (CIISB) research infrastructure project LM2015043 funded by MEYS is gratefully acknowledged for financially supporting our LC-MS/MS measurements at the Proteomics Core Facility. The support from the Czech Science Foundation project no. 15-21789S is also gratefully acknowledged.

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PHOSPHOGLYCOLIPID PROFILING OF BACTERIAL ENDOTOXINS

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Much interest is at present focused on bacterial endotoxins, also known as lipopolysaccharides (LPS), as they are responsible for the development of clinical symptoms of Gram-negative sepsis which is the leading cause of death in intensive care units. Endotoxicity is associated with the special phosphoglycolipid part of LPS, termed lipid A. Main challenges in the structural elucidation of lipid A arise from its amphiphilic character and inherent heterogeneity. A mass spectrometry-based *de novo* method combined with reversed-phase liquid chromatography for the detailed structural characterization of complex lipid A mixtures (obtained by mild acid hydrolysis of LPS) from different bacterial sources has been developed. Tandem mass spectrometric measurements were performed with an electrospray-ionisation quadrupole time-of-flight (ESI-Q-TOF) mass spectrometer in both negative- and positive-ionization modes in order to explore fragmentation pathways. It was found that characteristic product ions in the positive-ion mode could be used for the unambiguous assignment of the phosphorylation site, whereas the use of both ionization modes provided consistent and/or complementary information about the fatty acyl distribution between the two glucosamine moieties of lipid A. Since the immunostimulatory (advantageous) *vs.* proinflammatory (endotoxic) effect of the lipid A is closely related to the fine chemical structure, our relatively simple structural elucidation strategy could offer great potential in the bioanalysis of native lipid A samples and lipid A-based vaccines.

Keywords: lipid A, HPLC, tandem mass spectrometry, positive-ion mode, negative-ion mode

1. Introduction

Bacterial endotoxins are important initiators of clinical symptoms of Gram-negative sepsis, a serious medical condition with a high mortality rate among patients in intensive care units worldwide. Endotoxins, chemically lipopolysaccharides (LPS) or lipooligosaccharides (LOS), are found on the surface of Gram-negative bacteria. They are essentially composed of two regions: a heteropolysaccharide chain (divided into the oligosaccharide core and the O-side chain – this component is missing from LOS) that extends outwards from the bacterial cell surface, and a phosphoglycolipid, termed lipid A, which is anchored in the outer membrane (Fig. 1). Specifically, the endotoxic activity is related only to the lipidic domain of endotoxins.

With regard to the toxicity of lipid A the crucial factors are the acylation and phosphorylation status. This most biologically potent molecule, capable of inducing septic shock, consists of an asymmetrically hexa-acylated, bisphosphorylated glucosamine disaccharide [1]. Other lipid A species that deviate from this structure have lower endotoxicity or may even have beneficial effects. Consequently, the structural analysis of the lipid A moiety is of fundamental interest in terms of clinical aspects and vaccine development.

The structural characterization of lipid A poses a unique analytical challenge, as lipid A isolated from a single bacterial strain generally consists of a mixture of differently acylated and phosphorylated species. Furthermore, the relative abundances of the different structures can vary with growth conditions, e.g. temperature, culture

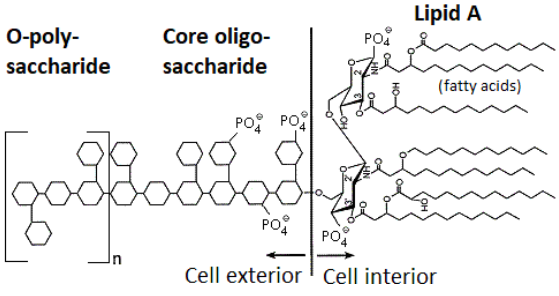

Figure 1: Schematic representation of the chemical structure of an enterobacterial outer membrane lipopolysaccharide (endotoxin).

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media and pH. Within this field, tandem mass spectrometry (MS/MS or MSn) has emerged as a core technology for the in-depth structural elucidation of lipid A species [2]. However, separation and simultaneous quantification of singular components by powerful separation methods prior to MS would be highly desirable to adequately characterize the heterogenic structures.

Here, a brief presentation of an alternative method to multiple-stage MS for the differentiation of various lipid A species – including isobars – within a bacterial sample is provided. Our method is based on a reversed-phase high-performance liquid chromatography (RP-HPLC) separation and online ESI-Q-TOF mass spectrometric detection of molecular species in native lipid A isolates. The exact structures of the lipid A molecules in the complex mixtures were deduced from specific fragmentation patterns obtained in both the negative- [3, 4] and positive-ion modes [5].

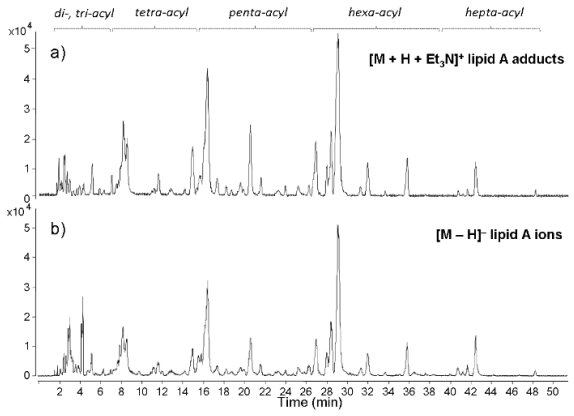

Figure 2: HPLC–MS base peak chromatograms of the native, heterogeneous *E. coli* O111 lipid A preparation acquired in the a) positive- and b) negative-ion modes.

2. Experimental

2.1 Chemicals and Reagents

Methanol, isopropyl alcohol, water (LC-MS grade), dichloromethane (HPLC, ≥99.9%), triethylamine (Et_3N) and acetic acid (AcOH) were purchased from Sigma-Aldrich (Steinheim, Germany).

2.2 Bacterial strains and lipid A isolation

The strains *Escherichia coli* O111 and *Proteus morganii* O34 were cultured in a laboratory fermenter, and then collected by centrifugation. The bacterial LPS was extracted from acetone-dried organisms by the traditional hot phenol-water procedure and lyophilized.

Lipid A was released from LPS by mild-acid hydrolysis with 1% (v/v) AcOH at 100°C for 1 h, then the solution was centrifuged (8000×g, 4°C, 20 mins). The sediment (containing lipid A) was washed four times with distilled water and lyophilized. Lipid A samples were dissolved in methanol/dichloromethane (95:5, v/v) at a final concentration of 0.5 mg/ml.

2.3 Liquid Chromatography – Mass Spectrometry Measurements

Separation and mass spectrometric detection of lipid A samples were performed with an 1290 Infinity Ultra-high performance liquid chromatography (UHPLC) system (Agilent Technologies, Waldbronn, Germany) coupled to a 6530 Accurate-Mass Q-TOF LC/MS system (Agilent Technologies, Singapore), using a Kinetex^{TM} core-shell C18 column (100 mm×4.6 mm, 2.6 µm particle size, Phenomenex). Mobile phase A was methanol/water (95:5, v/v) and mobile phase B was isopropyl alcohol, both containing 0.1% Et_3N and 0.1% AcOH (v/v). The injection volume was 3 µl. Separation was performed at a constant flow rate of 0.5 ml/min at 50°C. A linear gradient of 50 min started at 0% of mobile phase B and ramped up to 50% of mobile phase B, and was then maintained at 50% of mobile phase B for 2 mins.

Negative- and positive-ion mass spectra of the column eluate were recorded between *m/z* 100 and 3,200. The ESI ion source was operated using the following conditions: pressure of nebulizing gas (N_2), 30 psi; temperature and flow rate of drying gas (N_2), 325°C and 8 l/min, respectively; temperature and flow rate of sheath gas, 300°C and 11 l/min, respectively; capillary voltage, 4 kV; nozzle voltage, 2 kV; fragmentor potential, 100 V; and skimmer potential, 70 V. For the targeted MS/MS analysis, the following parameters were used: mass range, *m/z* 70–2000; acquisition rate, 333.3 ms/scan; quadrupole isolation width, 1.3 *m/z*; collision energy, 70–100 V; maximum number of precursor ions/cycle, 4; and active charge state of the precursor ion, 1.

3. Results and Analysis

3.1 HPLC-MS of bacterial lipid A

Crude lipid A preparations from *E. coli* O111 and *P. morganii* O34 bacteria were used as training sets for the development of the method. The HPLC separation conditions were optimized in order to find a balance between solubility, adequate separation and ionization of the intact lipid A's. Fig. 2 shows the HPLC–MS analysis in the positive- and negative-ion modes for the *E. coli* O111 lipid A isolate, only. It was found that the lipid A samples expressed extreme structural heterogeneity, showing the presence of numerous bis-, mono- and non-phosphorylated structures and diverse acylation states ranging from di- to hepta-acylation. The most abundant chromatographic peak from both bacteria was a hexa-acylated, monophosphorylated species. The elution order of the separated compounds was consistent with their relative hydrophobicity, defined mainly by the total num-

ber and length of hydrophobic acyl chains, followed by the number of polar phosphate groups. Several isomers of identical mass could be identified in which the length and/or position of the acyl chains varied.

Since the phosphoglycolipids are acidic in nature, they could readily be ionized as deprotonated ions, i.e. $[M - H]^-$ in the negative-ion mode, whereas their ionization in the positive-ion mode was enhanced by adduct formation with triethylamine used as an eluent additive, i.e. $[M + H + Et_3N]^+$ adducts were formed.

3.2 *Tandem mass spectrometric analysis of lipid A species*

In order to investigate the fragmentation behavior under low-energy collision-induced dissociation (CID) conditions of each separated lipid A precursor ion, ESI-MS/MS experiments were performed in both ionization modes. Based on previously published data on the acyl distribution of the C4'-monophosphorylated, hexa-acylated lipid A molecule from *E. coli* O111 [6], ions observed in the tandem mass spectra (not shown) of this lipid A species (appearing at t_R = 29.2 mins in Fig. 2) could be structurally assigned in both ionization modes [3,5]. Using this as a standard, the CID mass spectral profiles of other monophosphorylated lipid A species separated by chromatography were compared, and the fragmentation preferences for each of them, in both ionization modes, were explored. Fig. 3 presents an example of the correlation between the phosphoglycolipid structure and the fragmentation profiles in the case of the main hepta-acylated lipid A compound (t_R = 42.5 mins in Fig. 2) from *E. coli* O111. The site-specific cleavages are assigned with a number denoting the carbon of the glucosamine to which the substitution is attached, and a Greek letter designating the position of the bond which was cleaved.

In the tandem mass spectrum of the $[M + H + Et_3N]$+ triethylammonium adduct of this lipid A species (Fig. 3a), only a few ions resulting from the neutral loss of acyl chains are present, however, in that of the deprotonated form (Fig. 3b), the abundances of product ions in the higher *m/z* region straightforwardly indicated the consecutive dissociation order of the ester-linked fatty acids. Thus, their position could be assigned. Each tandem mass spectrum also exhibited diagnostic intra-ring (A-type ions in the negative-ion mode) or inter-ring (B- and Y-type ions in the positive-ion mode) cleavage products, which confirmed the substitution position of the fatty acids on the two glucosamine residues.

Fig. 4 shows the tandem mass spectra of the C1 phosphorylation isomer of this hepta-acylated lipid A species. The location of the single phosphoryl group (i.e. at C1 or C4') could be directly assessed from the positive-ion mode tandem mass spectra by calculating the mass difference between the *m/z* of the precursor ion and that of the product ion resulting from the cleavage of the 1α bond

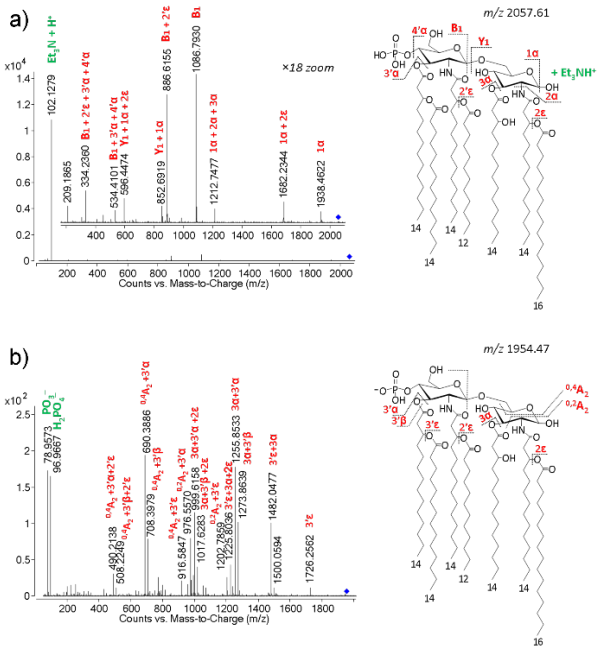

Figure 3: Tandem mass spectra in a) positive- and b) negative-ion modes and a depiction of the identified structure of the C4'-monophosphorylated hepta-acylated lipid A from the *E. coli* O111 bacterium. Site-specific cleavages are indicated next to the ion signals.

(compare Figs. 3a and 4a). Namely, the mass difference of 199 u corresponded to the combined loss of triethylamine and an orthophosphoric acid molecule, indicating that a phosphoric acid molecule had esterified the C1 hydroxyl group of the reducing end. On the other hand, a mass difference of 119 u resulted from the combined loss of triethylamine and a water molecule, indicating the presence of a free hydroxyl group at C1 (consequently, the phosphoryl group must be located at C4').

The change in the phosphorylation site also resulted in a distinctive tandem mass spectrum in the negative- ion mode (Fig. 4b). The main differences were the complete lack of A-type cross-ring fragments, and instead the appearance of Z-type inter-ring cleavage products. Furthermore, the intensities of the product ions resulting from the neutral loss of acyl chains were of about the same magnitude, indicating strong competition between the fatty acyl eliminations during the CID process, instead of their stepwise dissociation (compare with Fig. 3b).

The fragmentation behavior of non- and bis-phosphorylated lipid A species under both negative and positive CID conditions was investigated, as well. For these, a different sequencing of the acyl-chain cleavages could be observed in the negative-ion mode [3, 4], whereas similar fragmentation pathways to that of the monophosphorylated species in the positive-ion mode were identified [5]. Besides the A-type ions (nonreducing end), complementary X-type fragment ions (reducing end) appeared in the negative-ion mode tandem mass

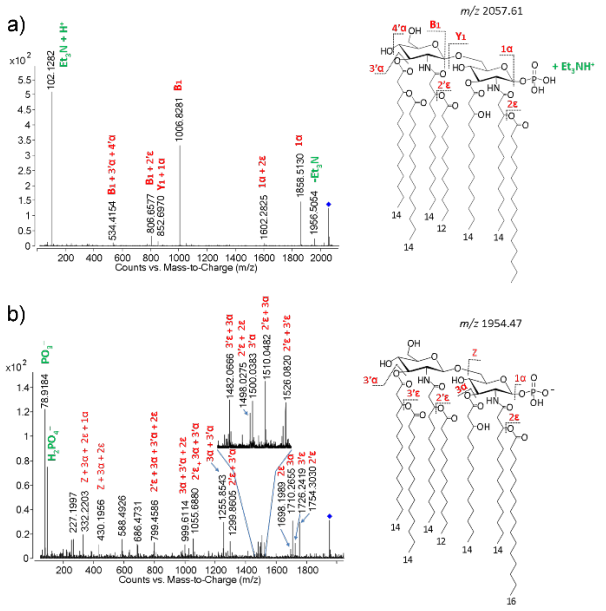

Figure 4: Tandem mass spectra in the a) positive- and b) negative-ion modes and depiction of the identified structure of the C1-monophosphorylated hepta-acylated lipid A from *E. coli* O111.

spectra of the nonphosphorylated lipid A species [4].

4. Discussion

The online HPLC–MS/MS methodology enabled the simultaneous structural characterization of both phosphorylated and nonphosphorylated lipid A variants within a single run. The main advantage of using the collision cell of a Q-TOF mass spectrometer was that several generations of precursor/product ions – that would otherwise be generated only at higher MS stages with ion-trap experiments – were observed simultaneously in the tandem mass spectra of the separated lipid A species. This facilitated new fragmentation rules to be set for the variety of phosphorylated and acylated lipid A compounds by a simple MS/MS experiment [3–5]. Specifically, the acylation profile of the non-, C4'-mono and bisphosphorylated lipid A species could be inferred partly from the positive- and fully from the negative-ion mode MS/MS analyses [3–5], whereas the complementary use of both ionization modes [3, 5] was needed for the full structural characterization of C1-monophosphorylated lipid A.

As an example, the complete structural elucidation strategy by MS/MS of a hepta-acylated C1'-monophosphorylated lipid A is as follows. First, observation of the 1α cleavage product in the positive-ion mode is essential to clarify the site of phosphorylation (*vide supra*). Next, the linkages at the $2'\varepsilon$ (only from the B-type ion) and 2ε positions can be determined from the positive-ion mode analysis. By knowing the fragmentation preference of the secondary ester bonds (which is $2'\varepsilon > 3'\varepsilon > 2\varepsilon$ [5]), substitutions at these sites can be deconvoluted from the negative-ion mode analysis by observing the $2'\varepsilon$, $3'\varepsilon$, 2ε, and 3α cleavage products in the upper *m/z* region and those resulting from the $3'\alpha$, $2'\varepsilon + 3'\varepsilon$, $2'\varepsilon + 2\varepsilon$, $2'\varepsilon + 3\alpha$, and $3'\varepsilon + 3\alpha$ cleavages displayed in the middle *m/z* region. Here, the dephosphorylated Z-type ion in the lower *m/z* region indirectly specifies the linkage at the 2α position. Finally, the remaining $2'\alpha$ substitution can be deduced from the intact B-type ion (positive-ion mode).

5. Conclusion

An efficient HPLC separation before the MS detection of natural lipid A mixtures has proven particularly useful in terms of revealing the diversity and relative amount of the various lipid A structures present in bacteria. Such investigations could help to explore the relative contributions of different lipid A structures to the overall activation of the innate immune system. Moreover, knowledge of the chemical and biological aspects of lipid A heterogeneity is crucial in order to design antimicrobial drugs which overcome the evasion strategy of the pathogen. Our technique could be well suited for phosphoglycolipid profiling from different bacterial strains or vaccine preparations.

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CAPILLARY ISOTACHOPHORESIS DETERMINATION OF TRACE OXIDIZED GLUTATHIONE IN BLOOD

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A capillary isotachophoresis (CITP) method performed in a column-coupling apparatus has been developed for the simultaneous determination of glutathione (GSH) and glutathione disulfide (GSSG) concentrations in blood samples. The determination of GSSG and GSH concentrations in biological samples is important because of their roles in oxidative stress. Different concentrations of a leading ion in the coupled columns (concentration cascade) and a large volume (37 μl) of the injected sample facilitated a GSSG concentration of between 2 and 25 μmol/l. A reaction between iodoacetate and GSH under alkaline conditions was used to prepare the sample in order to avoid oxidation of GSH to GSSG. This step eliminated the main source of systematic errors with regard to the determination of the GSSG concentration. A linear relationship (R^2=0.9969) between the zone length of S-(carboxymethyl)glutathione (the product of the reaction between GSH and iodoacetate) and the concentration of GSH (40-120 μmol/l) was obtained. The method was applied to the analysis of bovine blood samples that had been diluted by a factor of ten with satisfactory results.

Keywords: glutathione, glutathione disulfide, isotachophoresis, bovine blood

1. Introduction

Glutathione (GSH), a thiol containing tripeptide, plays an important role in the antioxidant system of eukaryotic cells [1]. Upon oxidation, GSH is transformed into glutathione disulfide (GSSG) [1]. The concentrations of GSH and GSSG and their molar ratio are indicators of cell functionality as well as oxidative stress [2]. Different aspects of the determination of GSH and GSSG concentrations in biological samples, including sample pretreatments, were recently reviewed [3–5]. The main problems are related to the non-enzymatic oxidation of GSH when the pH exceeds 7, enzymatic conversion of GSH, a need for the removal of proteins prior to the analysis, blocking of free thiol groups, reduction of disulfides, and derivatization of thiol groups [3,6]. Among a wide variety of analytical methods, capillary electromigration (CE) methods, mainly CE coupled with different detectors (UV absorbance [7–10], fluorescence [11] and laser-induced fluorescence (LIF) [12, 13], electrochemical [14, 15] and mass spectrometry [16], have been used to determine the concentration of glutathione [5, 17].

The aim of this work was to develop a capillary isotachophoresis (CITP) method that involves minimal sample pretreatment for the simultaneous determination of GSSG and GSH concentrations in bovine blood samples. Determination of trace analytes in regular CITP, when the quantitation is based on the measurement of the zone length, is not very common. In this work, two key problems were solved: (1) lowering the quantitation limit of the CITP method, and (2) stabilization of GSH without protein precipitation to ensure the accurate determination of the GSSG concentration.

2. Experimental

An EA 202A electrophoretic analyzer (Villa Labeco, Spišská Nová Ves, Slovakia) was used for CITP separations. This fully automated CE system was equipped with two columns. In the first column, a fluoroplastic capillary tube with an inside diameter (ID) of 800 μm and contactless conductivity detection were used. In the second column, a fused silica capillary with an ID of 300 μm, contactless conductivity and UV photometric detectors were used.

The CITP separations were performed using the electrolyte systems shown in Table 1 at a constant driving current, and in the anionic mode (electrode E3 in Fig. 1 used as a cathode). At the beginning and end of the day, the separation and electrolyte units as well as the sample loop of the autosampler were rinsed with deionized water

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Table 1: Electrolyte systems.

Electrolyte	Parameter	ES1	ES2
LE1	Leading ion	Chloride	
	Concentration [mmol/l]	10	10
	Counter ion	6-Aminocaproate	
	Concentration [mmol/l]	12	12
	EOF supressor	Methylhydroxy-ethylcellulose	
	Concentration [% w/V]	0.1	0.1
	pH	3.4	3.4
LE2	Leading ion	Chloride	
	Concentration [mmol/l]	5	2
	Counter ion	6-Aminocaproate	
	Concentration [mmol/l]	6	2.4
	EOF supressor	Methylhydroxy-ethylcellulose	
	Concentration [% w/V]	0.1	0.1
	pH	3.8	3.9
TE	Terminating ion	Caproate	
	Concentration [mmol/l]	20	20
	Counter ion	6-Aminocaproate	
	Concentration [mmol/l]	15	15
	EOF supressor	Methylhydroxy-ethylcellulose	
	Concentration [% w/V]	0.1	0.1
	pH	4.7	4.7

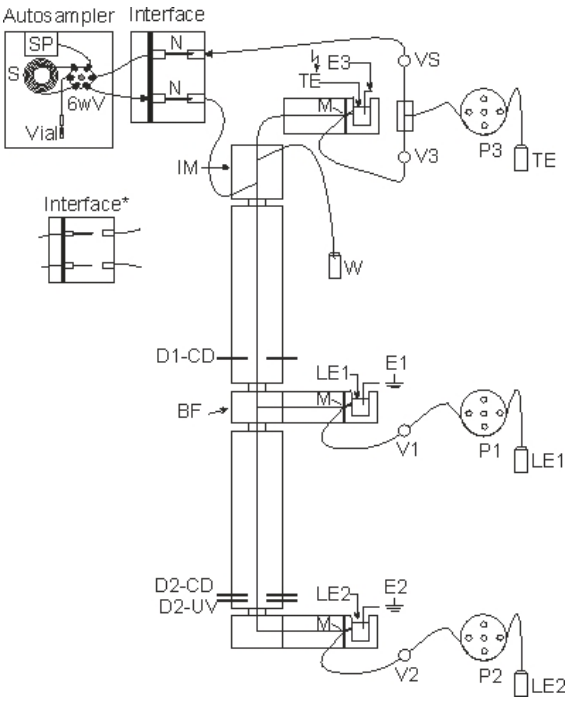

Figure 1: A scheme of the electrophoresis system. Autosampler: S – sample loop, SP – syringe pump, 6wV – 6-way valve. Interface: during the injection the septums are pierced by the needles (N). Interface*: during the separation the autosampler is disconnected from the analyzer. Separation and electrolyte unit: V1-V4 – pinch valves, E1-E3 – driving electrodes, P1-P3 – peristaltic pumps, IM – injection module, W – waste, D1-CD, D2-CD, D2-UV – contactless conductivity and absorbance detection cells, M – membrane, BF – bifurcation, TE, LE1, LE2 – terminating and leading electrolytes.

using built-in peristaltic pumps. Between analyses a relatively short rinsing procedure (ca. 1 min) with electrolyte solutions was used.

2.1 Samples

The bovine blood samples were collected using PVC taking set with an integrated needle HEMOS (Gama Group, České Budějovice, Czech Republic), and immediately transferred to the test tube containing K3-EDTA. The samples after dilution, required for hemolysis, were filtered prior to the analysis using a syringe filter with a glass fiber membrane and a pore size of 1 μm. During the dilution step a thiol-masking agent and NaOH were added to each sample.

3. Results and Discussion

3.1 Separation conditions

The combination of two columns with different IDs and the employment of a column-switching technique is beneficial for the CITP determination of analytes present in the multicomponent sample at low concentrations and/or at different concentration levels. The first (wider) capillary allowed the separation of sample constituents injected at a relatively high volume (37 μl). Typical macroconstituents, e.g. chloride and ethylenediaminetetraacetic acid (EDTA), migrated out of the separation path through a bifurcation block, and as a result they were removed from the separation compartment. During this stage of the separation the driving current flowed between electrodes E1 and E3 (Fig. 1).

The very small isotachophoresis (ITP) zones of analytes created in the first column were insufficient for their concentrations to be determined. As a result of switching the direction of the driving current through both columns (by connecting electrodes E2 and E3, Fig. 1), the separated constituents were transferred to the second column. The signal from D1-CD was used to determine an appropriate time to switch the current (Fig. 1).

In the second (narrower) capillary the ITP zones of analytes were prolonged. In addition, due to the low GSSG concentration in the samples of blood (not in excess of tens of μmol/l), their ITP zone length was further extended by a reduction in the concentration of leading ions (ES2, Table 1) in the second column (Fig. 2). A higher degree of sensitivity in ES2 is also evident from the parameters of regression equations for the analytes (Table 2).

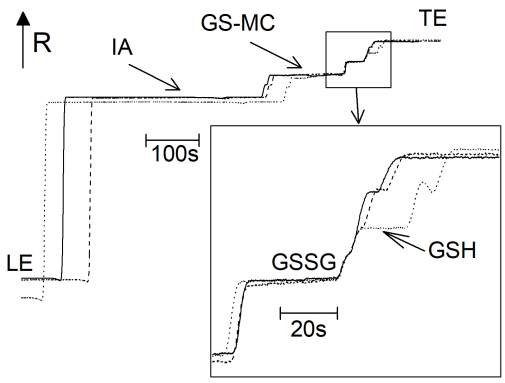

Figure 2: Isotachopherograms from the separations of GSSG and GSH performed in the electrolyte systems ES1 (a) and ES2 (b). The isotachopherograms were recorded by D2-CD (Fig. 1). The concentrations of GSSG and GSH in the injected sample were both 25 µmol/l.

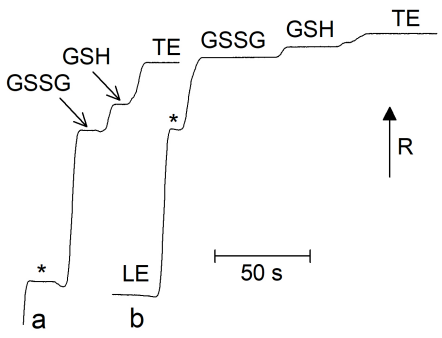

Figure 3: Isotachopherograms from the separation of the reaction mixture present in the electrolyte system ES2. The isotachopherograms were recorded by D2–CD. The mixture contained 15 µmol/l of GSSG, 80 µmol/l of GSH, 2 mmol/l of IA, 4 mmol/l of NaOH, and 10 % of TE. The sample was injected 5 mins (dot), 15 mins (dash) and 20 mins (solid) after the reagents were mixed.

3.2 *Stabilization of glutathione*

The stability of GSH and its oxidation to GSSG during the period between the collection of the sample and its analysis is the main source of systematic errors. In the sample of bovine blood a much higher concentration of GSSG and a lower concentration of GSH than expected was measured.

To avoid this problem, a thiol-masking agent was used, namely iodoacetic acid (IA) [18]. The substitution reaction between IA and GSH formed S-(carboxymethyl)glutathione (GS-MC).

Under the ITP separation conditions used, GS-MC migrated in front of GSSG (Fig. 3). The optimum conditions for the reaction between IA and GSH were determined by the ITP separations of reaction mixtures at different periods after the reagents were mixed. These experiments were conducted with both model and real samples. Under neutral and slightly alkaline conditions the reaction was very slow. An excess of IA (2 mmol/l) and the presence of NaOH (4 mmol/l) in the reaction mixture led to the fast (less than 20 mins.) and quantitative conversion of GSH to GS-MC (Figs. 3 and 4) without an increase in the concentration of GSSG.

For the purpose of a quantitative analysis, IA and NaOH was added to the sample of bovine blood immediately after its collection (during the sample dilution step required for hemolysis). Blood samples that had been diluted by a factor of ten were directly analyzed after their filtration through a syringe filter with a pore size of 1 µm. The concentrations of GSSG and GSH that were measured in the diluted blood samples were 4.4 µmol/l and 63.4 µmol/l, respectively. The average concentrations

Table 2: Parameters of regression equations.

Analyte	Range [µmol/l]	a [s l/µmol]	b [s]	R^2
GSSG[1]	5-50	0.67	1.45	0.999 7
GSSG[2]	2-25	1.66	2.01	0.9992
GSH[1]	10-50	0.48	0.25	0.9999
GSH[2]	10-50	1.24	0.60	0.9994
GSH (GS-MC)[2]	40-120	1.98	-6.60	0.9969

[1] Electrolyte system ES1 and [2] ES2 used for data evaluation. Regression equation: $Y = aX + b$.

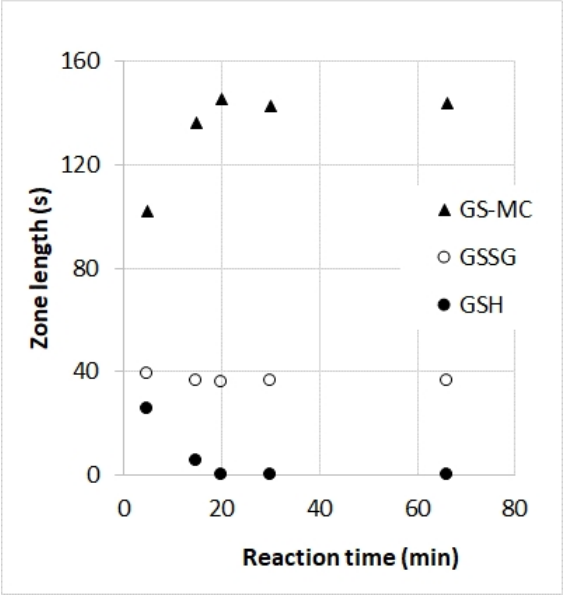

Figure 4: Dependence of the ITP zone length on the reaction time of the mixture containing 15 µmol/l GSSG, 80 µmol/l GSH, 2 mmol/l IA, 4 mmol/l NaOH and 10 % TE.

calculated from eight repetitive measurements of identical samples were in good agreement with those determined by enzymatic methods. The degrees of precision of the method, expressed by the relative standard deviation (RSD) values of the measured concentrations of GSSG and GSH, were 10.3% and 4.4%, respectively.

4. Conclusion

The sensitive and simultaneous determination of GSH and GSSG concentrations in entire samples of bovine blood is facilitated by the capillary isotachophoretic method developed. The simple and rapid preparation of blood samples, that only involves the masking of thiol group of GSH and the dilution and filtration of the sample, increases the accuracy of the GSSG concentration measured. No adverse effects caused by the proteins present in the real blood samples on the separation efficiency or detector response was observed. It can be assumed that this method is also suitable for the analysis of blood samples from other mammals.

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COLD PLASMA: THE WAY TO IMPROVE THE REPEATABILITY OF SALD ICP-MS ANALYSIS

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This work deals with the preparation of model biological microsamples for Substrate-Assisted Laser Desorption Inductively Coupled Plasma Mass Spectrometry (SALD ICP-MS). This technique provides the direct and fast analysis of liquid samples deposited onto polyethylene terephthalate (PET) plates (substrates) in the form of dried droplets with minimal sample preparation and submicrolitre sample volume requirements. Furthermore, SALD ICP-MS allows for the direct analysis of samples in organic solvents, which cannot be directly nebulised to ICP-MS. These benefits are, however, balanced out by the low degree of repeatability of the assay, which is typically about 30%. One of the approaches to increase the repeatability is a modification of the substrate surface by Diffuse Coplanar Surface Barrier Discharge (DCSBD) with the addition of an internal standard to the sample. Using this approach, a relative standard deviation (RSD) of less than 10% for model biological samples can be achieved. The only limitation of this technique is the occurrence of the so-called "aging effect", i.e. the gradual return of the modified physicochemical surface properties to their original state.

Keywords: SALD ICP-MS, Plasma Treatment, DCSBD, Microsample, Internal Standard

1. Introduction

The determination of elements in biological samples is often complicated by complex matrices, the presence of organic solvents, and trace amounts of the elements of interest. For this reason, it is necessary to select a technique that bridges the obstacles mentioned above. Such a technique is Substrate-Assisted Laser Desorption Inductively Coupled Plasma Mass Spectrometry (SALD ICP-MS), which is capable of analysing liquid samples of submicrolitre volumes in the form of dried droplets (stains) on a polyethylene terephthalate (PET) substrate [1–3]. The greatest difficulty of this technique is the low repeatability of the assay. This is caused mainly by the poorly reproducible deposition of droplets that consist of submicrolitre volumes and the inhomogeneity of the stains produced in terms of the area and thickness of the layer of solid residue in the stain.

The assay repeatability can be increased by increasing the surface energy of the substrate and using internal standards that can correct the mentioned effects.

One way to increase the surface energy, i.e. wettability of the substrate, is to expose it briefly to cold plasma, specifically Diffuse Coplanar Surface Barrier Discharge (DCSBD) [4,5].

The plasma system of the so-called DCSBD is composed of regularly alternating silver electrodes to which high alternate voltages are applied. The electrodes are located in a dielectric structure consisting of dielectric oil and thin ceramic plates. The most common arrangement of electrodes in this device is in the form of two intermeshing combs. Surface discharges are then generated on the surface of the ceramic plate, serving as a dielectric barrier. This electrode arrangement (plasma in contact with only inert and highly resistant ceramics) can consist of a theoretically infinite amount of 20×8 cm DCSBD units and thus, it is theoretically possible to achieve an infinite plasma area.

The principle of substrate surface treatment is based on the excitement and ionization of the species by a high voltage discharge and subsequent introduction of the resulting species onto the substrate surface in the form of radicals, ions and excited molecules.

A disadvantage of this technique over other physicochemical modifications is the gradual decrease in surface tension compared to its original value prior to the modification, or the so-called "aging effect" [6]; this period is in the order of several months. Factors influencing the qual-

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Table 1: Ablation parameter settings monitored in addition to the other isotopes of interest.

Isotope integration time	0.1 s
Flow rate of the He carrier gas	1.0 l·min^{-1}
Flow rate of the Ar auxiliary gas	0.6 l·min^{-1}
Flow rate of the Ar plasma gas	15 l·min^{-1}
Laser pulse repetition rate	10 Hz
Laser-beam waist	250 µm
Laser fluence	$\sim$ 0.75 J·cm^{-2}

ity of the modification are the voltage and frequency of the electromagnetic field, the composition of the gaseous atmosphere, the exposure time and the geometries of the sample and electrodes.

2. Experimental

In the first experiment, a DCSBD device (RPS400-Roplass plasma system 400 W, Roplass, Czech Republic) was used to increase the surface energy of the PET substrate (Bayer MaterialScience, Germany). The substrate surface was modified at 300 W for one, five and ten seconds. Subsequently, 1µl of 0.5 mM trisodium citrate (Sigma-Aldrich, USA) solution was deposited onto ten replicates of the PET substrate as a model biological sample. The contact angles were measured by the instrument (see System E, Advex Instruments, Czech Republic) using a technique called "sitting droplet on a solid foundation". The device consists of a charge-coupled device (CCD) camera placed on a sliding table in front of which a substrate plate is positioned. Using a micropipette, a 1µl droplet of the sample is deposited onto the substrate and the See System software calculates the contact angle based on the interpolation of the height and width of the arc that describes the shape of the drop. The size of the contact angle was evaluated immediately after modification of the substrate surface as well as after one, seven and 31 days.

Based on the previous experiment, where the aging of the modified surface was monitored by varying the contact angle and size of the stain, depending on the dropping time that elapsed from the surface modification, a complementary experiment was performed. 1mL of trisodium citrate sample was mixed with cadmium and indium standards (aqueous calibration solutions, certified reference materials (CRM), Analytika, Czech Republic) so that the resulting solution contained 400 ng·l^{-1} of these standards. Five replicates of the 200 nl droplet sample solutions prepared were immediately applied using a micropipette to the surface of the modified PET substrate as well as one, three and six days after the modification.

The Cd and In standards, chosen because of their similar atomic masses and first ionization energies, were used

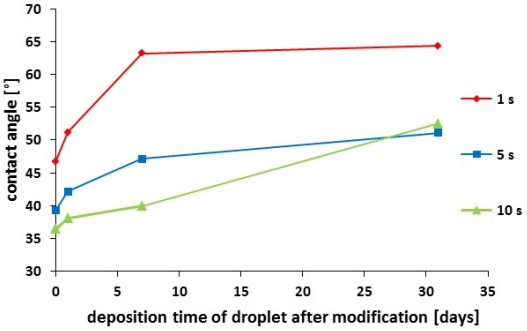

Figure 1: Dependence of the contact angle of droplets of 0.5 mM trisodium citrate solution on the PET surface modified by DCSBD after one, five and ten seconds; droplets were deposited immediately as well as one, seven and 31 days after the modification.

to correct the intensity of the monitored signals. The purpose of this experiment was to demonstrate whether aging of the substrate affects the intensities of the signals. At the same time, the size of the stains was measured. Ideally, the application conditions should be identical to the experiment where aging was observed by measuring contact angles. However, this was not possible due to different sample-volume requirements for the given measuring technique. 1 µl was required to measure contact angles and 0.2 µl for SALD ICP-MS.

The plate with samples was then inserted into the ablation cell (UP213 model, New Wave Research, Inc., USA) equipped with a 213-nm pulsed Nd:YAG laser, 3D positioning system and built-in CCD camera for visual control of the ablated samples. The dry aerosol created during ablation was analysed by the ICP mass spectrometer (Agilent 7500ce ICP-MS, Agilent, USA). In order to determine the beginning and end of the ablation process, the 13C isotope was isotope was monitored in addition to the other isotopes of interest.

For the ablation of the stains, a "zig-zag" ablation trajectory was selected with a 170 µm ablation line and a 160 µm·s^{-1} scanning speed. The remaining basic ablation parameters are shown in Table 1.

3. Results and discussion

First, the contact angle formed by 1 µl droplets of 0.5 mM trisodium citrate onto a PET substrate surface was evaluated immediately after modification of the substrate surface as well as after one, seven and 31 days. Ten replicates of the substrate surface were modified for one, five and ten seconds.

Fig. 1 shows that the droplets of 0.5 mM trisodium citrate solution that were applied to the substrate surface of PET modified by DCSBD for one second exhibited the greatest contact angle, hence the least wettability; i.e., a longer duration of DCSBD modification causes a higher degree of wettability. It is also evident from Fig. 1, how-

Table 2: Integrated SALD ICP-MS signals of ^{111}Cd and ^{115}In from stains of 0.5 mM trisodium citrate deposited at different times after PET surface modification.

Deposition time [days]		^{111}Cd	^{115}In	^{115}In / ^{111}Cd	Stain size [mm]
0	$\overline{X}$ [CPS]*	0.44	11	25	1.2±0.1
	RSD [%]	14	10	7.5	
1	$\overline{X}$ [CPS]*	1.4	31	22	1.6±0.2
	RSD [%]	34	38	7.2	
3	$\overline{X}$ [CPS]*	2.2	48	23	1.4±0.2
	RSD [%]	32	29	12	
6	$\overline{X}$ [CPS]*	2.8	58	22	1.2±0.3
	RSD [%]	34	24	19	

*CPS·10^6

ever, that the surfaces with the longest DCSBD modification times exhibited the fastest tendency to return to their original states in the long term.

In the following experiments, the aging effect of the substrate surface modified by DCSBD on the intensity of SALD ICP-MS signals was monitored. Five replicates of 200 nl droplets containing 0.5 mM trisodium citrate solution and 400 ng·l^{-1} of cadmium and indium standards were immediately deposited onto the modified PET surface as well as one, three and six days after modification.

From Table 2, it is apparent that the stain sizes, which should be theoretically dependent on the wettability rate and the aging effect of the modified substrate surface, do not affect the repeatability of the assay for the given samples. Furthermore, it is evident that the immediate average integrated intensity of the ^{111}Cd and ^{115}In signals from the stain ablation of the 0.5 mM trisodium citrate sample as well as one, three and six days after modification of the PET surface differed. This finding is contrary to the assumption that these values should be identical, because in all cases the same amount of sample solution was deposited and the spots were practically 100% ablated by visual inspection. The repeatability of the ablation was low and relative standard deviation (RSD) values exceeded 30%. Such high values of RSDs could be attributed, in particular, to the character of the sample itself, which was crystalline after drying, and the analytes as they were inhomogeneously distributed therein. However, when quantifying the signal intensity ratio of ^{115}In to ^{111}Cd, this inhomogeneity could be corrected, as a result the RSD values varied by around 7.5% for samples deposited immediately and one day after surface modification of the PET.

The samples on the treated surfaces exhibited the highest wettability and, therefore, it was possible to assume that the analytes were homogeneously dispersed in the stain. The ablation records in Figs. 2A and 2B also show a higher degree of homogeneity of the stains. Samples applied three and six days after modification exhibited higher corrected values of RSDs: 12 % and 19 %, respectively, mainly due to a higher level of inhomogeneity (Figs. 2C and 2D). Average ^{115}In/^{111}Cd integrated signal intensity values for the spots applied one, three and six days after surface modification could be used for quantification of the monitored analytes as the values almost coincided. However, the average value of the integrated signal from droplets applied to the substrate immediately after the modification differed. This could be caused by extreme changes in the physical properties of the substrate surface immediately after modification.

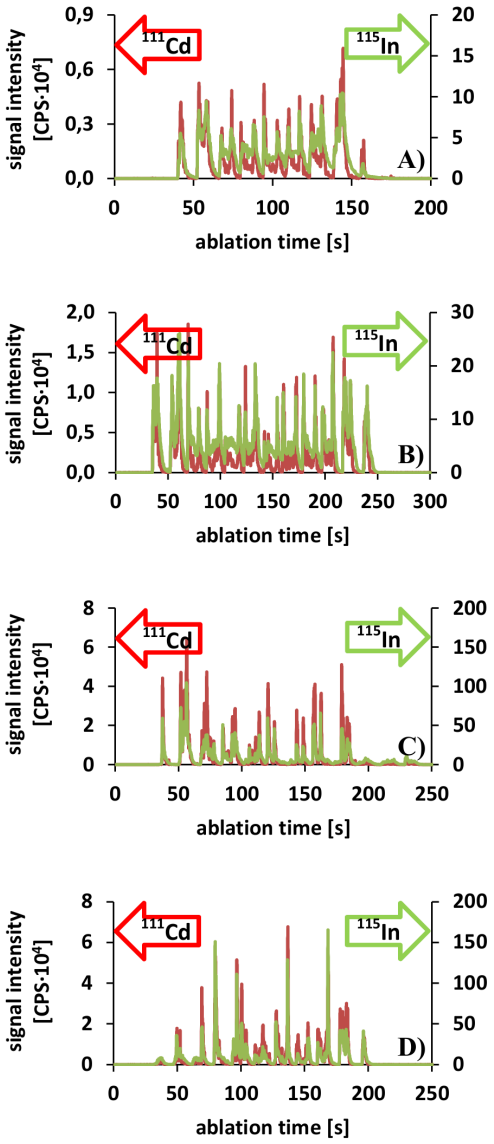

Figure 2: Ablation record of stains of 0.5 mM trisodium citrate deposited A) immediately, as well as B) 1, C) 3 and D) 6 days after PET substrate surface modification.

4. Conclusion

DCSBD technology was chosen for surface modification, i.e. to increase the energy and wettability of the sub-

strate surface. However, the disadvantage of this technique is the aging of the treated surface, i.e. the return of the modified physicochemical properties of the substrate surface to their original state prior to the modification. The experiments showed that the integrated signal intensities of the selected analytes, ^{111}Cd and ^{115}In, present in the desorbed spots of 0.5 mM trisodium citrate solution, that were deposited onto the substrate surface at various time intervals following its modification differed. Also, the repeatability of the analysis of individual samples expressed as RSDs changes with deposition time. RSD values were approximately 30% for 0.5 mM trisodium citrate samples. When quantifying the ratio of integrated ^{115}In/^{111}Cd signals, it was found that the values did not differ significantly from each other and could be used in quantitative analysis. Using internal standards, RSD values were reduced to less than 10% for trisodium citrate samples.

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CAPILLARY ELECTROPHORETIC ANALYSIS OF EXHALED BREATH CONDENSATE IN THE DIAGNOSIS OF GASTROESOPHAGEAL REFLUX DISEASE

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In this work, capillary electrophoresis with contactless conductometric detection (CCD) was used for the analysis of the ionic content of exhaled breath condensate (EBC) to differentiate between healthy individuals and patients with gastroesophageal reflux disease (GERD). The exhaled breath condensate was collected using a miniature sample collection device and the content analyzed using a separation electrolyte composed of 20 mM 2-(N-morpholino)ethanesulfonic acid, 20 mM L-histidine, 2 mM 18-Crown-6 and 30 μM cetyltrimethylammonium bromide. The separation of anions took less than 2.5 minutes, while the cations were separated in less than 1.5 minutes. The most significantly elevated ions in the group of patients suffering from gastroesophageal reflux disease were chloride, nitrate, propionate and butyrate. Although the number of subjects was too small to draw definite conclusions with regard to the discriminatory power of these ions, the pilot data are promising for EBC as a useful non-invasive alternative for other methods used in the diagnosis of gastroesophageal reflux disease.

Keywords: capillary electrophoresis, exhaled breath condensate, ionic analysis, diagnosis, gastroesophageal reflux disease

1. Introduction

Capillary electrophoresis is an attractive separation technique that is particularly suitable for the analysis of biological samples. Non-invasive sampling and the analysis of alternative biofluids are gaining scientific attention as these samples are acquired easily, can be obtained repeatedly without any particular stress to the patient, and often are formed of a simple matrix. One such sample is exhaled breath condensate (EBC).

EBC is obtained by cooling exhaled breath using suitable pieces of cooling equipment and apparatus. EBC is the aqueous part of the exhaled breath that mainly contains condensed water from breath and volatile compounds that are soluble in water, in addition to small respiratory droplets that provide information concerning the condition, inflammation and oxidative/nitrosative stress of the lungs. EBC was first used as a diagnostic sample by Sidorenko in the 1980s [1] and since then numerous articles on EBC have been published, including some recent reviews [2, 3]. EBC is attractive not only in terms of studying the condition of the lungs [4, 5], but possibly in the identification of other applications, for instance, in the assessment of inflammatory conditions of the trachea and esophagus. The latter is of particular importance because gastroesophageal reflux disease (GERD) is one of the most common diseases in the western world. Between 20 and 30% of the general population in Europe and USA will suffer from GERD [6] and about 60% of the adult population will experience some type of GERD during their lifespan. GERD is often diagnosed using invasive instruments such as 24-hour multichannel intraluminal impedance and pH monitoring (MII-pH) [7], during which the patient is required to continuously wear a narrow catheter in the esophagus. This device measures the spread and volume of gases, liquids as well as solids through the esophagus and evaluates the composition of refluxate. The data are eventually used to evaluate whether the person is suffering from the disease based on the number of recorded reflux episodes and the DeMeester score [8] which describes the severity of reflux.

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The use of MII-pH is, however, rather unpleasant and up to one third of subjects report discomfort. In this paper, the replacement of the above-mentioned MII-pH method with a significantly less invasive technique for analyzing EBC was attempted. EBC was collected using a specially designed miniature sampler [4, 9, 10] that was previously developed in our laboratory. EBC samples were collected from several healthy volunteers and a group of patients suffering from symptoms attributed to gastroesophageal reflux disease. Capillary electrophoresis was used to analyze the ionic content of EBC samples and differences in selected ion concentrations were identified between the groups.

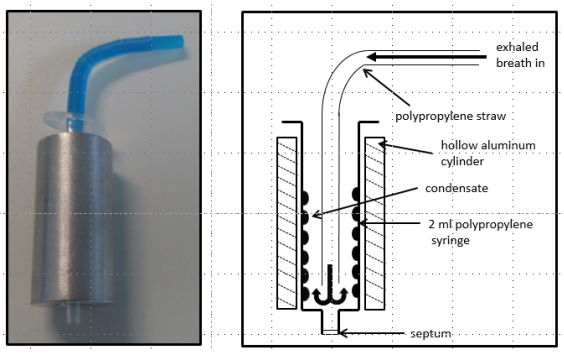

Figure 1: The photograph and scheme of the EBC sampler used in this work.

2. Experimental

2.1 Instrumentation

An in-house-built CE instrument was used for all electrophoretic separations at ± 15 kV using a high voltage power supply unit (Spellman CZE2000R Start Spellman, Pulborough, UK). Two Pt wires (with an outer diameter (OD) of 0.5 mm, 3 cm in length, Advent Research Materials Ltd., Eynsham, UK) were inserted into the electrolyte vials to serve as electrodes. Fused-silica (FS) capillaries (with an inner diameter (ID) of 50 μm, OD of 375 μm, 39 cm in total length, Polymicro Technologies, Phoenix, AZ, USA) were used for the separation. All CE experiments were performed at ambient temperature. A custom-made contactless conductometric detector (CCD) (Version 5.06, ADMET s.r.o., Prague, Czech Republic) operating at a frequency of 1.8432 MHz and a voltage of 50 V_{p-p} was used for the detection of the separated analytes. Data were collected using an Orca 2800 24-bit A/D converter and ECOMAC software ver. 0.254 (ECOM spol s.r.o., Prague, Czech Republic). In all experiments, hydrodynamic injection was applied to the samples, consisting of the elevation of the sample vial to a height of 10 cm for 20 s.

The sampler for the collection of EBC has been developed in our group previously [9]. Briefly, it was constructed from a 2 ml syringe (B. Braun Melsungen AG, Melsungen, Germany) cooled by a 5 cm-long hollow aluminum cylinder with an OD of 2.5 cm and an ID identical to the OD of the syringe (see Fig. 1).

The cylinder was kept in dry ice at -80 °C prior to the collection of EBC and an insulation sleeve was used during its collection. A straw with an OD of 6 mm, and a wall thickness of 0.2 mm (purchased in a local store) was used to exhale the air through the sampler. The end of the syringe was enclosed with a parafilm septum to avoid loss of EBC.

2.2 Chemicals

All chemicals were of reagent grade (Sigma-Aldrich, Steinheim, Germany) and deionized (DI) water (Purite, Neptune, Watrex, Prague, Czech Republic) was used for the preparation of stock solutions and dilutions. Background electrolytes (BGEs) for CE measurements were prepared daily by diluting 100 mM of stock solutions of 2-(N-morpholino)ethanesulfonic acid (MES), L-histidine (L-His) and 18-Crown-6 to the required concentrations. Cetyltrimethylammonium bromide (CTAB) was prepared as a 10 mM stock solution in 5 % acetonitrile.

The separation electrolyte that was used for separation and quantitation of inorganic anions, cations and organic acids consisted of 20 mM MES, 20 mM L-His, 2 mM 18-Crown-6 and 30 μM CTAB.

2.3 Capillary-conditioning procedure

Prior to its first use, the separation capillary was preconditioned by flushing it with 0.1 M NaOH for 30 mins., then with DI water for 30 mins. and finally with a solution of BGEs for 10 mins. Between two successive injections, the capillary was flushed with the BGE solution for 1 min. At the end of a working day, the capillaries were washed with DI water for 15 mins., followed by the application of a vacuum for 5 mins. before being stored dry overnight.

2.4 Sample-collection procedure

The EBC was collected in the morning, the subjects did not eat or drink beforehand. The EBC was collected using the previously described EBC-sampler. The subject was asked to exhale deeply 3-5 times into the sampler, but depending on the lung capacity of each, the number of exhalations was modified to collect approximately 100 μl of their EBC. After the sampling of EBC was completed, the condensate from the walls of the syringe was forced to the tip of the syringe by the plunger and transferred to the sample vial for CE analysis. Part of the EBC sample was also used in another experiment published elsewhere, in which the pH was measured using a microelectrode.

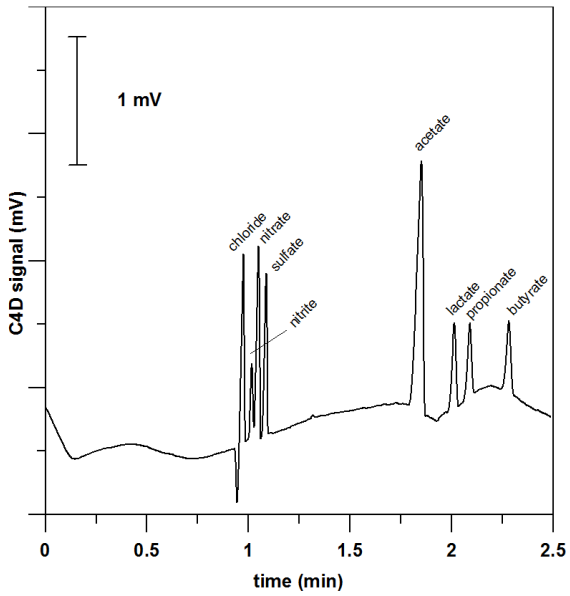

Figure 2: Separation of a model mixture consisting of 4 inorganic anions and 4 organic acids. BGE: 20 mM MES/L-His, 2 mM 18-Crown-6, 30 µM CTAB. HV: positive +15 kV, C4D detection. Ion concentrations in the parentheses in µM: acetate (200); chloride, nitrate, lactate, propionate, butyrate (25); nitrite, sulfate (10).

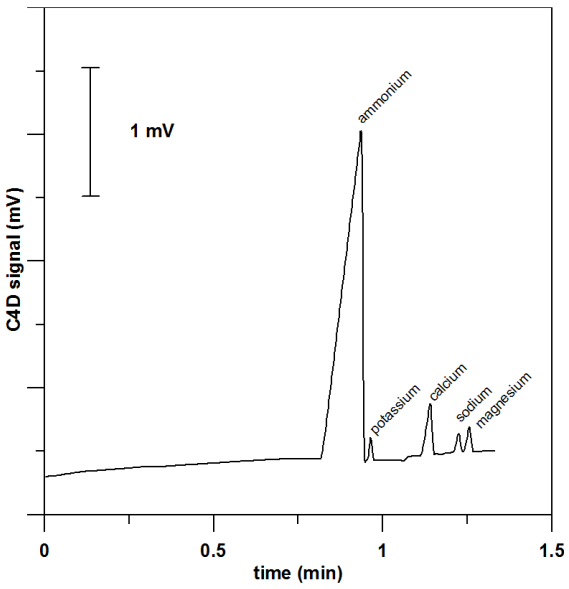

Figure 3: Separation of a model mixture of 5 inorganic cations. BGE: 20 mM MES/L-His, 2 mM 18-Crown-6, 30 µM CTAB. HV: negative -15 kV, C4D detection. Ion concentrations in the parentheses in µM: ammonium (1000); calcium (25); potassium, sodium and magnesium (10).

2.5 Selection of patients and healthy individuals

In this initial screening, a group of healthy individuals and a group of patients diagnosed with asthma and pulmonary fibrosis were selected. The healthy individuals had no history of GERD or heartburn and were free from other related symptoms. The healthy individuals selected were from approximately the same age group as the patients, i.e., the average age of healthy subjects was 48 and that of patients was 56. The patients suffered from asthma and pulmonary fibrosis and were from the Department of Pulmonary Diseases and Tuberculosis at the Faculty Hospital Brno.

3. Results and Analysis

3.1 Electrolyte selection

To separate the ionic content of the EBC, the BGEs composed of MES and L-His was chosen as this electrolyte allows for the analysis of anions, organic acids, and cations with good degrees of sensitivity. MES and L-His fulfill the criteria for suitable coions and counterions in CE separation using contactless conductivity detection (C4D). The conductance of the respective BGE components is low, thus analytes including small organic acids (acetate, lactate, butyrate) will be detected as positive peaks. The addition of 18-Crown-6 to the separation electrolyte improves the resolution of K^+ and NH_4^+ cations and does not influence the selectivity of other measured cations to a great extent, nor does it influence the separation of anions and organic acids. CTAB is added to the separation electrolyte to decrease the electroosmotic flow (EOF).

It should be noted here that although this separation electrolyte allows for the simultaneous separation of anions and cations using dual-opposite end injection [11], in this work anions and cations were determined separately by switching the high-voltage polarity. The separation of a model mixture consisting of 8 anions in less than 2.5 mins. is shown in Fig. 2.

The ion concentrations were selected to be similar to those found in EBC samples. The separation of cations using the same conditions but using reverse-polarity voltage is shown in Fig. 3. The ammonium cation is usually present at a concentration 100 times greater than other cations in the EBC, its concentration was thus also increased to 1000 µM to reflect the expected concentration. Note that even with this significant excess the separation of NH_4^+ from K^+ is sufficient and the BGE composition can be used for real sample analysis.

3.2 Analysis of anions in EBC samples

From each subject, the EBC sample was collected as described previously. About 50 µl of the sample was transferred into an Eppendorf tube and hydrodynamically injected into the CE system. The EBC samples were analyzed first for anions and organic acids using positive HV polarity and then for cations using negative HV polarity,

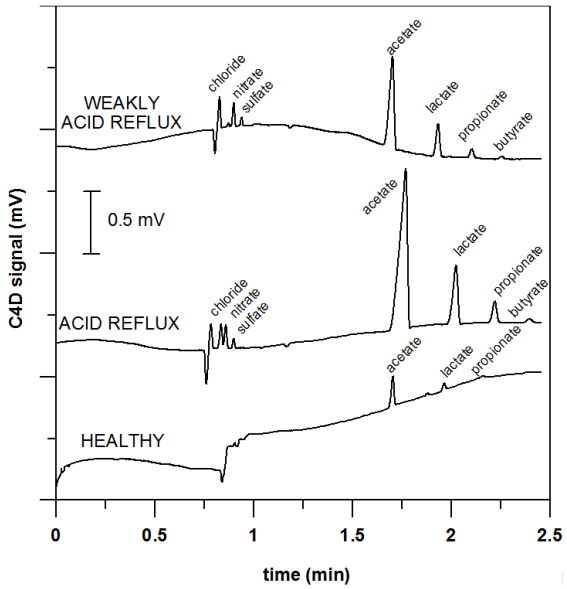

Figure 4: Separation of anions in EBC samples of healthy persons as well as patients with acid reflux or weakly acid reflux. BGE: 20 mM MES/L-His, 2 mM 18-Crown-6, 30 µM CTAB. HV: positive +15 kV, C4D detection.

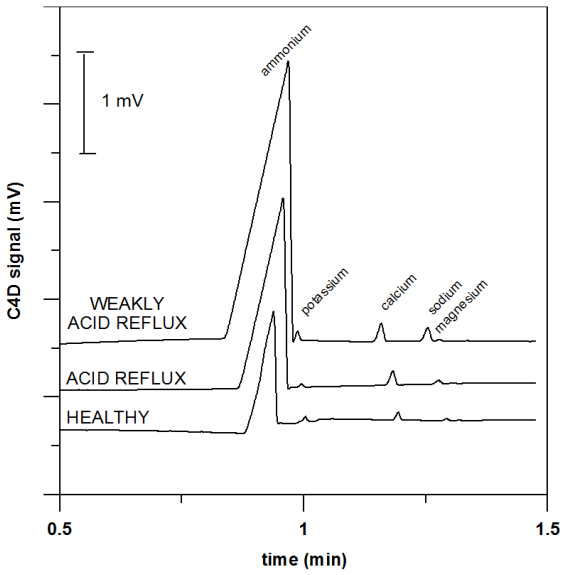

Figure 5: Separation of cations in EBC samples of healthy persons as well as those with acid reflux and weakly acid reflux. BGE: 20 mM MES/L-His, 2 mM 18-Crown-6, 30 µM CTAB. HV: negative -15 kV, C4D detection.

with the HV electrode placed in the detection vial. The concentrations of all 13 ions in the samples were evaluated using calibration curves measured with standard solutions. The parameters such as the calibration curve, regression coefficient (R^2) and concentration range are shown in Table 1. In this initial screening the analysis of several samples from the healthy group and patients that were diagnosed as having the acid reflux or weakly acid reflux was attempted and the ions that would be significantly different in these groups identified.

A series of electropherograms of anions and organic acids in a healthy individual, patient with acid reflux and patient with weakly acid reflux is shown in Fig. 4. One can clearly see that the concentrations of several anions, most notably chloride, nitrate and organic acids, are significantly higher in the group of patients than in that of the healthy volunteers. Some small inorganic anions such as nitrite and nitrate are often found in the EBC of patients with respiratory diseases as markers of nitrosative stress. On the other hand, the presence of an increased concentration of chloride and organic acids may be a significant step towards the diagnosis of GERD.

3.3 Analysis of cations in EBC samples

The analysis of cations in the same samples can be seen in Fig. 5. Although higher concentrations of the ammonium cation were found in patients compared to healthy individuals, the content was not significantly different from that of healthy individuals, and this applied for other cations (K^+, Na^+, Ca^{2+}, Mg^{2+}) as well.

4. Discussion

A limited number of subjects were tested and the determination of whether a simple EBC sample could be used as a surrogate for more invasive, expensive and tedious diagnostic methods attempted. The analysis of anions showed some promising results with regard to the small inorganic anions (chloride and nitrate) collected and the concentration of organic acids that were elevated in the patients

Table 1: Calibration equations of all separated anions, cations and organic acids. BGE: 20 mM MES/L-His, 2 mM 18-Crown-6, 30 µM CTAB. HV: positive +15 kV or negative -15 kV, C4D detection.

Ion	Calibration equation	R^2	range (µM)
Cl^-	$y = 0.0278x + 0.0258$	0.9957	0.6-25
NO_2^-	$y = 0.0270x + 0.0023$	0.9994	0.6-25
NO_3^-	$y = 0.0315x + 0.0166$	0.9987	0.6-25
SO_4^{2-}	$y = 0.0656x + 0.0143$	0.9980	0.25-10
acetate	$y = 0.0156x + 0.0200$	0.9995	1-200
lactate	$y = 0.0207x + 0.0097$	0.9996	0.6-25
propionate	$y = 0.0189x + 0.0024$	0.9983	0.6-25
butyrate	$y = 0.0188x - 0.0001$	0.9990	0.6-25
NH_4^+	$y = 0.0211x - 0.1437$	0.9993	25-1000
K^+	$y = 0.0347x + 0.0013$	0.9994	0.25-10
Ca^{2+}	$y = 0.0528x + 0.0059$	0.9995	0.6-25
Na^+	$y = 0.0356x + 0.0079$	0.9996	0.25-10
Mg^{2+}	$y = 0.0618x - 0.0054$	0.9995	0.25-10

suffering from GERD symptoms, compared to healthy volunteers. Unfortunately, with regard to the analysis of cations, the differences were not significant and cations were deemed unsuitable.

5. Conclusion

Capillary electrophoretic analysis of EBC was used for the first time in an attempt to distinguish the groups of patients with GERD from healthy individuals. CE with C4D was used for the analysis of small inorganic anions, cations and organic acids present in the EBC samples. Although it has been shown that selected samples had elevated concentrations of chloride, nitrate, butyrate and propionate, the number of subjects was too small to draw definite conclusions concerning the discriminatory power of these ions. It also seems that although there is a difference between groups of healthy individuals and sufferers of acid reflux/weakly acid reflux, the results with regard to acid reflux and weakly acid reflux are very similar and these two groups cannot be distinguished based on the current data. A larger scale clinical study, in which the EBC of patients and healthy individuals is scrutinized with regard to the ion content, pH and concentration of other biomarkers (for instance pepsin in saliva) to obtain statistically significant results for evaluation is currently being undertaken. Nevertheless, the initial data is somewhat promising for EBC as a useful non-invasive alternative for other methods used in GERD diagnostics.

Symbols

BGE	background electrolyte
CE	capillary electrophoresis
C4D	contactless conductivity detection
CTAB	cetyltrimethylammonium bromide
DI	deionized
EBC	exhaled breath condensate
GERD	gastroesophageal reflux disease
L-His	L-histidine
ID	inner diameter
MES	2-(N-morpholino)ethanesulfonic acid
OD	outer diameter

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STUDY OF VARIOUS PARAMETERS THAT INFLUENCE THE CONTENT OF EXHALED BREATH CONDENSATE USED IN THE DIAGNOSIS OF GASTROESOPHAGEAL REFLUX DISEASE

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In this work, various parameters that influence the ionic content and pH of exhaled breath condensate in terms of the non-invasive diagnosis of gastroesophageal reflux disease were studied. Exhaled breath condensate samples were collected using a miniature and inexpensive sampling device. Capillary electrophoresis with contactless conductometric detection was used to monitor the ionic content of exhaled breath condensate. Background electrolyte composed of 20 mM of 2-(N-Morpholino)ethanesulfonic acid, 20 mM of L-Histidine, 2 mM of 18-Crown-6 and 30 μM of cetyltrimethylammonium bromide facilitated the rapid separation of anions and cations, both in less than 2 minutes. The possibility of contamination of the exhaled breath condensate by saliva is discussed in detail. The day-to-day repeatability (n=5) of the ionic content and pH of the exhaled breath condensate was studied and was satisfactory, reflecting mainly the physiological variability.

Keywords: capillary electrophoresis, exhaled breath condensate, ionic analysis, pH, gastroesophageal reflux disease

1. Introduction

Gastroesophageal reflux disease (GERD) is a disease caused by the backflow of gastric contents into the esophagus due to the failure of physiological antireflux mechanisms and can lead to symptoms such as a chronic cough, globus sensation, laryngitis, pharyngitis, rhinosinusitis, otitis media, bronchial asthma, chronic obstructive pulmonary disease, sleep apnea and noncardiac chest pain [1, 2]. Currently, no suitable, non-invasive diagnostic method for GERD is applicable in clinical practice. Nowadays, the gold standard with regard to the diagnosis of GERD is a 24-hour multichannel intraluminal impedance-pH technique (MII-pH) that is rather invasive [3]. Non-invasive sampling is becoming more important. Exhaled breath condensate (EBC) as one of several non-invasive samples can be easily obtained by the cooling and subsequent condensation of exhaled breath. The EBC is composed mainly of water, however, it also contains volatile and non-volatile compounds (inorganic ions, organic acids) that can be useful as biomarkers of various diseases [4].

The identification of ions in EBC samples is easily achieved by capillary electrophoresis (CE). The main advantage of this method is that it is able to cope with a minute volume of samples and analyses are often very rapid. Another parameter that is often used to diagnose GERD is pH, measured by a conventional MII-pH probe, which is both invasive and costly. The pH can also be measured in collected samples of EBC [5] which is non-invasive and cheaper. Monitoring various parameters of EBC in patients suffering from symptoms of extraesophageal/gastroesophageal reflux disease is an attractive, simple and non-invasive approach that could be used as a surrogate for other, more invasive diagnostic methods. However, to obtain relevant results, various parameters that can affect the results such as sampling procedures, effects of food and drinks and the intraday variability of individuals have to be studied prior to the use of EBC sampling in diagnostics.

2. Experimental

2.1 Instrumentation

A purpose-built CE instrument was used for all CE analyses. The separation voltage was provided by a high-voltage power-supply unit (DX250, EMCO High Voltage, Sutter Creek, CA, USA). A custom-made contactless conductivity detector (C4D, Version 5.06, ADMET

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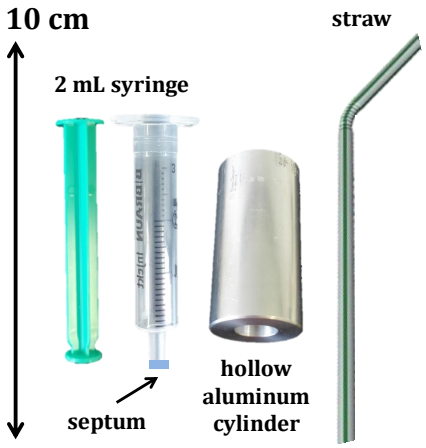

Figure 1: The photo of the components used to build a miniature EBC sampling device.

s.r.o., Prague, Czech Republic) operating at a frequency of 1.8432 MHz and a voltage of 50 V_{p-p} using a 24-bit A/D converter (ORCA 2800, ECOM s.r.o., Prague, Czech Republic) was used to detect separated analytes and collect data. CE analyses were performed in fused-silica capillaries (inner diameter (ID) of 50 μm, outer diameter (OD) of 375 μm, total length of 40 cm, effective length of 17 cm, MicroQuartz GmbH, Munich, Germany). Injection of a standard solution and samples was performed hydrodynamically. Two Pt wires (OD of 0.5 mm, length of 3 cm, Advent Research Materials Ltd., Eynsham, England) were inserted into the electrolyte vials to serve as electrodes. All CE experiments were performed at ambient temperature.

To measure the pH of the EBC samples, CO_2 present in these samples was removed by a stream of N_2 gas (nitrogen generator, RP-ZE-N2L3000). The pH was measured using a pH microelectrode (MI-410 Microcombination pH Probe, Microelectrodes, Inc., Bedford, New Hampshire, USA) and pH-meter (Orion Star^{TM} A111 pH Benchtop Meter, Thermo Fisher Scientific, Waltham, Massachusetts, USA).

EBC samples were collected from the volunteers using an EBC sampler previously developed by our group [6,7]. Briefly, the sampler for collection of EBC was constructed from a 2-mL syringe (B. Braun Melsungen AG, Melsungen, Germany) and a 5 cm-long hollow aluminum cylinder (see Fig. 1) with an outer diameter (OD) of 2.5 cm and an inner diameter (ID) precisely matching the outer diameter of the syringe to facilitate efficient cooling of the walls. The cylinder was kept in a deep freezer at -17°C and an insulation sleeve was used during collection to maintain the temperature for the whole duration of the sampling procedure. Another aim of this insulation sleeve was to prevent frostbite during collection of the sample. Straw (purchased from a local store) with an OD of 6 mm and wall thickness of 0.2 mm was used to exhale the air through the sampler. The end of the syringe was enclosed with a parafilm septum to avoid EBC loss.

2.2 Chemicals

All chemicals were of reagent grade, and deionized (DI) water was used for preparation and dilution of the stock solution. Stock solutions (10 mM) consisting of inorganic anions and cations were prepared from their sodium and chloride salts (NaCl, $NaNO_3$, $NaNO_2$, Na_2SO_4, KCl, $CaCl_2$, $MgCl_2$) with the exception of ammonium, the stock solution of which was prepared from ammonium fluoride (all from PLIVA-Lachema, Brno, Czech Republic). The stock solutions of anions from organic acids were prepared from lithium acetate dihydrate, lithium lactate, propionic acid and butyric acid (all from Sigma-Aldrich, Steinheim, Germany).

The background electrolyte (BGE) for CE measurements was prepared daily by diluting 100 mM stock solutions of 2-(N-Morpholino)ethanesulfonic acid (MES), L-Histidine (His) and 100 mM 18-Crown-6 (all Sigma–Aldrich) to the required concentration. Hexadecyltrimethylammonium bromide (CTAB, Sigma–Aldrich) was prepared as 10 mM stock solution in 5 % acetonitrile and was added to the BGE to yield the final concentration of 30 μM.

2.3 Capillary conditioning

Prior to its first use, the separation capillary was preconditioned by flushing it with 0.1 M NaOH for 30 mins, DI water for 30 mins and BGE solution for 20 mins. Between two successive injections, the capillary was flushed with BGE solution for 1 min. At the end of a working day, the capillaries were washed with DI water for 15 mins and stored in DI water overnight.

3. Results and Analysis

3.1 EBC sampling procedure

The collection of EBC consists of several steps: deep exhalation until the lungs were as empty as possible, followed by rapid inhalation (1-2 s) and finally gradual complete exhalation into the sampling device (8-10 s). This procedure was suggested by Almstrand et al. [8] and yielded the highest concentration of respiratory droplets and, therefore, the highest concentrations of non-volatile substances present in EBC. The volunteer was asked to exhale three times into the sampling device according to the proposed procedure. After the EBC sampling had been completed, the condensate from the syringe walls was wiped with the plunger of the syringe as far as the tip of the syringe and transferred to the sample vial for CE analysis. Often, only one exhalation (30 μL of the sample) was sufficient to obtain a sizable amount of EBC for CE analysis. During the wiping procedure, the condensate collected from the walls is naturally mixed and

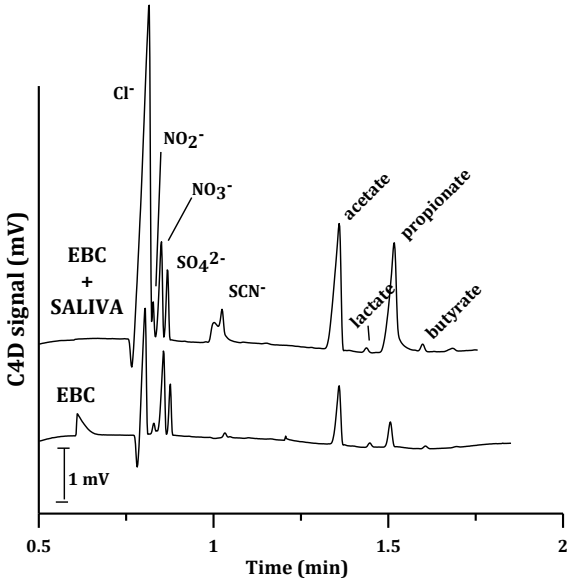

Figure 2: Anionic separation of an EBC sample and an EBC sample spiked with human saliva. BGE: 20 mM MES/His, 2 mM 18-Crown-6, 30 µM CTAB. HV: positive +15 kV, C4D detection. Injection: hydrodynamic for 30 s at a height of 10 cm.

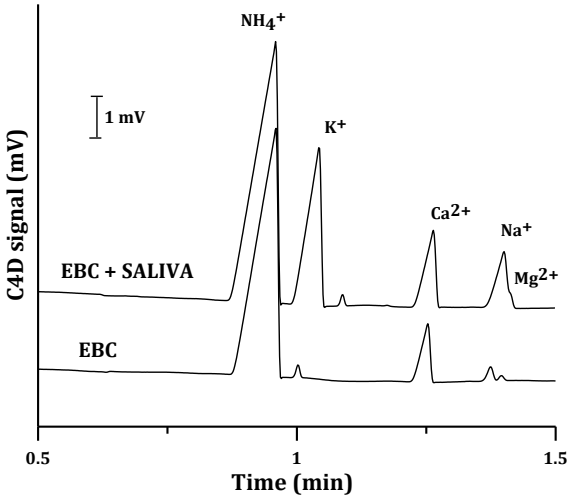

Figure 3: Cationic separation of an EBC sample and an EBC sample spiked with human saliva. BGE: 20 mM MES/His, 2 mM 18-Crown-6, 30 µM CTAB. HV: negative -15 kV, C4D detection. Injection: hydrodynamic for 30 s at a height of 10 cm.

a representative EBC sample is thus obtained. To maintain the simplicity, low costs and wide availability of the components necessary for the EBC sampler, a saliva trap was not included. Therefore, one can occasionally expect interference from saliva droplets.

This can happen especially when sampling of long durations is applied or EBC is collected from patients with serious lung diseases and/or children. Nevertheless, saliva contamination is very rare and happened in ca. 1 in every 100 samples. Contaminated samples were easily identified and excluded from further investigations. Fig. 2 shows the CE separation of anions from two EBC samples; an EBC sample obtained from 3 deep exhalations ($\approx$ 100 µL of EBC) and the same EBC sample spiked with 1 µL of human saliva (to simulate saliva contamination). The EBC sample spiked with saliva displayed a huge increase in the concentration of all anions which significantly exceeded the upper limits of concentrations found in healthy individuals or patients suffering from various lung diseases [9, 10]. Another even more suitable indicator is the thiocyanate, normally present in human saliva [11] but not in EBC.

Cationic analysis of the same two samples (Fig. 3) exhibited the highest increase in sodium and potassium, again way above the normal levels as far as the concentrations of healthy people and sufferers are concerned. Other cations also exhibited increases.

3.2 *Day-to-day repeatability of the ionic content of EBC*

In the next part of the study, the day-to-day repeatability of the ionic content of EBC (over the following 5 days) obtained from three healthy persons (#1, #2 and #3) was studied. Samples were collected in the morning, before breakfast, and all volunteers were asked not to drink or eat before the sampling, the only exception was the consumption of tap water. EBC samples were obtained from single exhalations by using the EBC sampler. All samples were analyzed by CE-C4D and peak areas of all ions in the electropherograms were integrated. The resultant peak areas (PA) for selected ions (ammonium, sodium, acetate and nitrite) were plotted against each of the five

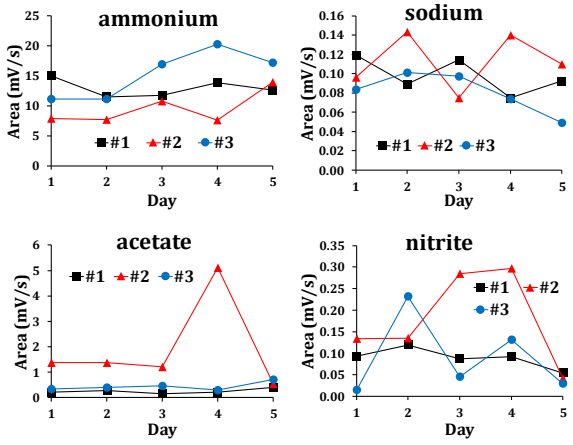

Figure 4: PA of selected ions found in EBC from a day-to-day study consisting of 5 days. Separation and detection conditions as in Figs. 2 and 3.

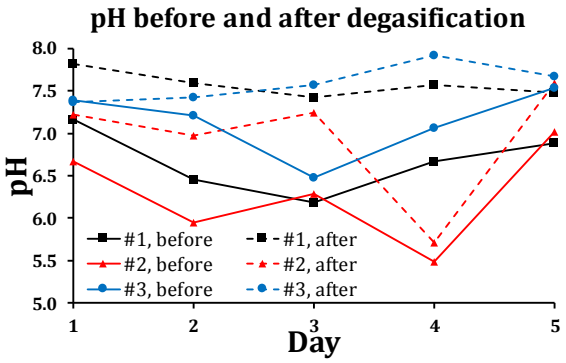

Figure 5: The trend over 5 days in the pH of EBC samples obtained from three healthy volunteers. The pH of EBC before (solid line) and after degasification with N_2 (dashed line).

days (Fig. 4). From this figure, a sufficient repeatability of all ions present in EBC samples over the five days can be seen. Although the spread might seem large, this is caused mainly by the physiological variability of the ion concentration in EBC.

3.3 Day-to-day repeatability of the pH of EBC

Except for cations and anions, which were measured by CE-C4D, other parameters, e.g. exhaled breath volume (measured by spirometry) or the pH of EBC, can be necessary for standardization and diagnostic purposes [12, 13]. The pH of EBC samples was measured and the current experimental setup allowed for pH measurements to be made from as little as 10 µL of EBC. The pH of EBC obtained from a single exhalation can thus be easily measured. The pH of EBC samples obtained in the previous section (day-to-day repeatability) was measured before and after degasification with N_2 (1.8 atm for 5 mins.), and the resultant data can be seen in Fig. 5. It is known that the degasification of liquids by an inert gas, such as N_2, can remove dissolved gases, e.g. CO_2 [14]. Our experiments support this premise since an increase in the pH of all samples was observed after degasification with N_2. Moreover, degasification of the EBC samples significantly increased the repeatability of pH values over the 5 days of the day-to-day study.

3.4 Other parameters that influence the ionic content of EBC and the pH

The influence of various foods, drinks and meals on the ionic content and pH of EBC was also examined. Two EBC control samples (taken in the morning, before breakfast during which only water could be drunk) were obtained from one healthy person. These samples were compared with the two EBC samples from the same day and after one of the following foods/drinks/meals – breakfast, lunch, chocolate, coffee, orange juice, milk, chewing gum or after cleaning teeth with toothpaste or

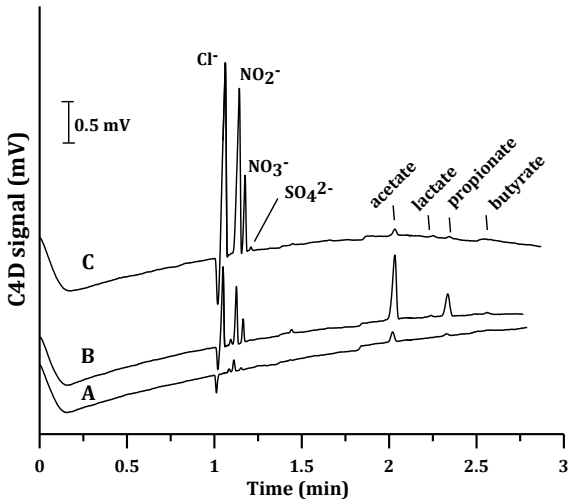

Figure 6: Separation of anions in different EBC samples. EBC samples collected in the morning (A), after the ingestion of orange juice (B), and after chewing a piece of chewing gum (C). BGE: 20 mM MES/His, 2 mM 18-Crown-6, 30 µM CTAB. HV: positive +12.4 kV, C4D detection. Injection: hydrodynamic for 20 s at a height of 12.5 cm.

dentifrice. The time lag between each of the influences and sample collections was 10 minutes. Examples of the separation of anions and cations in EBC samples collected in the morning, after the ingestion of orange juice and chewing a piece of chewing gum are shown in Figs. 6 and 7. Overall, significant differences were found between samples and, therefore, a time lag of at least one

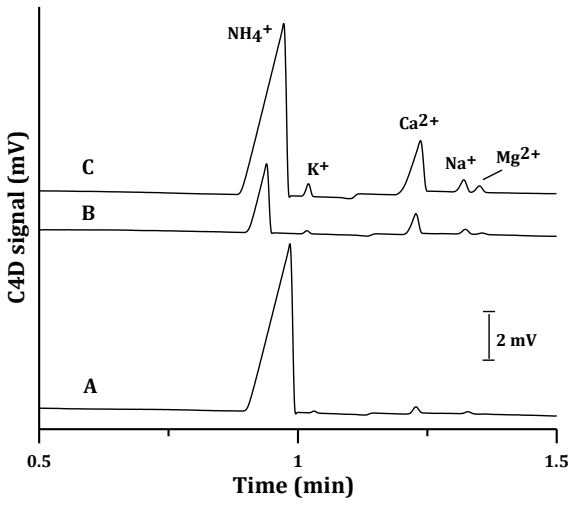

Figure 7: Separation of cations in different EBC samples. EBC samples collected in the morning (A), after the ingestion of orange juice (B), and after chewing a piece of chewing gum (C). BGE: 20 mM MES/His, 2 mM 18-Crown-6, 30 µM CTAB. HV: negative -14.5 kV, C4D detection. Injection: hydrodynamic for 20 s at a height of 12.5 cm.

hour following the flushing of the mouth with DI water was suggested prior to the EBC collection to obtain a relevant EBC sample.

4. Conclusion

In this work, capillary electrophoresis with contactless conductometric detection was used to study the day-to-day repeatability of the ionic content of EBC. The pH of EBC before and after degasification with a stream of N_2 was studied by a pH microelectrode over five consecutive days. The influence of various foods and drinks on the ionic content of EBC was investigated and exhibited significant differences between EBC samples. It has been shown that EBC is influenced by various parameters. Therefore, healthy individuals or patients should provide their EBC samples in the morning before breakfast/brushing teeth or at least one hour after consuming any food or drink last followed by flushing of the mouth with DI water.

Symbols

CE	capillary electrophoresis
C4D	contactless conductivity detection
CTAB	cetyltrimethylammonium bromide
DI	deionized
EBC	exhaled breath condensate
His	L-Histidine
ID	inner diameter
MES	2-(N-Morpholino)ethanesulfonic acid
OD	outer diameter

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ANALYSIS AND IDENTIFICATION OF POLYPHENOLIC COMPOUNDS IN GREEN FOODS USING A COMBINATION OF HPLC-ESI-IT-TOF-MS/MS

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The beneficial effects of green foods on human health are well known. These benefits are mainly due to polyphenol content. The aim of this work was the development and utilization of an advanced mass spectrometry (MS) technique combined with high-performance liquid chromatography (HPLC) for the analysis and identification of polyphenolic compounds in green foods. HPLC-ESI-MS/MS analyses of selected extracts prepared from various types of green food (*Urtica dioica L.* and *Allium ursinum L.*) and their polyphenolic profiles were performed by an LCMS-IT-TOF analyzer equipped with electrospray ionization. HPLC separation was conducted by a Kinetex XB-C18 column (100×2.1 mm; 2.6 μm) using gradient elution (water + 0.1% formic acid: acetonitrile + 0.1% formic acid) with a flow rate of 0.2 mL/min. The MS1-MS3 analyses were performed within a 50-1000 m/z range in both positive and negative ionization modes. The total analysis time was 20 minutes and injected volumes were 2 μL and 5 μL, respectively.

Keywords: polyphenolic compounds, green food, HPLC-MS analysis, identification

1. Introduction

From a chemical point of view, antioxidants are phenolic compounds derived from simple phenols up to complex polyphenols. An antioxidant is a substance whose molecules limit the activity of oxygenated compounds, namely reduce their likelihood or convert them into a less reactive state. Antioxidants limit the oxidation process in the body or in the mixtures in which they are found. In food, they extend their expiration date. In the body, they reduce the likelihood of certain types of diseases [1].

The main reason for monitoring the presence and activity of antioxidants is their beneficial effect on the human organism. Their health benefits include antioxidant, antibacterial, anticarcinogenic, anti-allergenic and anti-inflammatory properties [2–4]. Antioxidants in green foods belong to a group of low-molecular-weight natural antioxidants whose scientific name is phenolic compounds [5]. Green foods are characterized into four basic groups: herbs and spices, fruits, vegetables and nuts.

Despite the fact that numerous polyphenolic compounds have been detected in green food, there is still a need for new reliable analytical methods. The most common procedures for the identification and determination of polyphenolic compounds in green food are based on sample pretreatment by liquid-liquid extraction followed by separation and identification using high-performance liquid chromatography coupled with tandem mass spectrometry (HPLC–MS/MS) [6]. Several authors are concerned with the identification and characterization of phenolic compounds in green foods using a combination of high-performance liquid chromatography and mass spectrometry with electrospray ionization (HPLC-ESI-MS) and atmospheric pressure chemical ionization (HPLC-APCI-MS). For example, HPLC-ESI-MS was used to separate and identify phenolic acids in custard apples [7], tannins in chestnut samples [8], phenolic compounds in groundnuts [9], tomatoes [10], spinach [11], and gingerol-related species (gingerols and shogaols) in ginger [12]. The HPLC-APCI-MS/MS method was used to identify carotenoids in samples of mango and citruses [13]. The HPLC-ESI-MS method was also used to analyze samples of nettles [14] and bear's garlic [15].

The aim of this work was to analyze and identify polyphenolic compounds in selected samples of green food using a combination of high-performance liquid chromatography and high-resolution mass spectrometry techniques.

2. Experimental

2.1 Instrumentation

All HPLC-ESI-MS/MS analyses were performed by using a Shimadzu LCMS-IT-TOF^{TM} mass spectrometer (Shimadzu, Kyoto, Japan). This mass spectrometer combines an electrospray ionization (ESI), a 3D quadrupole ion trap (IT) and an orthogonal acceleration time-of-flight

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analyzer (TOF) to provide both a high degree of sensitivity and resolution of ions. The HPLC part consisted of an autosampler (SIL-20A), high pressure pumps (LC-20AD), a thermostat (CTO-20A), a diode array detector (SPD-M20A), a degasser together with a mixing system (DGU-20A5) and system controller (CBM-20A). LCMS Solutions v. 3.5.1 (Shimadzu) software was used for data acquisition and evaluation.

Chromatographic separation of the polyphenolic compounds was performed on a Kinetex XB-C18 column (100×2.1 mm; 2.6 µm) (Phenomenex, Torrance, CA, USA). Acidified water and acetonitrile (both with an addition of 0.1 % or 10 mmol/L of formic acid) were used as mobile phases. Gradient elution of the mobile phase was used. The flow rate of the mobile phase was 0.2 mL/min. The gradient program was 0 min.: 5% B; 3 min.: 10% B; 8 min.: 40% B; 10 min.: 60% B; 11 min.: 90% B; 12 min.: 90% B; 12.1 min.: 5% B; and 20 min.: 5% B. The chromatographic column was thermostated at 40 °C. All MS analyses were performed in both positive and negative ionization modes. The voltages at the ESI capillaries were $+4.5$ kV and -3.5 kV. The range of recorded values was set at $50 - 1000$ m/z. The temperatures of the Curved Desolvation Line (CDL) capillary and heat block were set at 200 °C. The injected volume of the sample was 2 µL and 5 µL, respectively.

2.2 Chemicals

The following chemicals were used to prepare the solutions and mobile phases: water for LC-MS, acetonitrile for LC-MS (both from LiChrosolv, Merck, Darmstadt, Germany), and formic acid for LC-MS (Sigma-Aldrich, Steinheim, Germany). Standard selected polyphenolic compounds were obtained from Sigma-Aldrich.

2.3 Sampling and sample preparation

The samples of nettle (*Urtica dioica L.*) were collected in March 2017 in the geographical area of Hruštín, after which they were dried and stored in a dry and dark place. Samples of bear's garlic (*Allium ursinum L.*) were collected in March 2017 in the geographical area of Bratislava, after which they were dried and stored in a dry and dark place. Prior to the analysis, the extracts of each sample were prepared as follows: approximately 0.5 g of each was weighed and 50 mL of water was poured onto them at 97 °C. The extraction times were 5, 10 and 15 mins. The extracts were then filtered through filter paper and cooled to room temperature. After cooling, the samples were filtered through a syringe microfilter (PVDF with pore size 0.22 µm) and then analyzed using HPLC-ESI-MS/MS or stored in a refrigerator at 4 °C for a maximum of 24 hours.

3. Results and Discussion

The first part of the experimental work focused on the optimization of conditions for the chromatographic separation of phenolic compounds in selected extracts prepared from different plant species. After HPLC separation, optimization with regard to the composition of the mobile phase in terms of the ionization of the analytes was performed. To confirm the effect with regard to the composition of the mobile phase on the resulting MS signal, a simple experiment was proposed. HPLC-ESI-MS/MS analysis of the bear's garlic and nettle extracts used mobile phases, which varied according to only the concentration of the added formic acid. Gradient elution utilized the following mobile phases with the addition of different concentrations of formic acid: 0.1% formic acid in water (A) / 0.1% formic acid in acetonitrile (B), and 10 mmol/L formic acid in water (A) / 10 mmol/L formic acid in acetonitrile (B). For illustrative purposes, Figs. 1 and 2 show the results from the HPLC-ESI-MS analysis of the 5-minute- long extraction of nettles. After comparing these results with the results of measuring a 5-minute-long extraction of nettles with the addition of 0.1% (26.5 mmol/L) formic acid to the mobile phases, it is clear that the use of a higher concentration of formic acid is more appropriate for the analysis of nettle extracts (promoting the protonation of molecules).

A similar procedure was used to select the appropriate composition of the mobile phases for HPLC-ESI-MS analysis of the garlic extract. The results of the 15-minute-long extraction of bear's garlic with the addition of 0.1% formic acid to the mobile phases can be seen in Fig. 3. The results of the 15-minute-long extraction of garlic with the addition of 10 mmol/L formic acid are recorded in Fig. 4 and suggest this is preferable for the

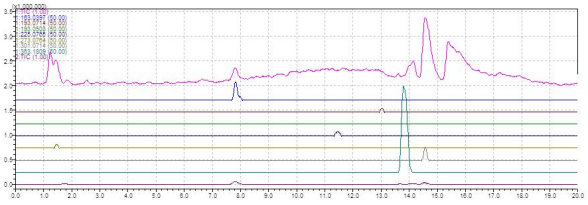

Figure 1: HPLC-ESI-MS analysis of the nettle extract (5 min extraction) with the addition of 10 mmol/L formic acid to the mobile phases. TIC (total ion current) in the positive mode – Top: TIC in the negative mode – Bottom and Middle: EIC (extracted ion chromatogram) records of identified phenolic compounds included.

Figure 2: HPLC-ESI-MS analysis of the nettle extract (5 min extraction) with the addition of 0.1% formic acid to the mobile phases. TIC in the positive mode – Top: TIC in the negative mode – Bottom and Middle: EIC records of identified phenolic compounds included.

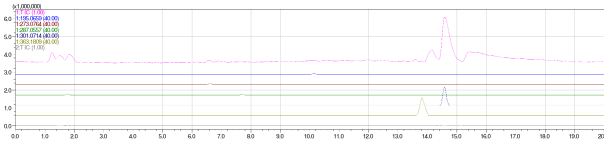

Figure 3: HPLC-ESI-MS analysis of the garlic extract (15 min extraction) with the addition of 0.1% formic acid to the mobile phases. TIC in the positive mode – Top: TIC in the negative mode – Bottom and Middle: EIC records of identified phenolic compounds included.

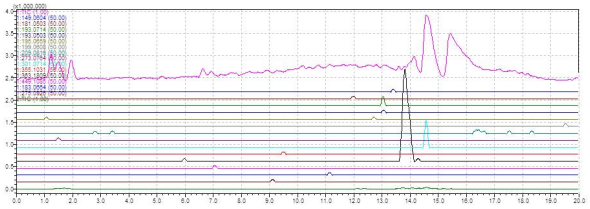

Figure 4: HPLC-ESI-MS analysis of the bear's garlic extract (15 min extraction) with the addition of 10 mmol/L formic acid to the mobile phases. TIC in the positive mode – Top: TIC in the negative mode – Bottom and Middle: EIC records of identified phenolic compounds included.

analysis of the garlic samples. The final conditions for chromatographic separation and mass spectrometric detection are described in detail in the experimental section.

On the basis of the data gathered from HPLC-ESI-MS analyses, it is possible to obtain a better overview of the composition of individual extracts prepared from different plant species as well as information on the nature and structure of the phenolic compounds contained therein. The characterization and identification of individual polyphenolic compounds in plant extracts was performed by the analysis of the MS1-MS2 spectra that were recorded.

The MS1 and MS2 spectra of the coumaric acid in the sample of the nettle extract can be seen in Fig. 5. In the MS1 spectrum an ion with m/z 163.0471 related to the coumarone $[M\text{-}H]^-$ ion is observed. In the MS2 spectrum obtained by the fragmentation of coumaric acid, the ion with m/z 119.0612, which was formed by cleaving the carboxyl group from the coumaric acid molecule, is visible. Another analyte found in the nettle sample was

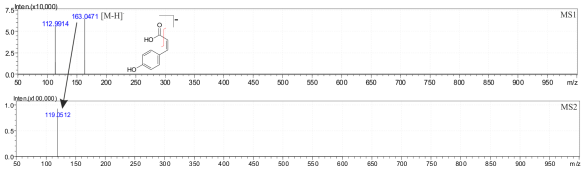

Figure 5: MS1 and MS2 spectra corresponding to coumaric acid obtained by HPLC-ESI-MS/MS analysis of the nettle extract (15 min extraction) in the negative ionization mode.

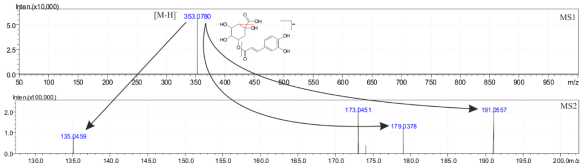

Figure 6: MS1 and MS2 spectra of the corresponding chlorogenic acid obtained by HPLC-ESI-MS/MS analysis of the nettle extract (15 min extraction) in the negative ionization mode.

chlorogenic acid, the presence of which was confirmed by the MS1 and MS2 spectra shown in Fig. 6. The MS1 spectrum is dominated by the ion with m/z 353.0780 corresponding to the $[M\text{-}H]^-$ ion of chlorogenic acid. In the MS2 spectrum obtained by the fragmentation of the ion with m/z 353.0780, the ion with m/z 191.0557, which was obtained by cleaving $\text{-}C_9O_3H_7$ from the chlorogenic acid molecule, is identified. In this spectrum another ion with m/z 179.0378, which was formed by cleaving $\text{-}C_7O_5H_6$ from the chlorogenic acid molecule, is observed. In the MS2 spectrum an ion with m/z 173.0451 is also seen, which corresponds to this cleavage. An ion with m/z 135.0459 in MS2 seen in the chlorogenic acid spectrum was probably generated by cleaving the carboxyl group from the ion m/z 179.0378.

As in the case of the HPLC-ESI-HRMS analysis of nettle extracts, tandem mass spectrometry was used to identify polyphenolic substances in garlic extracts. As an illustration, the MS1 and MS2 chlorogenic acid spectra are presented in Fig. 7. In the MS1 spectrum the molecular ion $[M\text{-}H]^-$ with m/z 353.0844, which is associated with chlorogenic acid, in a sample of the garlic extract is seen. In the MS2 spectrum the ions with m/z 191.0496 and 179.0298, which were formed from the precursor ion (m/z 353.0844) and belong to the illustrated chlorogenic acid fragments, are observed.

The identification of each polyphenolic compound was possible by recording the retention time as well as mass spectra of samples and authentic standards.

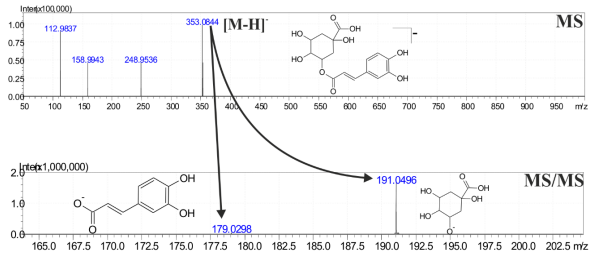

Figure 7: MS and MS/MS spectra corresponding to chloroformic acid obtained by HPLC-ESI-MS/MS analysis of the bear's garlic extract (10 min extraction) in the negative ionization mode.

4. Conclusion

In our experimental work, the use of advanced mass spectrometry techniques in combination with high-performance liquid chromatography-mass spectrometry (HPLC-MS^n) for the analysis and identification of polyphenolic compounds in extracts prepared from different types of green foods was our focus. From the HPLC-MS^n data obtained by analyzing extracts from different types of green food, it was possible to obtain information about the composition of the analyzed extracts as well as information about the nature and structure of the polyphenolic compounds represented therein. Using the developed HPLC-ESI-IT-TOF-MS method, the presence of 13 antioxidants in the sample of nettle extracts and 16 antioxidants in the sample of garlic extracts was confirmed. Based on the results obtained, it can be stated that the developed HPLC-MSn method is a suitable tool for the characterization and identification of polyphenolic compounds in extracts prepared from green foods.

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SILICA MONOLITHIC CAPILLARY COLUMNS FOR HILIC SEPARATIONS

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The monolithic capillary columns (0.1 mm × 150 mm) prepared by the acidic hydrolysis of tetramethoxysilane (TMOS) in the presence of polyethylene glycol (PEG) and urea were modified by zwitterionic stationary phases and evaluated under HILIC separation conditions by employing a mixture containing nucleosides and nucleotides. The polymeric layer of zwitterions did not affect the high separation efficiency of the original silica monolith. The prepared zwitterionic columns exhibited high separation efficiencies in a range 61,000–289,000 theoretical plates/m for a 2-methacryloyloxyethyl phosphorylcholine-based stationary phase and in a range 59,000–135,000 theoretical plates/m for a [2-(methacryloyloxy)ethyl]dimethyl-(3-sulfopropyl)ammonium hydroxide-based stationary phase under optimal separation conditions. The grafted layer of zwitterions on the silica monolithic surface also significantly improved the separation selectivity to compounds of interest.

Keywords: HILIC, silica monolith, nucleoside, nucleotide, capillary chromatography

1. Introduction

Silica-based monolithic columns were developed by Nakanishi and co-workers in the early 1990s [1, 2]. The preparation procedure of the columns has been improved over the years [3]. The unique structure of silica-based monoliths facilitates a high degree of permeability of separation beds and good mass transfer kinetics leading to a high separation efficiency. Thus, liquid chromatography has been identified as an alternative to particle packed columns.

While silica-based monolithic columns dedicated to reversed-phase separations are well established, the new stationary phases of hydrophilic interaction chromatography (HILIC) separation are still a focus of research. Concerning the published papers, the preparation of silica-based monoliths via the so-called "one-pot" process prevails. On the other hand, the chemical modification of preformed silica monoliths offers a well-defined silica skeleton of almost the same permeability and separation efficiency for all designed stationary phases.

This contribution presents a characterization of silica-based monolithic capillary columns prepared by employing the second approach. The zwitterionic monomers [2-(methacryloyloxy)ethyl]dimethyl-(3-sulfopropyl)ammonium hydroxide (MEDSA) and 2-methacryloyloxyethyl phosphorylcholine (MEPC) were used to prepare the desired stationary phases. The prepared columns were evaluated under HILIC separation conditions by employing a mixture containing nucleosides and nucleotides.

2. Experimental

2.1 Material and Instrumentation

MEDSA and MEPC monolithic capillary columns were prepared following a protocol outlined in our previous study [4]. In order to obtain a stable polymeric layer of MEPC on the silica-based monolith, the polymerization time was prolonged to 6 hours.

Toluene, adenosine, uridine, cytidine and nucleotides – adenosine monophosphate (AMP), adenosine diphosphate (ADP), adenosine triphosphate (ATP), cytidine monophosphate (CMP), cytidine diphosphate (CDP), uridine monophosphate (UMP), and uridine diphosphate (UDP) – were purchased from Sigma–Aldrich (Prague, Czech Republic). Acetic acid, ammonium hydroxide and acetonitrile (ACN) (CHROMASOLV, HPLC gradient grade) were obtained from Riedel-de Haën (Seelze, Germany).

High-performance liquid chromatography (HPLC) equipment consisted of two identical syringe pumps (100DM model with a D-series controller, Teledyne ISCO, Lincoln, Nebraska, USA) directly connected to a static nano mixer (60 nl volume) (Upchurch Scientific, Oak Harbor, Washington, USA). The injection was performed by an electrically actuated E90-220 injection valve with a 60 nl internal loop (Valco Instruments, Houston, Texas, USA) and a T-splitter with a restrictor (fused

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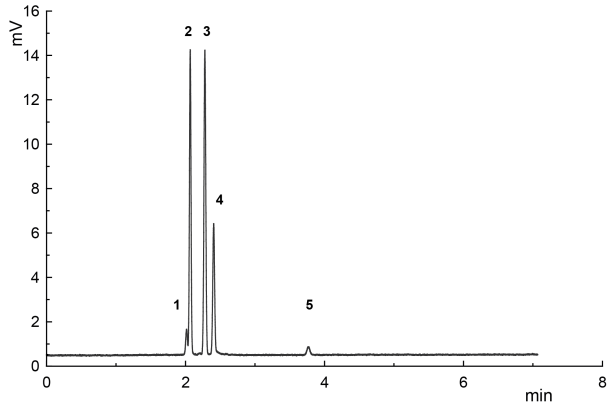

Figure 1: HILIC separation of four purine bases in a bare silica monolithic capillary column. Mobile phase: 95% (v/v) ACN, 5% (v/v) 100 mM ammonium acetate pH=4.5; flow rate 500 nL/min; UV detection at 210 nm. Peak identification: 1 – toluene (unretained compound), 2 – xanthine, 3 – caffeine, 4 – theobromine, and 5 – hypoxanthine.

silica capillary 0.025 mm × 150 mm in length). The inlet section of the monolithic column was inserted through the body of the splitter into the injection valve to minimize extra-column dispersion.

The outlet of the monolithic column was connected to a Spectra 100 UV/Vis detector (Thermo Separation Products, Waltham, Massachusetts, USA) via a 0.035 mm × 120 mm-long fused silica capillary (with an optical bubble cell window inner diameter (ID) 0.110 mm) using a polytetrafluoroethylene (PTFE) sleeve. UV detection was performed at 210 nm. The detector signal was processed by the chromatography station software Clarity (DataApex, Prague, Czech Republic).

3. Results and Discussion

3.1 Chromatographic properties of the bare silica monolith

The prepared bare silica monolithic capillary columns were evaluated under HILIC conditions by employing a mixture containing toluene as an unretained compound and four purine bases, namely xanthine, caffeine, theobromine and hypoxanthine. The obtained chromatogram

Table 1: The retention factor (k), the height equivalent to the theoretical plate (H), the number of theoretical plates per meter (N), and peak asymmetry (As) obtained on the silica monolithic column. See Fig. 1 for separation conditions.

Compound	k	H/μm	N	As
Toluene	–	7.4	135,185	0.67
Xanthine	0.03	4.5	222,547	0.88
Caffeine	0.13	5.8	172,795	1.00
Theobromine	0.19	5.2	192,528	1.13
Hypoxanthine	0.87	4.8	209,601	1.33

Table 2: The retention factor (k), number of theoretical plates per meter (N), and peak asymmetry (As) obtained on the MEPC column. See Fig. 2 for the separation conditions.

Compound	k	N	As
Toluene	–	138,955	1.0
Adenosine	0.16	201,048	1.0
Uridine	0.25	289,537	1.1
Cytidine	0.41	244,314	0.9
AMP	1.24	206,697	1.3
UMP	1.66	183,465	1.5
CMP	2.50	157,760	1.4
ADP	3.98	122,169	2.0
UDP	5.34	112,389	2.4
CDP	7.58	81,476	2.8
ATP	9.73	61,462	2.1

where all purine bases are baseline separated and have narrow symmetrical peaks is presented in Fig. 1.

The prepared column exhibits a high degree of separation efficiency reaching values of between 135,000 and 220,000 theoretical plates/m which corresponds to the minimum plate height within the range of 4.5–7.4 µm, as outlined in Table 1.

3.2 HILIC separation of nucleosides and nucleotides

The commercially available methacrylate-based monomers MEDSA and MEPC were selected for the preparation of zwitterionic stationary phases. The bare silica monolith prepared in a 0.1 mm ID fused silica capillary was modified by 3-(trimethoxysilyl)propyl methacrylate and a polymer layer, with the appropriate zwitterion grafted onto it. The suitability of these columns for the isocratic HILIC separation of nucleosides and nucleotides was studied.

Chromatograms for selected compounds obtained on MEDSA (Fig. 2A) and MEPC (Fig. 2B) capillary columns are presented in Fig. 2. An enhanced degree of separation was obtained on the MEPC column (Fig. 2B) where all 11 compounds present in a sample mixture were well separated within 25 mins. The value of peak asymmetry (As) reflects the change in the molecular shape of the analyte and increases slightly as the number of phosphate groups increases in the relevant nucleotide, as presented in Table 2.

On the other hand, the separation efficiency decreases along with the number of phosphate groups in the molecule of nucleotide exhibit lower values of N compared to nucleosides. A similar trend was also observed on the MEDSA-modified monolithic capillary column.

The MEDSA-modified monolithic column exhibits a poorer separation efficiency than the MEPC column for all nucleosides and nucleotides, even the number of theoretical plates per meter for toluene is comparable, as illustrated in Table 3. The MEDSA column also exhibits

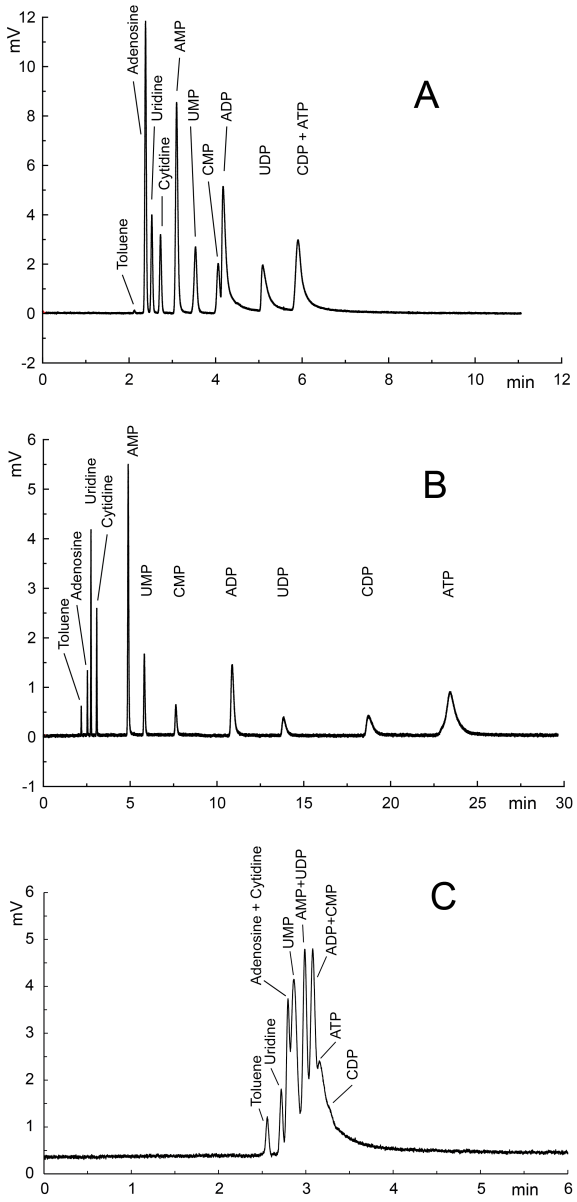

Figure 2: Separation of nucleosides and nucleotides on prepared MEDSA (A), MEPC (B) and bare silica (C) monolithic columns. Mobile phase: 70% (v/v) ACN/30% (v/v) 50 mM ammonium acetate pH = 4.5; flow rate 500 nl/min; UV detection at 210 nm.

a lower retention factor for selected compounds, especially for di- and triphosphates of the relevant nucleosides. This can be explained by the fact that monomers used for column modification differ in terms of their terminal groups, which affects the selectivity of the prepared stationary phases. The MEDSA monolithic capillary column contains negatively charged terminal sulfo groups which decrease the retention factor of negatively charged nucleotides. On the other hand, the MEPC monomer provides the stationary phase with a slightly positive charge originating from the phosphorylcholine functional group which improves the retention factor of negatively charged

Table 3: The retention factor (k), number of theoretical plates per meter (N), and peak asymmetry (As) obtained on the MEDSA column. See Fig. 2 for the separation conditions.

Compound	k	N	As
Toluene	–	131,000	1.2
Adenosine	0.12	135,641	1.1
Uridine	0.19	129,895	1.2
Cytidine	0.28	130,754	1.2
AMP	0.46	101,991	1.5
UMP	0.66	89,032	1.0
CMP	0.91	90,838	1.1
ADP	0.97	71,440	1.7
UDP	1.40	48,371	5.1
CDP	1.78	49,250	3.1
ATP	1.78	59,378	3.5

nucleotides under the HILIC separation conditions, e.g. the retention factor k for ATP is approximately 5 times higher on the MEPC column compared to the MEDSA column.

The separation of nucleosides and nucleotides on a bare silica monolithic capillary column is shown in Fig. 2C. The bare silica monolith does not exhibit a sufficient degree of selectivity to compounds of the target. All compounds are eluted from the column within 1 min and accompanied with the coelution of most compounds.

4. Conclusion

A silica-based monolith in a capillary format coated with zwitterionic polymer layers is a good alternative for the analysis of complex mixtures containing highly polar compounds such as nucleosides and nucleotides. In particular, the MEPC stationary phase containing positively charged terminal groups enables the highly selective HILIC separation of nucleosides and nucleotides to be achieved. The utilization of such a stationary phase with regard to the analysis of real samples, where usually other highly polar-interfering species originating from complex matrices are present and complicate the analysis, should be evaluated in the future.

Acknowledgement

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DETERMINATION OF HOMOCYSTEINE IN URINE AND SALIVA BY MICROCHIP ELECTROPHORESIS

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An online combination of isotachophoresis (ITP) and capillary zone electrophoresis (CZE) was employed on a microchip with a column-coupling technology for the determination of homocysteine in various body fluids. ITP with its high concentration ability was used as a sample pretreatment and injection technique for CZE, which facilitated the rapid and sensitive determination of homocysteine. The resolution of the analyte from other constituents present in real complex samples was enhanced by discrete spacers, which were added to the injected sample. A solid-phase microextraction (SPME) pretreatment technique based on silver- and barium-form resins was used prior to the ITP-CZE analysis to remove high concentrations of chloride and sulfate naturally present in the analyzed samples. The combination of the micro-pretreatment and microelectrophoresis techniques facilitated the determination of trace concentrations of homocysteine in samples of urine and saliva.

Keywords: homocysteine, body fluids, microchip electrophoresis, solid-phase microextraction

1. Introduction

Homocysteine (Hcy) is a non-proteinogenic sulfur-containing amino acid whose metabolism is at the intersection of two metabolic pathways: remethylation and transsulfuration. In remethylation, Hcy acquires a methyl group from N5-methyltetrahydrofolate or from betaine to form methionine. The reaction with N5-methyltetrahydrofolate occurs in all tissues and is vitamin B12-dependent, whereas the reaction with betaine is confined mainly to the liver and is vitamin B12-independent. A considerable proportion of methionine is then activated by ATP to form S-adenosylmethionine [1].

Increased concentration levels of Hcy in body fluids are considered to be an important risk factor or marker of various diseases, particularly cardiovascular ones [2]. In review papers [3–5], individual methods of determining Hcy in biological samples are summarized. Analytical methods using high-performance liquid chromatography (HPLC) to separate Hcy are some of the most commonly used. HPLC methods for the quantification of Hcy utilize derivatization procedures as well as procedures with non-derivatized Hcy by electrochemical detection (ECD). The growing interest in clinical analyses has generated increased attention due to the rapid determination of Hcy using automated methods. For this reason, the immunoassay of Hcy in plasma has become a preferred analytical approach [5].

Recently several procedures have been published for the determination of Hcy by capillary electrophoresis (CE). CE compared to HPLC is more advantageous in terms of the need for a very small sample volume, good resolution, short analysis time, simplicity of automation and elimination of various (toxic) solvents. This is evidenced by many of the works that have dealt with the determination of Hcy in body fluids, e.g. in plasma [6–18], serum [13] or urine [16, 19]. Most of them dealt with the use of laser-induced fluorescence (LIF) as a detection technique, however, UV detection was also used.

A miniaturized form of CE, microchip electrophoresis (MCE), was used for the separation and detection of Hcy and glutathione. The analysis time on the glass microchip using amperometric detection was less than 80 s [6]. A polydimethylsiloxane (PDMS) microchip produced by a simple photolithographic technique facilitated the rapid separation of HCy and cysteine [20]. MCE analyses are more favorable than CE because they are considerably shorter, achieve higher degrees of separation efficiency and, in particular, reduce overall costs associated with chemical consumption and waste production.

This paper deals with the development of a new method for the determination of Hcy in various body fluids on a microchip with coupled-channels (CC). The

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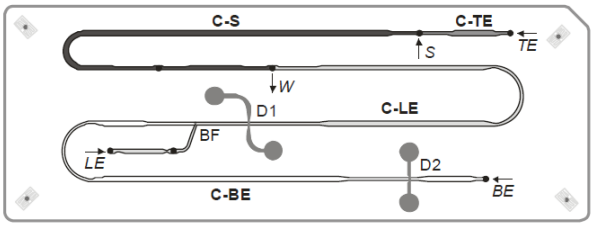

Figure 1: A scheme of the CC microchip with conductivity detection. C-LE = the first (ITP) separation channel (volume of 4.5 μL) filled with a leading electrolyte; C-BE = the second (CZE) separation channel (volume of 4.3 μL) filled with a background electrolyte; C-TE = the third channel (volume of 0.8 μL) filled with a terminating electrolyte; C-S = sample injection channel (volume of 9.9 μL); D1, D2 = Pt conductivity sensors; BF = bifurcation section; BE, LE, TE, S = inlets for the background, leading, terminating and sample solutions to the microchip channels, respectively; W = an outlet channel for the waste container.

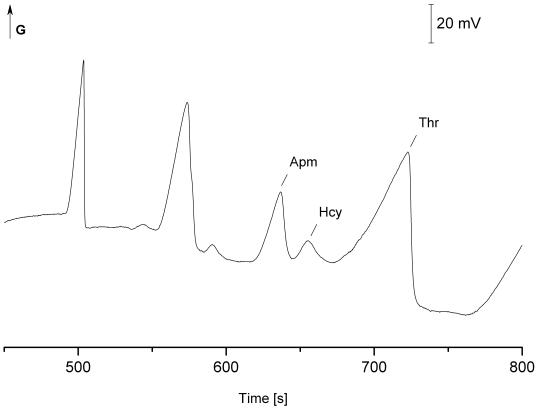

Figure 2: Electropherogram from the ITP-CZE separation of Hcy in the presence of discrete spacers. The sample injected onto the microchip: 100 μmol/L Apm, 15 μmol/L Hcy, 150 μmol/L Thr in a 50 % terminating electrolyte. Apm = aminopimelate; Hcy = homocysteine; Thr = threonine; G = conductivity.

CC technology employed on the microchip with contact conductivity detection enables online capillary zone electrophoresis (CZE) to be coupled with the isotachophoresis (ITP) sample pretreatment. Samples of urine and saliva were simplified by solid-phase microextraction (SPME) prior to the ITP-CZE separations on the microchip. SPME based on silver- and barium-form resins provided a high degree of compatibility with MCE and a high level of selectivity, whilst simultaneously removing a huge amount of chloride and sulfate in the analyzed samples of body fluids.

2. Experimental

ITP-CZE separations were carried out on a poly(methyl methacrylate) microchip (*Fig.1*) with the CC technique and integrated conductivity detection sensors (IonChip^{TM} 3.0, Merck, Darmstadt, Germany).

Chemicals used for the preparation of electrolyte solutions and model samples were obtained from Sigma-Aldrich (Bratislava, Slovakia), Fluka Chemika-Biochemika (Buchs, Switzerland), Serva Electrophoresis GmbH (Heidelberg, Germany) and Erba Lachema s.r.o. (Brno, Czech Republic). Samples of urine and saliva were collected from volunteers. Before the analysis, the samples were homogenized and analyzed after being diluted appropriately with deionized water, and pretreated by SPME to remove chloride and sulfate. Solid-phase extraction (SPE) microcolumns of 0.5 mL in volume containing silver- and barium-form resins (Alltech, Grace Davison Discovery Sciences, Deerfield, Illinois, USA) were used for this purpose.

3. Results and Discussion

ITP-CZE separations were performed in a hydrodynamically closed separation system with suppressed electroosmotic flow. These working conditions effectively reduce the fluctuations in the total migration velocity of the analytes which is crucial to obtain reproducible results, especially on the microchip with short separation paths [21]. In the ITP separations, a leading electrolyte with a pH of 9.1 and glycine as a terminating electrolyte were used. In the CZE step of the ITP-CZE technique, the pH of the background electrolyte was 9.8.

The ITP realized in the first separation channel on the microchip (Fig. 1) preconcentrates the analyte and other constituents of the sample for the CZE separation step. On the other hand, the close migration configuration of the constituents in the ITP stage of the ITP-CZE technique can be a limiting factor for achieving the required resolution of the analyte from matrix constituents, especially on the microchip with short separation channels. In such a case, discrete spacers (DSs) are effectively used to define the fraction of the sample transferred to the second CZE channel when the CC technique is employed. Then, the constituents of the sample migrating outside of the mobility interval defined by the DSs are removed from the separation system. In this way, ITP works as an online sample clean-up technique prior to the CZE. In our case aminopimelate and threonine were used as the front and rear DSs, and the undesirable constituents of the sample were electrophoretically removed from the separation system by column switching prior to the CZE separation realized in the second channel (Fig. 2).

A 1.4 μmol/L limit of detection (LOD) for Hcy was obtained by the ITP-CZE technique on the microchip. A relatively large volume of the sample that was injected onto the microchip (9.9 μl) contributed to achieving the low value of LOD when a universal and relatively low-sensitive conductivity detector was used. Under the working and separation conditions employed, the reproducible migration velocities (the relative standard devi-

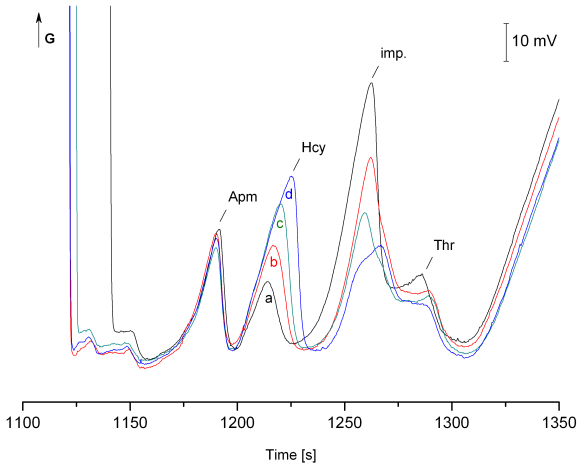

Figure 3: Electropherograms from ITP-CZE analyses of urine samples after SPME pretreatment with various additions of Hcy. Sample injected on the microchip: urine diluted twice, 400 µmol/L Apm, 200 µmol/L Thr in 50 % terminating electrolyte with (a) 0; (b) 10; (c) 20; (d) 40 µmol/L Hcy. Apm = aminopimelate; Hcy = homocysteine; Thr = threonine; imp. = impurity; G = conductivity.

ations (RSDs) of migration times were between 0.5 and 1.2 %) and determinations of trace concentrations of Hcy (RSDs of peak areas were 1.2 %) were achieved.

Considering the high concentration levels of chloride and sulfate in urine and saliva samples, these interfering anions were removed before ITP-CZE analysis from real samples. For this purpose, SPME using silver- and barium-form resins was conducted. The content of Hcy in samples of body fluid was evaluated by the method of standard addition using ITP-CZE analysis after SPME pretreatment. The Hcy concentrations in the tested samples of urine and saliva were approximately 22 µmol/L and 6 µmol/L (Fig. 3), respectively.

4. Conclusion

The SPME-ITP-CZE method performed on the microchip with conductivity detection and the CC technique enabled the fast (total analysis time of approximately 20 mins.) and reliable determination of the trace concentrations of Hcy in biological samples such as urine and saliva.

Acknowledgement

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EDITORIAL PREFACE TO SPECIAL ISSUE DEDICATED TO THE 14TH INTERNATIONAL INTERDISCIPLINARY MEETING ON BIOANALYSIS

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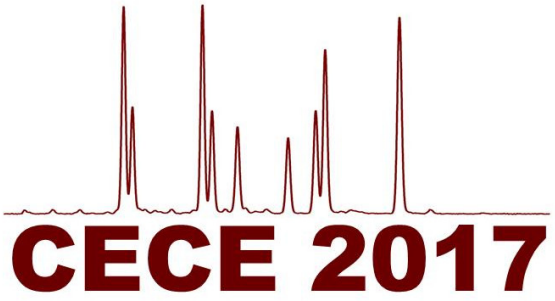
The CECE-2017, 14th International Interdisciplinary Meeting on Bioanalysis was held in Veszprém, Hungary on 8-11 October, 2017. The symposium was organized by the MTA-PE Translational Glycomics Research Group, MUKKI, University of Pannonia. The funding idea of this conference series was "bring together scientists from different disciplines who may not meet at other meetings". Following this initiative, 17 world-class invited speakers delivered stimulating lectures not only about cutting-edge bioanalysis but also about other hot topics, such as aerosol chemistry, etc. The informal atmosphere of the meeting supported direct networking between well-established senior researchers and young scientists at the beginning of their carrier. The first two days of invited lectures was extended with CECE Junior, a dedicated day for PhD students and postdocs, providing them an opportunity to present their results and catch the attention of colleagues both from academia and industry. More than 120 registered participants attended the event during the three days.

The scientific program was preceded by a unique Hungarian social event, a sausage making exercise on Sunday. This national tradition was an exceptional get-together party, where invited speakers, participants, students, sponsors and organizers made spicy and tasty sausage under strict quality control by the father of CECE series, Frantisek Foret.

As an accompanying section, the Horváth Csaba Memorial Exhibition was reopened on the occasion of opening an exhibition for another great Hungarian chromatographer Professor Ervin sz. Kováts. The joint display was dubbed as Hungarian "Chromatography" Mafia Exhibition and these two good friends are together as examples for the next generations.



Overall, the program was of very high scientific level. In this symposium issue, selected contributions are published after normal peer-review process covering very diverse topics of the bioanalytical field.

At this point, the chair András Guttman and co-chair Herbert Lindner would like to thank the hard work of colleagues and students namely Brigitta Mészáros, Beáta Borza, László Hajba, Márton Szigeti, Balázs Reider and Gábor Járvás from the Hungarian side and Bettina Sarg and Klaus Faserl from the Austrian side.

Last but not least, this event would not have happened without the financial support of our sponsors – SCIEX, Unicam Hungary, Thermo-Fischer Scientific, Bio-Science, Agilent, Kromat, Watrex, Waters, University of Pannonia, City of Veszprém and the Hungarian Society of Separation Sciences.

In 2018 the series will return to its home town Brno on 15-17 October, 2018, see you there!

András Guttman and Herbert Lindner

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SEMIQUANTITATIVE ASSESSMENT OF DISHEVELLED-3 PHOSPHORYLATION STATUS BY MASS SPECTROMETRY

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The focus of this paper is the human Dishevelled 3 protein (hDvl3), an essential component of the Wnt signalling pathway that contributes to their regulation. Mass spectrometry-based analysis of hDvl3 phosphorylations induced by eight associated kinases was performed revealing several dozens of phosphorylation sites. The main outcome of this study was the description of Dvl phosphorylation "patterns" induced by individual kinases.

Keywords: phosphorylation, Dishevelled 3, mass spectrometry, CK1ε, NEK2

1. Introduction

Reversible protein phosphorylation belongs to posttranslational modification which is of high biological significance mainly due to its role in regulating cellular processes such as gene expression, cell division, signal transduction, metabolism, differentiation, apoptosis, etc. [1]

Dishevelled-3 (Dvl3) is a protein involved in the Wnt signalling pathways and gets heavily phosphorylated in response to pathway activation by Wnt ligands [2].

In our study, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) separation of hDvl3 immunoprecipitates was combined with titanium dioxide phosphopeptide enrichment followed by liquid chromatography-tandem mass spectrometry (LC-MS/MS). Data were processed using Skyline software to obtain a semiquantitative assessment with regard to the occupancy of phosphorylation sites induced by individual kinases.

2. Experimental

2.1 Cell culture and transfection

The cell line HEK293 was used for this study. Cells were seeded in dishes 15 cm in diameter and transfected at a confluence of 60%. Two days after transfection, after reaching confluence, they were harvested for immunoprecipitation. A chilled NP-40 lysis buffer with protease inhibitors, dithiothreitol (DTT), phosphatase inhibitors and N-ethylmaleimide (NEM) was used for cell lysis. The lysate was incubated with the antibody Anti FLAG M2.

2.2 Gel electrophoresis, protein digestion and phosphopeptide enrichment

Immunoprecipitates were separated by SDS–PAGE, fixed with acetic acid in methanol, and stained with Coomassie Brilliant Blue for 1 hour then partially destained.

Corresponding 1-D bands were excised. After destaining, the proteins in gel pieces were incubated with 10 mM of DTT at 56 °C for 45 mins. After the removal of DTT excess samples were incubated with 55 mM of iodoacetamide (IAA) at room temperature in darkness for 30 mins, then an alkylation solution was removed and gel pieces were hydrated for 45 mins at 4 °C in a digestion solution (5 ng/µl trypsin, sequencing grade, Promega, Fitchburg, Wisconsin, USA, in 25mM ammonium bicarbonate (AmBic)). The trypsin digestion proceeded for 2 hours at 37 °C on a Thermomixer (at 750 rpm; Eppendorf AG, Hamburg, Germany). Subsequently, the tryptic digests were cleaved by chymotrypsin (5 ng/µl, sequencing grade, Roche, Basel, Switzerland, in 25mM AmBic) for 2 hours at 37 °C. Digested peptides were extracted from gels using 50% acetonitrile (ACN) solution with 2.5% formic acid (FA) and concentrated in a SpeedVac concentrator (Eppendorf AG, Hamburg, Germany). The aliquot (1/10) of the sample was transferred to an autosampler vial and concentrated under a vacuum. Water was used to produce 15 µl of the peptide solution which was directly

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PHOSPHOGLYCOLIPID PROFILING OF BACTERIAL ENDOTOXINS

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Much interest is at present focused on bacterial endotoxins, also known as lipopolysaccharides (LPS), as they are responsible for the development of clinical symptoms of Gram-negative sepsis which is the leading cause of death in intensive care units. Endotoxicity is associated with the special phosphoglycolipid part of LPS, termed lipid A. Main challenges in the structural elucidation of lipid A arise from its amphiphilic character and inherent heterogeneity. A mass spectrometry-based *de novo* method combined with reversed-phase liquid chromatography for the detailed structural characterization of complex lipid A mixtures (obtained by mild acid hydrolysis of LPS) from different bacterial sources has been developed. Tandem mass spectrometric measurements were performed with an electrospray-ionisation quadrupole time-of-flight (ESI-Q-TOF) mass spectrometer in both negative- and positive-ionization modes in order to explore fragmentation pathways. It was found that characteristic product ions in the positive-ion mode could be used for the unambiguous assignment of the phosphorylation site, whereas the use of both ionization modes provided consistent and/or complementary information about the fatty acyl distribution between the two glucosamine moieties of lipid A. Since the immunostimulatory (advantageous) *vs.* proinflammatory (endotoxic) effect of the lipid A is closely related to the fine chemical structure, our relatively simple structural elucidation strategy could offer great potential in the bioanalysis of native lipid A samples and lipid A-based vaccines.

Keywords: lipid A, HPLC, tandem mass spectrometry, positive-ion mode, negative-ion mode

1. Introduction

Bacterial endotoxins are important initiators of clinical symptoms of Gram-negative sepsis, a serious medical condition with a high mortality rate among patients in intensive care units worldwide. Endotoxins, chemically lipopolysaccharides (LPS) or lipooligosaccharides (LOS), are found on the surface of Gram-negative bacteria. They are essentially composed of two regions: a heteropolysaccharide chain (divided into the oligosaccharide core and the O-side chain – this component is missing from LOS) that extends outwards from the bacterial cell surface, and a phosphoglycolipid, termed lipid A, which is anchored in the outer membrane (Fig. 1). Specifically, the endotoxic activity is related only to the lipidic domain of endotoxins.

With regard to the toxicity of lipid A the crucial factors are the acylation and phosphorylation status. This most biologically potent molecule, capable of inducing septic shock, consists of an asymmetrically hexa-acylated, bisphosphorylated glucosamine disaccharide [1]. Other lipid A species that deviate from this structure have lower endotoxicity or may even have beneficial effects. Consequently, the structural analysis of the lipid A moiety is of fundamental interest in terms of clinical aspects and vaccine development.

The structural characterization of lipid A poses a unique analytical challenge, as lipid A isolated from a single bacterial strain generally consists of a mixture of differently acylated and phosphorylated species. Furthermore, the relative abundances of the different structures can vary with growth conditions, e.g. temperature, culture

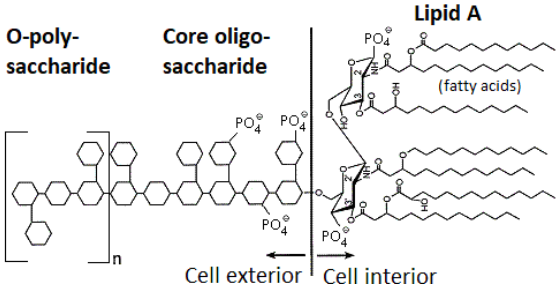

Figure 1: Schematic representation of the chemical structure of an enterobacterial outer membrane lipopolysaccharide (endotoxin).

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media and pH. Within this field, tandem mass spectrometry (MS/MS or MSn) has emerged as a core technology for the in-depth structural elucidation of lipid A species [2]. However, separation and simultaneous quantification of singular components by powerful separation methods prior to MS would be highly desirable to adequately characterize the heterogenic structures.

Here, a brief presentation of an alternative method to multiple-stage MS for the differentiation of various lipid A species – including isobars – within a bacterial sample is provided. Our method is based on a reversed-phase high-performance liquid chromatography (RP-HPLC) separation and online ESI-Q-TOF mass spectrometric detection of molecular species in native lipid A isolates. The exact structures of the lipid A molecules in the complex mixtures were deduced from specific fragmentation patterns obtained in both the negative- [3, 4] and positive-ion modes [5].

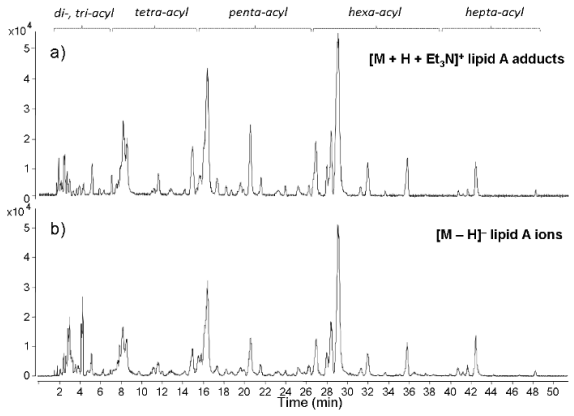

Figure 2: HPLC–MS base peak chromatograms of the native, heterogeneous *E. coli* O111 lipid A preparation acquired in the a) positive- and b) negative-ion modes.

2. Experimental

2.1 Chemicals and Reagents

Methanol, isopropyl alcohol, water (LC-MS grade), dichloromethane (HPLC, ≥99.9%), triethylamine (Et_3N) and acetic acid (AcOH) were purchased from Sigma-Aldrich (Steinheim, Germany).

2.2 Bacterial strains and lipid A isolation

The strains *Escherichia coli* O111 and *Proteus morganii* O34 were cultured in a laboratory fermenter, and then collected by centrifugation. The bacterial LPS was extracted from acetone-dried organisms by the traditional hot phenol-water procedure and lyophilized.

Lipid A was released from LPS by mild-acid hydrolysis with 1% (v/v) AcOH at 100°C for 1 h, then the solution was centrifuged (8000×g, 4°C, 20 mins). The sediment (containing lipid A) was washed four times with distilled water and lyophilized. Lipid A samples were dissolved in methanol/dichloromethane (95:5, v/v) at a final concentration of 0.5 mg/ml.

2.3 Liquid Chromatography – Mass Spectrometry Measurements

Separation and mass spectrometric detection of lipid A samples were performed with an 1290 Infinity Ultra-high performance liquid chromatography (UHPLC) system (Agilent Technologies, Waldbronn, Germany) coupled to a 6530 Accurate-Mass Q-TOF LC/MS system (Agilent Technologies, Singapore), using a Kinetex^{TM} core-shell C18 column (100 mm×4.6 mm, 2.6 µm particle size, Phenomenex). Mobile phase A was methanol/water (95:5, v/v) and mobile phase B was isopropyl alcohol, both containing 0.1% Et_3N and 0.1% AcOH (v/v). The injection volume was 3 µl. Separation was performed at a constant flow rate of 0.5 ml/min at 50°C. A linear gradient of 50 min started at 0% of mobile phase B and ramped up to 50% of mobile phase B, and was then maintained at 50% of mobile phase B for 2 mins.

Negative- and positive-ion mass spectra of the column eluate were recorded between *m/z* 100 and 3,200. The ESI ion source was operated using the following conditions: pressure of nebulizing gas (N_2), 30 psi; temperature and flow rate of drying gas (N_2), 325°C and 8 l/min, respectively; temperature and flow rate of sheath gas, 300°C and 11 l/min, respectively; capillary voltage, 4 kV; nozzle voltage, 2 kV; fragmentor potential, 100 V; and skimmer potential, 70 V. For the targeted MS/MS analysis, the following parameters were used: mass range, *m/z* 70–2000; acquisition rate, 333.3 ms/scan; quadrupole isolation width, 1.3 *m/z*; collision energy, 70–100 V; maximum number of precursor ions/cycle, 4; and active charge state of the precursor ion, 1.

3. Results and Analysis

3.1 HPLC-MS of bacterial lipid A

Crude lipid A preparations from *E. coli* O111 and *P. morganii* O34 bacteria were used as training sets for the development of the method. The HPLC separation conditions were optimized in order to find a balance between solubility, adequate separation and ionization of the intact lipid A's. Fig. 2 shows the HPLC–MS analysis in the positive- and negative-ion modes for the *E. coli* O111 lipid A isolate, only. It was found that the lipid A samples expressed extreme structural heterogeneity, showing the presence of numerous bis-, mono- and non-phosphorylated structures and diverse acylation states ranging from di- to hepta-acylation. The most abundant chromatographic peak from both bacteria was a hexa-acylated, monophosphorylated species. The elution order of the separated compounds was consistent with their relative hydrophobicity, defined mainly by the total num-

ber and length of hydrophobic acyl chains, followed by the number of polar phosphate groups. Several isomers of identical mass could be identified in which the length and/or position of the acyl chains varied.

Since the phosphoglycolipids are acidic in nature, they could readily be ionized as deprotonated ions, i.e. $[M - H]^-$ in the negative-ion mode, whereas their ionization in the positive-ion mode was enhanced by adduct formation with triethylamine used as an eluent additive, i.e. $[M + H + Et_3N]^+$ adducts were formed.

3.2 *Tandem mass spectrometric analysis of lipid A species*

In order to investigate the fragmentation behavior under low-energy collision-induced dissociation (CID) conditions of each separated lipid A precursor ion, ESI-MS/MS experiments were performed in both ionization modes. Based on previously published data on the acyl distribution of the C4'-monophosphorylated, hexa-acylated lipid A molecule from *E. coli* O111 [6], ions observed in the tandem mass spectra (not shown) of this lipid A species (appearing at t_R = 29.2 mins in Fig. 2) could be structurally assigned in both ionization modes [3,5]. Using this as a standard, the CID mass spectral profiles of other monophosphorylated lipid A species separated by chromatography were compared, and the fragmentation preferences for each of them, in both ionization modes, were explored. Fig. 3 presents an example of the correlation between the phosphoglycolipid structure and the fragmentation profiles in the case of the main hepta-acylated lipid A compound (t_R = 42.5 mins in Fig. 2) from *E. coli* O111. The site-specific cleavages are assigned with a number denoting the carbon of the glucosamine to which the substitution is attached, and a Greek letter designating the position of the bond which was cleaved.

In the tandem mass spectrum of the $[M + H + Et_3N]$+ triethylammonium adduct of this lipid A species (Fig. 3a), only a few ions resulting from the neutral loss of acyl chains are present, however, in that of the deprotonated form (Fig. 3b), the abundances of product ions in the higher *m/z* region straightforwardly indicated the consecutive dissociation order of the ester-linked fatty acids. Thus, their position could be assigned. Each tandem mass spectrum also exhibited diagnostic intra-ring (A-type ions in the negative-ion mode) or inter-ring (B- and Y-type ions in the positive-ion mode) cleavage products, which confirmed the substitution position of the fatty acids on the two glucosamine residues.

Fig. 4 shows the tandem mass spectra of the C1 phosphorylation isomer of this hepta-acylated lipid A species. The location of the single phosphoryl group (i.e. at C1 or C4') could be directly assessed from the positive-ion mode tandem mass spectra by calculating the mass difference between the *m/z* of the precursor ion and that of the product ion resulting from the cleavage of the 1α bond

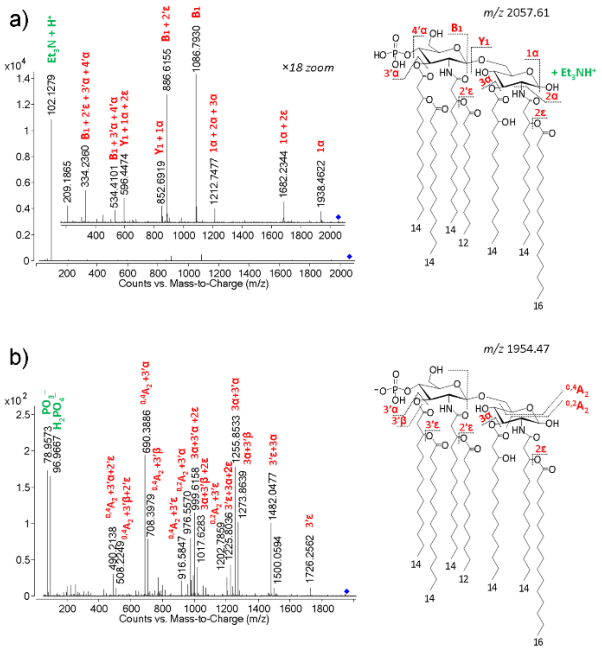

Figure 3: Tandem mass spectra in a) positive- and b) negative-ion modes and a depiction of the identified structure of the C4'-monophosphorylated hepta-acylated lipid A from the *E. coli* O111 bacterium. Site-specific cleavages are indicated next to the ion signals.

(compare Figs. 3a and 4a). Namely, the mass difference of 199 u corresponded to the combined loss of triethylamine and an orthophosphoric acid molecule, indicating that a phosphoric acid molecule had esterified the C1 hydroxyl group of the reducing end. On the other hand, a mass difference of 119 u resulted from the combined loss of triethylamine and a water molecule, indicating the presence of a free hydroxyl group at C1 (consequently, the phosphoryl group must be located at C4').

The change in the phosphorylation site also resulted in a distinctive tandem mass spectrum in the negative- ion mode (Fig. 4b). The main differences were the complete lack of A-type cross-ring fragments, and instead the appearance of Z-type inter-ring cleavage products. Furthermore, the intensities of the product ions resulting from the neutral loss of acyl chains were of about the same magnitude, indicating strong competition between the fatty acyl eliminations during the CID process, instead of their stepwise dissociation (compare with Fig. 3b).

The fragmentation behavior of non- and bis-phosphorylated lipid A species under both negative and positive CID conditions was investigated, as well. For these, a different sequencing of the acyl-chain cleavages could be observed in the negative-ion mode [3, 4], whereas similar fragmentation pathways to that of the monophosphorylated species in the positive-ion mode were identified [5]. Besides the A-type ions (nonreducing end), complementary X-type fragment ions (reducing end) appeared in the negative-ion mode tandem mass

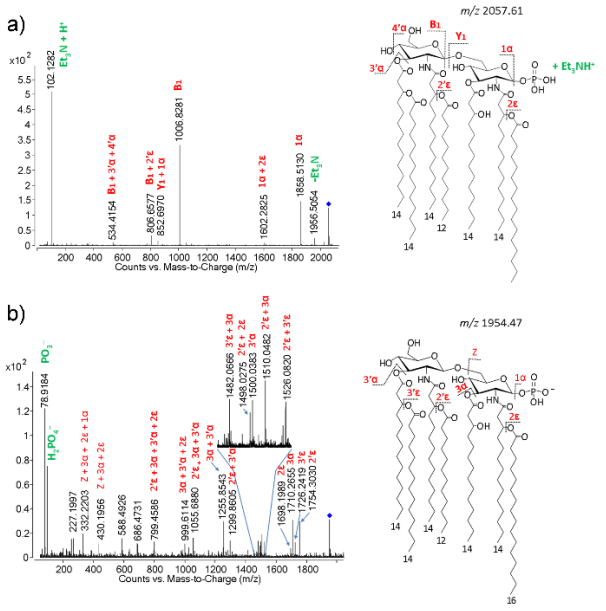

Figure 4: Tandem mass spectra in the a) positive- and b) negative-ion modes and depiction of the identified structure of the C1-monophosphorylated hepta-acylated lipid A from *E. coli* O111.

spectra of the nonphosphorylated lipid A species [4].

4. Discussion

The online HPLC–MS/MS methodology enabled the simultaneous structural characterization of both phosphorylated and nonphosphorylated lipid A variants within a single run. The main advantage of using the collision cell of a Q-TOF mass spectrometer was that several generations of precursor/product ions – that would otherwise be generated only at higher MS stages with ion-trap experiments – were observed simultaneously in the tandem mass spectra of the separated lipid A species. This facilitated new fragmentation rules to be set for the variety of phosphorylated and acylated lipid A compounds by a simple MS/MS experiment [3–5]. Specifically, the acylation profile of the non-, C4'-mono and bisphosphorylated lipid A species could be inferred partly from the positive- and fully from the negative-ion mode MS/MS analyses [3–5], whereas the complementary use of both ionization modes [3, 5] was needed for the full structural characterization of C1-monophosphorylated lipid A.

As an example, the complete structural elucidation strategy by MS/MS of a hepta-acylated C1'-monophosphorylated lipid A is as follows. First, observation of the 1α cleavage product in the positive-ion mode is essential to clarify the site of phosphorylation (*vide supra*). Next, the linkages at the $2'\varepsilon$ (only from the B-type ion) and 2ε positions can be determined from the positive-ion mode analysis. By knowing the fragmentation preference of the secondary ester bonds (which is $2'\varepsilon > 3'\varepsilon > 2\varepsilon$ [5]), substitutions at these sites can be deconvoluted from the negative-ion mode analysis by observing the $2'\varepsilon$, $3'\varepsilon$, 2ε, and 3α cleavage products in the upper *m/z* region and those resulting from the $3'\alpha$, $2'\varepsilon + 3'\varepsilon$, $2'\varepsilon + 2\varepsilon$, $2'\varepsilon + 3\alpha$, and $3'\varepsilon + 3\alpha$ cleavages displayed in the middle *m/z* region. Here, the dephosphorylated Z-type ion in the lower *m/z* region indirectly specifies the linkage at the 2α position. Finally, the remaining $2'\alpha$ substitution can be deduced from the intact B-type ion (positive-ion mode).

5. Conclusion

An efficient HPLC separation before the MS detection of natural lipid A mixtures has proven particularly useful in terms of revealing the diversity and relative amount of the various lipid A structures present in bacteria. Such investigations could help to explore the relative contributions of different lipid A structures to the overall activation of the innate immune system. Moreover, knowledge of the chemical and biological aspects of lipid A heterogeneity is crucial in order to design antimicrobial drugs which overcome the evasion strategy of the pathogen. Our technique could be well suited for phosphoglycolipid profiling from different bacterial strains or vaccine preparations.

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CAPILLARY ISOTACHOPHORESIS DETERMINATION OF TRACE OXIDIZED GLUTATHIONE IN BLOOD

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A capillary isotachophoresis (CITP) method performed in a column-coupling apparatus has been developed for the simultaneous determination of glutathione (GSH) and glutathione disulfide (GSSG) concentrations in blood samples. The determination of GSSG and GSH concentrations in biological samples is important because of their roles in oxidative stress. Different concentrations of a leading ion in the coupled columns (concentration cascade) and a large volume (37 μl) of the injected sample facilitated a GSSG concentration of between 2 and 25 μmol/l. A reaction between iodoacetate and GSH under alkaline conditions was used to prepare the sample in order to avoid oxidation of GSH to GSSG. This step eliminated the main source of systematic errors with regard to the determination of the GSSG concentration. A linear relationship (R^2=0.9969) between the zone length of S-(carboxymethyl)glutathione (the product of the reaction between GSH and iodoacetate) and the concentration of GSH (40-120 μmol/l) was obtained. The method was applied to the analysis of bovine blood samples that had been diluted by a factor of ten with satisfactory results.

Keywords: glutathione, glutathione disulfide, isotachophoresis, bovine blood

1. Introduction

Glutathione (GSH), a thiol containing tripeptide, plays an important role in the antioxidant system of eukaryotic cells [1]. Upon oxidation, GSH is transformed into glutathione disulfide (GSSG) [1]. The concentrations of GSH and GSSG and their molar ratio are indicators of cell functionality as well as oxidative stress [2]. Different aspects of the determination of GSH and GSSG concentrations in biological samples, including sample pretreatments, were recently reviewed [3–5]. The main problems are related to the non-enzymatic oxidation of GSH when the pH exceeds 7, enzymatic conversion of GSH, a need for the removal of proteins prior to the analysis, blocking of free thiol groups, reduction of disulfides, and derivatization of thiol groups [3,6]. Among a wide variety of analytical methods, capillary electromigration (CE) methods, mainly CE coupled with different detectors (UV absorbance [7–10], fluorescence [11] and laser-induced fluorescence (LIF) [12, 13], electrochemical [14, 15] and mass spectrometry [16], have been used to determine the concentration of glutathione [5, 17].

The aim of this work was to develop a capillary isotachophoresis (CITP) method that involves minimal sample pretreatment for the simultaneous determination of GSSG and GSH concentrations in bovine blood samples. Determination of trace analytes in regular CITP, when the quantitation is based on the measurement of the zone length, is not very common. In this work, two key problems were solved: (1) lowering the quantitation limit of the CITP method, and (2) stabilization of GSH without protein precipitation to ensure the accurate determination of the GSSG concentration.

2. Experimental

An EA 202A electrophoretic analyzer (Villa Labeco, Spišská Nová Ves, Slovakia) was used for CITP separations. This fully automated CE system was equipped with two columns. In the first column, a fluoroplastic capillary tube with an inside diameter (ID) of 800 μm and contactless conductivity detection were used. In the second column, a fused silica capillary with an ID of 300 μm, contactless conductivity and UV photometric detectors were used.

The CITP separations were performed using the electrolyte systems shown in Table 1 at a constant driving current, and in the anionic mode (electrode E3 in Fig. 1 used as a cathode). At the beginning and end of the day, the separation and electrolyte units as well as the sample loop of the autosampler were rinsed with deionized water

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COLD PLASMA: THE WAY TO IMPROVE THE REPEATABILITY OF SALD ICP-MS ANALYSIS

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This work deals with the preparation of model biological microsamples for Substrate-Assisted Laser Desorption Inductively Coupled Plasma Mass Spectrometry (SALD ICP-MS). This technique provides the direct and fast analysis of liquid samples deposited onto polyethylene terephthalate (PET) plates (substrates) in the form of dried droplets with minimal sample preparation and submicrolitre sample volume requirements. Furthermore, SALD ICP-MS allows for the direct analysis of samples in organic solvents, which cannot be directly nebulised to ICP-MS. These benefits are, however, balanced out by the low degree of repeatability of the assay, which is typically about 30%. One of the approaches to increase the repeatability is a modification of the substrate surface by Diffuse Coplanar Surface Barrier Discharge (DCSBD) with the addition of an internal standard to the sample. Using this approach, a relative standard deviation (RSD) of less than 10% for model biological samples can be achieved. The only limitation of this technique is the occurrence of the so-called "aging effect", i.e. the gradual return of the modified physicochemical surface properties to their original state.

Keywords: SALD ICP-MS, Plasma Treatment, DCSBD, Microsample, Internal Standard

1. Introduction

The determination of elements in biological samples is often complicated by complex matrices, the presence of organic solvents, and trace amounts of the elements of interest. For this reason, it is necessary to select a technique that bridges the obstacles mentioned above. Such a technique is Substrate-Assisted Laser Desorption Inductively Coupled Plasma Mass Spectrometry (SALD ICP-MS), which is capable of analysing liquid samples of submicrolitre volumes in the form of dried droplets (stains) on a polyethylene terephthalate (PET) substrate [1–3]. The greatest difficulty of this technique is the low repeatability of the assay. This is caused mainly by the poorly reproducible deposition of droplets that consist of submicrolitre volumes and the inhomogeneity of the stains produced in terms of the area and thickness of the layer of solid residue in the stain.

The assay repeatability can be increased by increasing the surface energy of the substrate and using internal standards that can correct the mentioned effects.

One way to increase the surface energy, i.e. wettability of the substrate, is to expose it briefly to cold plasma, specifically Diffuse Coplanar Surface Barrier Discharge (DCSBD) [4,5].

The plasma system of the so-called DCSBD is composed of regularly alternating silver electrodes to which high alternate voltages are applied. The electrodes are located in a dielectric structure consisting of dielectric oil and thin ceramic plates. The most common arrangement of electrodes in this device is in the form of two intermeshing combs. Surface discharges are then generated on the surface of the ceramic plate, serving as a dielectric barrier. This electrode arrangement (plasma in contact with only inert and highly resistant ceramics) can consist of a theoretically infinite amount of 20×8 cm DCSBD units and thus, it is theoretically possible to achieve an infinite plasma area.

The principle of substrate surface treatment is based on the excitement and ionization of the species by a high voltage discharge and subsequent introduction of the resulting species onto the substrate surface in the form of radicals, ions and excited molecules.

A disadvantage of this technique over other physicochemical modifications is the gradual decrease in surface tension compared to its original value prior to the modification, or the so-called "aging effect" [6]; this period is in the order of several months. Factors influencing the qual-

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Table 2: Integrated SALD ICP-MS signals of ^{111}Cd and ^{115}In from stains of 0.5 mM trisodium citrate deposited at different times after PET surface modification.

Deposition time [days]		^{111}Cd	^{115}In	^{115}In / ^{111}Cd	Stain size [mm]
0	$\overline{X}$ [CPS]*	0.44	11	25	1.2±0.1
	RSD [%]	14	10	7.5	
1	$\overline{X}$ [CPS]*	1.4	31	22	1.6±0.2
	RSD [%]	34	38	7.2	
3	$\overline{X}$ [CPS]*	2.2	48	23	1.4±0.2
	RSD [%]	32	29	12	
6	$\overline{X}$ [CPS]*	2.8	58	22	1.2±0.3
	RSD [%]	34	24	19	

*CPS·10^6

ever, that the surfaces with the longest DCSBD modification times exhibited the fastest tendency to return to their original states in the long term.

In the following experiments, the aging effect of the substrate surface modified by DCSBD on the intensity of SALD ICP-MS signals was monitored. Five replicates of 200 nl droplets containing 0.5 mM trisodium citrate solution and 400 ng·l^{-1} of cadmium and indium standards were immediately deposited onto the modified PET surface as well as one, three and six days after modification.

From Table 2, it is apparent that the stain sizes, which should be theoretically dependent on the wettability rate and the aging effect of the modified substrate surface, do not affect the repeatability of the assay for the given samples. Furthermore, it is evident that the immediate average integrated intensity of the ^{111}Cd and ^{115}In signals from the stain ablation of the 0.5 mM trisodium citrate sample as well as one, three and six days after modification of the PET surface differed. This finding is contrary to the assumption that these values should be identical, because in all cases the same amount of sample solution was deposited and the spots were practically 100% ablated by visual inspection. The repeatability of the ablation was low and relative standard deviation (RSD) values exceeded 30%. Such high values of RSDs could be attributed, in particular, to the character of the sample itself, which was crystalline after drying, and the analytes as they were inhomogeneously distributed therein. However, when quantifying the signal intensity ratio of ^{115}In to ^{111}Cd, this inhomogeneity could be corrected, as a result the RSD values varied by around 7.5% for samples deposited immediately and one day after surface modification of the PET.

The samples on the treated surfaces exhibited the highest wettability and, therefore, it was possible to assume that the analytes were homogeneously dispersed in the stain. The ablation records in Figs. 2A and 2B also show a higher degree of homogeneity of the stains. Samples applied three and six days after modification exhibited higher corrected values of RSDs: 12 % and 19 %, re-

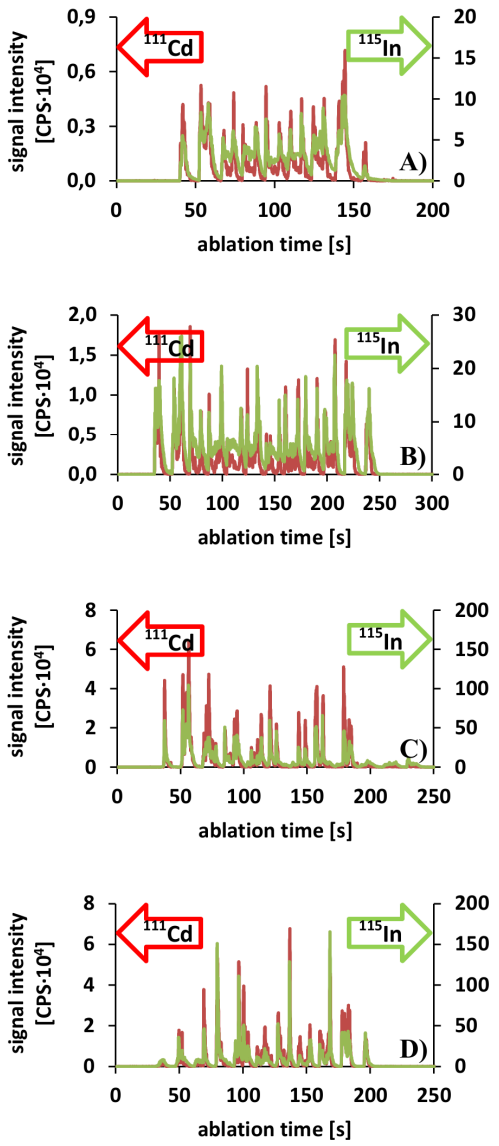

Figure 2: Ablation record of stains of 0.5 mM trisodium citrate deposited A) immediately, as well as B) 1, C) 3 and D) 6 days after PET substrate surface modification.

spectively, mainly due to a higher level of inhomogeneity (Figs. 2C and 2D). Average ^{115}In/^{111}Cd integrated signal intensity values for the spots applied one, three and six days after surface modification could be used for quantification of the monitored analytes as the values almost coincided. However, the average value of the integrated signal from droplets applied to the substrate immediately after the modification differed. This could be caused by extreme changes in the physical properties of the substrate surface immediately after modification.

4. Conclusion

DCSBD technology was chosen for surface modification, i.e. to increase the energy and wettability of the sub-

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CAPILLARY ELECTROPHORETIC ANALYSIS OF EXHALED BREATH CONDENSATE IN THE DIAGNOSIS OF GASTROESOPHAGEAL REFLUX DISEASE

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In this work, capillary electrophoresis with contactless conductometric detection (CCD) was used for the analysis of the ionic content of exhaled breath condensate (EBC) to differentiate between healthy individuals and patients with gastroesophageal reflux disease (GERD). The exhaled breath condensate was collected using a miniature sample collection device and the content analyzed using a separation electrolyte composed of 20 mM 2-(N-morpholino)ethanesulfonic acid, 20 mM L-histidine, 2 mM 18-Crown-6 and 30 μM cetyltrimethylammonium bromide. The separation of anions took less than 2.5 minutes, while the cations were separated in less than 1.5 minutes. The most significantly elevated ions in the group of patients suffering from gastroesophageal reflux disease were chloride, nitrate, propionate and butyrate. Although the number of subjects was too small to draw definite conclusions with regard to the discriminatory power of these ions, the pilot data are promising for EBC as a useful non-invasive alternative for other methods used in the diagnosis of gastroesophageal reflux disease.

Keywords: capillary electrophoresis, exhaled breath condensate, ionic analysis, diagnosis, gastroesophageal reflux disease

1. Introduction

Capillary electrophoresis is an attractive separation technique that is particularly suitable for the analysis of biological samples. Non-invasive sampling and the analysis of alternative biofluids are gaining scientific attention as these samples are acquired easily, can be obtained repeatedly without any particular stress to the patient, and often are formed of a simple matrix. One such sample is exhaled breath condensate (EBC).

EBC is obtained by cooling exhaled breath using suitable pieces of cooling equipment and apparatus. EBC is the aqueous part of the exhaled breath that mainly contains condensed water from breath and volatile compounds that are soluble in water, in addition to small respiratory droplets that provide information concerning the condition, inflammation and oxidative/nitrosative stress of the lungs. EBC was first used as a diagnostic sample by Sidorenko in the 1980s [1] and since then numerous articles on EBC have been published, including some recent reviews [2, 3]. EBC is attractive not only in terms of studying the condition of the lungs [4, 5], but possibly in the identification of other applications, for instance, in the assessment of inflammatory conditions of the trachea and esophagus. The latter is of particular importance because gastroesophageal reflux disease (GERD) is one of the most common diseases in the western world. Between 20 and 30% of the general population in Europe and USA will suffer from GERD [6] and about 60% of the adult population will experience some type of GERD during their lifespan. GERD is often diagnosed using invasive instruments such as 24-hour multichannel intraluminal impedance and pH monitoring (MII-pH) [7], during which the patient is required to continuously wear a narrow catheter in the esophagus. This device measures the spread and volume of gases, liquids as well as solids through the esophagus and evaluates the composition of refluxate. The data are eventually used to evaluate whether the person is suffering from the disease based on the number of recorded reflux episodes and the DeMeester score [8] which describes the severity of reflux.

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STUDY OF VARIOUS PARAMETERS THAT INFLUENCE THE CONTENT OF EXHALED BREATH CONDENSATE USED IN THE DIAGNOSIS OF GASTROESOPHAGEAL REFLUX DISEASE

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In this work, various parameters that influence the ionic content and pH of exhaled breath condensate in terms of the non-invasive diagnosis of gastroesophageal reflux disease were studied. Exhaled breath condensate samples were collected using a miniature and inexpensive sampling device. Capillary electrophoresis with contactless conductometric detection was used to monitor the ionic content of exhaled breath condensate. Background electrolyte composed of 20 mM of 2-(N-Morpholino)ethanesulfonic acid, 20 mM of L-Histidine, 2 mM of 18-Crown-6 and 30 μM of cetyltrimethylammonium bromide facilitated the rapid separation of anions and cations, both in less than 2 minutes. The possibility of contamination of the exhaled breath condensate by saliva is discussed in detail. The day-to-day repeatability (n=5) of the ionic content and pH of the exhaled breath condensate was studied and was satisfactory, reflecting mainly the physiological variability.

Keywords: capillary electrophoresis, exhaled breath condensate, ionic analysis, pH, gastroesophageal reflux disease

1. Introduction

Gastroesophageal reflux disease (GERD) is a disease caused by the backflow of gastric contents into the esophagus due to the failure of physiological antireflux mechanisms and can lead to symptoms such as a chronic cough, globus sensation, laryngitis, pharyngitis, rhinosinusitis, otitis media, bronchial asthma, chronic obstructive pulmonary disease, sleep apnea and noncardiac chest pain [1, 2]. Currently, no suitable, non-invasive diagnostic method for GERD is applicable in clinical practice. Nowadays, the gold standard with regard to the diagnosis of GERD is a 24-hour multichannel intraluminal impedance-pH technique (MII-pH) that is rather invasive [3]. Non-invasive sampling is becoming more important. Exhaled breath condensate (EBC) as one of several non-invasive samples can be easily obtained by the cooling and subsequent condensation of exhaled breath. The EBC is composed mainly of water, however, it also contains volatile and non-volatile compounds (inorganic ions, organic acids) that can be useful as biomarkers of various diseases [4].

The identification of ions in EBC samples is easily achieved by capillary electrophoresis (CE). The main advantage of this method is that it is able to cope with a minute volume of samples and analyses are often very rapid. Another parameter that is often used to diagnose GERD is pH, measured by a conventional MII-pH probe, which is both invasive and costly. The pH can also be measured in collected samples of EBC [5] which is non-invasive and cheaper. Monitoring various parameters of EBC in patients suffering from symptoms of extraesophageal/gastroesophageal reflux disease is an attractive, simple and non-invasive approach that could be used as a surrogate for other, more invasive diagnostic methods. However, to obtain relevant results, various parameters that can affect the results such as sampling procedures, effects of food and drinks and the intraday variability of individuals have to be studied prior to the use of EBC sampling in diagnostics.

2. Experimental

2.1 Instrumentation

A purpose-built CE instrument was used for all CE analyses. The separation voltage was provided by a high-voltage power-supply unit (DX250, EMCO High Voltage, Sutter Creek, CA, USA). A custom-made contactless conductivity detector (C4D, Version 5.06, ADMET

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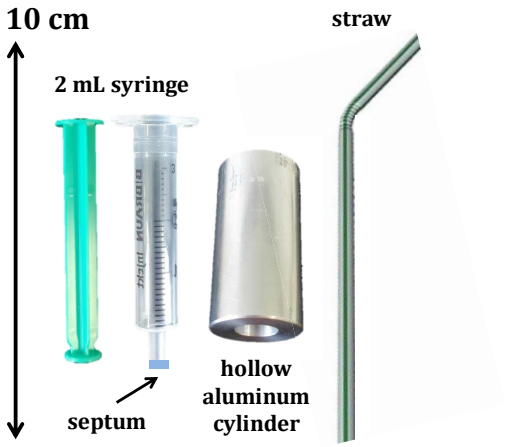

Figure 1: The photo of the components used to build a miniature EBC sampling device.

s.r.o., Prague, Czech Republic) operating at a frequency of 1.8432 MHz and a voltage of 50 V_{p-p} using a 24-bit A/D converter (ORCA 2800, ECOM s.r.o., Prague, Czech Republic) was used to detect separated analytes and collect data. CE analyses were performed in fused-silica capillaries (inner diameter (ID) of 50 μm, outer diameter (OD) of 375 μm, total length of 40 cm, effective length of 17 cm, MicroQuartz GmbH, Munich, Germany). Injection of a standard solution and samples was performed hydrodynamically. Two Pt wires (OD of 0.5 mm, length of 3 cm, Advent Research Materials Ltd., Eynsham, England) were inserted into the electrolyte vials to serve as electrodes. All CE experiments were performed at ambient temperature.

To measure the pH of the EBC samples, CO_2 present in these samples was removed by a stream of N_2 gas (nitrogen generator, RP-ZE-N2L3000). The pH was measured using a pH microelectrode (MI-410 Microcombination pH Probe, Microelectrodes, Inc., Bedford, New Hampshire, USA) and pH-meter (Orion Star^{TM} A111 pH Benchtop Meter, Thermo Fisher Scientific, Waltham, Massachusetts, USA).

EBC samples were collected from the volunteers using an EBC sampler previously developed by our group [6,7]. Briefly, the sampler for collection of EBC was constructed from a 2-mL syringe (B. Braun Melsungen AG, Melsungen, Germany) and a 5 cm-long hollow aluminum cylinder (see Fig. 1) with an outer diameter (OD) of 2.5 cm and an inner diameter (ID) precisely matching the outer diameter of the syringe to facilitate efficient cooling of the walls. The cylinder was kept in a deep freezer at -17°C and an insulation sleeve was used during collection to maintain the temperature for the whole duration of the sampling procedure. Another aim of this insulation sleeve was to prevent frostbite during collection of the sample. Straw (purchased from a local store) with an OD of 6 mm and wall thickness of 0.2 mm was used to exhale the air through the sampler. The end of the syringe was enclosed with a parafilm septum to avoid EBC loss.

2.2 Chemicals

All chemicals were of reagent grade, and deionized (DI) water was used for preparation and dilution of the stock solution. Stock solutions (10 mM) consisting of inorganic anions and cations were prepared from their sodium and chloride salts (NaCl, $NaNO_3$, $NaNO_2$, Na_2SO_4, KCl, $CaCl_2$, $MgCl_2$) with the exception of ammonium, the stock solution of which was prepared from ammonium fluoride (all from PLIVA-Lachema, Brno, Czech Republic). The stock solutions of anions from organic acids were prepared from lithium acetate dihydrate, lithium lactate, propionic acid and butyric acid (all from Sigma-Aldrich, Steinheim, Germany).

The background electrolyte (BGE) for CE measurements was prepared daily by diluting 100 mM stock solutions of 2-(N-Morpholino)ethanesulfonic acid (MES), L-Histidine (His) and 100 mM 18-Crown-6 (all Sigma–Aldrich) to the required concentration. Hexadecyltrimethylammonium bromide (CTAB, Sigma–Aldrich) was prepared as 10 mM stock solution in 5 % acetonitrile and was added to the BGE to yield the final concentration of 30 μM.

2.3 Capillary conditioning

Prior to its first use, the separation capillary was preconditioned by flushing it with 0.1 M NaOH for 30 mins, DI water for 30 mins and BGE solution for 20 mins. Between two successive injections, the capillary was flushed with BGE solution for 1 min. At the end of a working day, the capillaries were washed with DI water for 15 mins and stored in DI water overnight.

3. Results and Analysis

3.1 EBC sampling procedure

The collection of EBC consists of several steps: deep exhalation until the lungs were as empty as possible, followed by rapid inhalation (1-2 s) and finally gradual complete exhalation into the sampling device (8-10 s). This procedure was suggested by Almstrand et al. [8] and yielded the highest concentration of respiratory droplets and, therefore, the highest concentrations of non-volatile substances present in EBC. The volunteer was asked to exhale three times into the sampling device according to the proposed procedure. After the EBC sampling had been completed, the condensate from the syringe walls was wiped with the plunger of the syringe as far as the tip of the syringe and transferred to the sample vial for CE analysis. Often, only one exhalation (30 μL of the sample) was sufficient to obtain a sizable amount of EBC for CE analysis. During the wiping procedure, the condensate collected from the walls is naturally mixed and

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ANALYSIS AND IDENTIFICATION OF POLYPHENOLIC COMPOUNDS IN GREEN FOODS USING A COMBINATION OF HPLC-ESI-IT-TOF-MS/MS

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The beneficial effects of green foods on human health are well known. These benefits are mainly due to polyphenol content. The aim of this work was the development and utilization of an advanced mass spectrometry (MS) technique combined with high-performance liquid chromatography (HPLC) for the analysis and identification of polyphenolic compounds in green foods. HPLC-ESI-MS/MS analyses of selected extracts prepared from various types of green food (*Urtica dioica L.* and *Allium ursinum L.*) and their polyphenolic profiles were performed by an LCMS-IT-TOF analyzer equipped with electrospray ionization. HPLC separation was conducted by a Kinetex XB-C18 column (100×2.1 mm; 2.6 μm) using gradient elution (water + 0.1% formic acid: acetonitrile + 0.1% formic acid) with a flow rate of 0.2 mL/min. The MS1-MS3 analyses were performed within a 50-1000 m/z range in both positive and negative ionization modes. The total analysis time was 20 minutes and injected volumes were 2 μL and 5 μL, respectively.

Keywords: polyphenolic compounds, green food, HPLC-MS analysis, identification

1. Introduction

From a chemical point of view, antioxidants are phenolic compounds derived from simple phenols up to complex polyphenols. An antioxidant is a substance whose molecules limit the activity of oxygenated compounds, namely reduce their likelihood or convert them into a less reactive state. Antioxidants limit the oxidation process in the body or in the mixtures in which they are found. In food, they extend their expiration date. In the body, they reduce the likelihood of certain types of diseases [1].

The main reason for monitoring the presence and activity of antioxidants is their beneficial effect on the human organism. Their health benefits include antioxidant, antibacterial, anticarcinogenic, anti-allergenic and anti-inflammatory properties [2–4]. Antioxidants in green foods belong to a group of low-molecular-weight natural antioxidants whose scientific name is phenolic compounds [5]. Green foods are characterized into four basic groups: herbs and spices, fruits, vegetables and nuts.

Despite the fact that numerous polyphenolic compounds have been detected in green food, there is still a need for new reliable analytical methods. The most common procedures for the identification and determination of polyphenolic compounds in green food are based on sample pretreatment by liquid-liquid extraction followed by separation and identification using high-performance liquid chromatography coupled with tandem mass spectrometry (HPLC–MS/MS) [6]. Several authors are concerned with the identification and characterization of phenolic compounds in green foods using a combination of high-performance liquid chromatography and mass spectrometry with electrospray ionization (HPLC-ESI-MS) and atmospheric pressure chemical ionization (HPLC-APCI-MS). For example, HPLC-ESI-MS was used to separate and identify phenolic acids in custard apples [7], tannins in chestnut samples [8], phenolic compounds in groundnuts [9], tomatoes [10], spinach [11], and gingerol-related species (gingerols and shogaols) in ginger [12]. The HPLC-APCI-MS/MS method was used to identify carotenoids in samples of mango and citruses [13]. The HPLC-ESI-MS method was also used to analyze samples of nettles [14] and bear's garlic [15].

The aim of this work was to analyze and identify polyphenolic compounds in selected samples of green food using a combination of high-performance liquid chromatography and high-resolution mass spectrometry techniques.

2. Experimental

2.1 Instrumentation

All HPLC-ESI-MS/MS analyses were performed by using a Shimadzu LCMS-IT-TOF^{TM} mass spectrometer (Shimadzu, Kyoto, Japan). This mass spectrometer combines an electrospray ionization (ESI), a 3D quadrupole ion trap (IT) and an orthogonal acceleration time-of-flight

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analyzer (TOF) to provide both a high degree of sensitivity and resolution of ions. The HPLC part consisted of an autosampler (SIL-20A), high pressure pumps (LC-20AD), a thermostat (CTO-20A), a diode array detector (SPD-M20A), a degasser together with a mixing system (DGU-20A5) and system controller (CBM-20A). LCMS Solutions v. 3.5.1 (Shimadzu) software was used for data acquisition and evaluation.

Chromatographic separation of the polyphenolic compounds was performed on a Kinetex XB-C18 column (100×2.1 mm; 2.6 µm) (Phenomenex, Torrance, CA, USA). Acidified water and acetonitrile (both with an addition of 0.1 % or 10 mmol/L of formic acid) were used as mobile phases. Gradient elution of the mobile phase was used. The flow rate of the mobile phase was 0.2 mL/min. The gradient program was 0 min.: 5% B; 3 min.: 10% B; 8 min.: 40% B; 10 min.: 60% B; 11 min.: 90% B; 12 min.: 90% B; 12.1 min.: 5% B; and 20 min.: 5% B. The chromatographic column was thermostated at 40 °C. All MS analyses were performed in both positive and negative ionization modes. The voltages at the ESI capillaries were $+4.5$ kV and -3.5 kV. The range of recorded values was set at $50 - 1000$ m/z. The temperatures of the Curved Desolvation Line (CDL) capillary and heat block were set at 200 °C. The injected volume of the sample was 2 µL and 5 µL, respectively.

2.2 Chemicals

The following chemicals were used to prepare the solutions and mobile phases: water for LC-MS, acetonitrile for LC-MS (both from LiChrosolv, Merck, Darmstadt, Germany), and formic acid for LC-MS (Sigma-Aldrich, Steinheim, Germany). Standard selected polyphenolic compounds were obtained from Sigma-Aldrich.

2.3 Sampling and sample preparation

The samples of nettle (*Urtica dioica L.*) were collected in March 2017 in the geographical area of Hruštín, after which they were dried and stored in a dry and dark place. Samples of bear's garlic (*Allium ursinum L.*) were collected in March 2017 in the geographical area of Bratislava, after which they were dried and stored in a dry and dark place. Prior to the analysis, the extracts of each sample were prepared as follows: approximately 0.5 g of each was weighed and 50 mL of water was poured onto them at 97 °C. The extraction times were 5, 10 and 15 mins. The extracts were then filtered through filter paper and cooled to room temperature. After cooling, the samples were filtered through a syringe microfilter (PVDF with pore size 0.22 µm) and then analyzed using HPLC-ESI-MS/MS or stored in a refrigerator at 4 °C for a maximum of 24 hours.

3. Results and Discussion

The first part of the experimental work focused on the optimization of conditions for the chromatographic separation of phenolic compounds in selected extracts prepared from different plant species. After HPLC separation, optimization with regard to the composition of the mobile phase in terms of the ionization of the analytes was performed. To confirm the effect with regard to the composition of the mobile phase on the resulting MS signal, a simple experiment was proposed. HPLC-ESI-MS/MS analysis of the bear's garlic and nettle extracts used mobile phases, which varied according to only the concentration of the added formic acid. Gradient elution utilized the following mobile phases with the addition of different concentrations of formic acid: 0.1% formic acid in water (A) / 0.1% formic acid in acetonitrile (B), and 10 mmol/L formic acid in water (A) / 10 mmol/L formic acid in acetonitrile (B). For illustrative purposes, Figs. 1 and 2 show the results from the HPLC-ESI-MS analysis of the 5-minute- long extraction of nettles. After comparing these results with the results of measuring a 5-minute-long extraction of nettles with the addition of 0.1% (26.5 mmol/L) formic acid to the mobile phases, it is clear that the use of a higher concentration of formic acid is more appropriate for the analysis of nettle extracts (promoting the protonation of molecules).

A similar procedure was used to select the appropriate composition of the mobile phases for HPLC-ESI-MS analysis of the garlic extract. The results of the 15-minute-long extraction of bear's garlic with the addition of 0.1% formic acid to the mobile phases can be seen in Fig. 3. The results of the 15-minute-long extraction of garlic with the addition of 10 mmol/L formic acid are recorded in Fig. 4 and suggest this is preferable for the

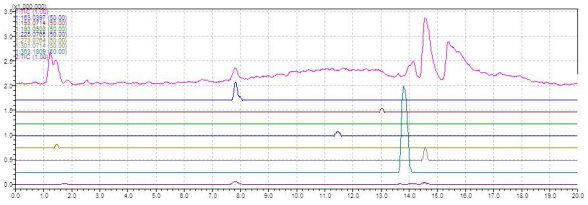

Figure 1: HPLC-ESI-MS analysis of the nettle extract (5 min extraction) with the addition of 10 mmol/L formic acid to the mobile phases. TIC (total ion current) in the positive mode – Top: TIC in the negative mode – Bottom and Middle: EIC (extracted ion chromatogram) records of identified phenolic compounds included.

Figure 2: HPLC-ESI-MS analysis of the nettle extract (5 min extraction) with the addition of 0.1% formic acid to the mobile phases. TIC in the positive mode – Top: TIC in the negative mode – Bottom and Middle: EIC records of identified phenolic compounds included.

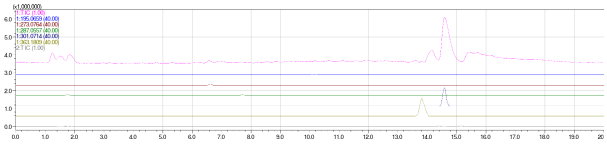

Figure 3: HPLC-ESI-MS analysis of the garlic extract (15 min extraction) with the addition of 0.1% formic acid to the mobile phases. TIC in the positive mode – Top: TIC in the negative mode – Bottom and Middle: EIC records of identified phenolic compounds included.

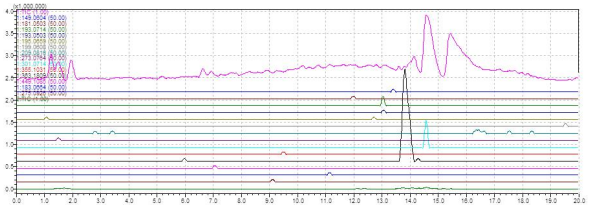

Figure 4: HPLC-ESI-MS analysis of the bear's garlic extract (15 min extraction) with the addition of 10 mmol/L formic acid to the mobile phases. TIC in the positive mode – Top: TIC in the negative mode – Bottom and Middle: EIC records of identified phenolic compounds included.

analysis of the garlic samples. The final conditions for chromatographic separation and mass spectrometric detection are described in detail in the experimental section.

On the basis of the data gathered from HPLC-ESI-MS analyses, it is possible to obtain a better overview of the composition of individual extracts prepared from different plant species as well as information on the nature and structure of the phenolic compounds contained therein. The characterization and identification of individual polyphenolic compounds in plant extracts was performed by the analysis of the MS1-MS2 spectra that were recorded.

The MS1 and MS2 spectra of the coumaric acid in the sample of the nettle extract can be seen in Fig. 5. In the MS1 spectrum an ion with m/z 163.0471 related to the coumarone $[M\text{-}H]^-$ ion is observed. In the MS2 spectrum obtained by the fragmentation of coumaric acid, the ion with m/z 119.0612, which was formed by cleaving the carboxyl group from the coumaric acid molecule, is visible. Another analyte found in the nettle sample was

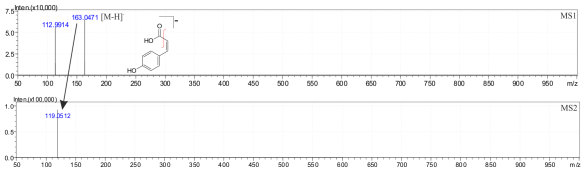

Figure 5: MS1 and MS2 spectra corresponding to coumaric acid obtained by HPLC-ESI-MS/MS analysis of the nettle extract (15 min extraction) in the negative ionization mode.

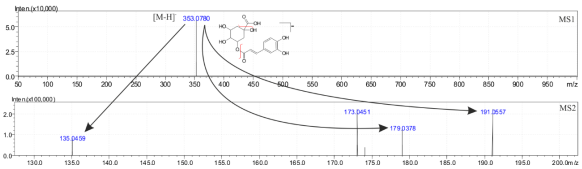

Figure 6: MS1 and MS2 spectra of the corresponding chlorogenic acid obtained by HPLC-ESI-MS/MS analysis of the nettle extract (15 min extraction) in the negative ionization mode.

chlorogenic acid, the presence of which was confirmed by the MS1 and MS2 spectra shown in Fig. 6. The MS1 spectrum is dominated by the ion with m/z 353.0780 corresponding to the $[M\text{-}H]^-$ ion of chlorogenic acid. In the MS2 spectrum obtained by the fragmentation of the ion with m/z 353.0780, the ion with m/z 191.0557, which was obtained by cleaving $\text{-}C_9O_3H_7$ from the chlorogenic acid molecule, is identified. In this spectrum another ion with m/z 179.0378, which was formed by cleaving $\text{-}C_7O_5H_6$ from the chlorogenic acid molecule, is observed. In the MS2 spectrum an ion with m/z 173.0451 is also seen, which corresponds to this cleavage. An ion with m/z 135.0459 in MS2 seen in the chlorogenic acid spectrum was probably generated by cleaving the carboxyl group from the ion m/z 179.0378.

As in the case of the HPLC-ESI-HRMS analysis of nettle extracts, tandem mass spectrometry was used to identify polyphenolic substances in garlic extracts. As an illustration, the MS1 and MS2 chlorogenic acid spectra are presented in Fig. 7. In the MS1 spectrum the molecular ion $[M\text{-}H]^-$ with m/z 353.0844, which is associated with chlorogenic acid, in a sample of the garlic extract is seen. In the MS2 spectrum the ions with m/z 191.0496 and 179.0298, which were formed from the precursor ion (m/z 353.0844) and belong to the illustrated chlorogenic acid fragments, are observed.

The identification of each polyphenolic compound was possible by recording the retention time as well as mass spectra of samples and authentic standards.

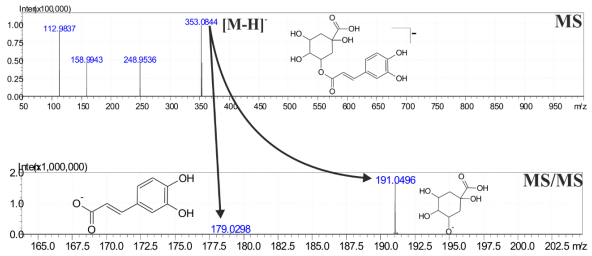

Figure 7: MS and MS/MS spectra corresponding to chloroformic acid obtained by HPLC-ESI-MS/MS analysis of the bear's garlic extract (10 min extraction) in the negative ionization mode.

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SILICA MONOLITHIC CAPILLARY COLUMNS FOR HILIC SEPARATIONS

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The monolithic capillary columns (0.1 mm × 150 mm) prepared by the acidic hydrolysis of tetramethoxysilane (TMOS) in the presence of polyethylene glycol (PEG) and urea were modified by zwitterionic stationary phases and evaluated under HILIC separation conditions by employing a mixture containing nucleosides and nucleotides. The polymeric layer of zwitterions did not affect the high separation efficiency of the original silica monolith. The prepared zwitterionic columns exhibited high separation efficiencies in a range 61,000–289,000 theoretical plates/m for a 2-methacryloyloxyethyl phosphorylcholine-based stationary phase and in a range 59,000–135,000 theoretical plates/m for a [2-(methacryloyloxy)ethyl]dimethyl-(3-sulfopropyl)ammonium hydroxide-based stationary phase under optimal separation conditions. The grafted layer of zwitterions on the silica monolithic surface also significantly improved the separation selectivity to compounds of interest.

Keywords: HILIC, silica monolith, nucleoside, nucleotide, capillary chromatography

1. Introduction

Silica-based monolithic columns were developed by Nakanishi and co-workers in the early 1990s [1, 2]. The preparation procedure of the columns has been improved over the years [3]. The unique structure of silica-based monoliths facilitates a high degree of permeability of separation beds and good mass transfer kinetics leading to a high separation efficiency. Thus, liquid chromatography has been identified as an alternative to particle packed columns.

While silica-based monolithic columns dedicated to reversed-phase separations are well established, the new stationary phases of hydrophilic interaction chromatography (HILIC) separation are still a focus of research. Concerning the published papers, the preparation of silica-based monoliths via the so-called "one-pot" process prevails. On the other hand, the chemical modification of preformed silica monoliths offers a well-defined silica skeleton of almost the same permeability and separation efficiency for all designed stationary phases.

This contribution presents a characterization of silica-based monolithic capillary columns prepared by employing the second approach. The zwitterionic monomers [2-(methacryloyloxy)ethyl]dimethyl-(3-sulfopropyl)ammonium hydroxide (MEDSA) and 2-methacryloyloxyethyl phosphorylcholine (MEPC) were used to prepare the desired stationary phases. The prepared columns were evaluated under HILIC separation conditions by employing a mixture containing nucleosides and nucleotides.

2. Experimental

2.1 Material and Instrumentation

MEDSA and MEPC monolithic capillary columns were prepared following a protocol outlined in our previous study [4]. In order to obtain a stable polymeric layer of MEPC on the silica-based monolith, the polymerization time was prolonged to 6 hours.

Toluene, adenosine, uridine, cytidine and nucleotides – adenosine monophosphate (AMP), adenosine diphosphate (ADP), adenosine triphosphate (ATP), cytidine monophosphate (CMP), cytidine diphosphate (CDP), uridine monophosphate (UMP), and uridine diphosphate (UDP) – were purchased from Sigma–Aldrich (Prague, Czech Republic). Acetic acid, ammonium hydroxide and acetonitrile (ACN) (CHROMASOLV, HPLC gradient grade) were obtained from Riedel-de Haën (Seelze, Germany).

High-performance liquid chromatography (HPLC) equipment consisted of two identical syringe pumps (100DM model with a D-series controller, Teledyne ISCO, Lincoln, Nebraska, USA) directly connected to a static nano mixer (60 nl volume) (Upchurch Scientific, Oak Harbor, Washington, USA). The injection was performed by an electrically actuated E90-220 injection valve with a 60 nl internal loop (Valco Instruments, Houston, Texas, USA) and a T-splitter with a restrictor (fused

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DETERMINATION OF HOMOCYSTEINE IN URINE AND SALIVA BY MICROCHIP ELECTROPHORESIS

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An online combination of isotachophoresis (ITP) and capillary zone electrophoresis (CZE) was employed on a microchip with a column-coupling technology for the determination of homocysteine in various body fluids. ITP with its high concentration ability was used as a sample pretreatment and injection technique for CZE, which facilitated the rapid and sensitive determination of homocysteine. The resolution of the analyte from other constituents present in real complex samples was enhanced by discrete spacers, which were added to the injected sample. A solid-phase microextraction (SPME) pretreatment technique based on silver- and barium-form resins was used prior to the ITP-CZE analysis to remove high concentrations of chloride and sulfate naturally present in the analyzed samples. The combination of the micro-pretreatment and microelectrophoresis techniques facilitated the determination of trace concentrations of homocysteine in samples of urine and saliva.

Keywords: homocysteine, body fluids, microchip electrophoresis, solid-phase microextraction

1. Introduction

Homocysteine (Hcy) is a non-proteinogenic sulfur-containing amino acid whose metabolism is at the intersection of two metabolic pathways: remethylation and transsulfuration. In remethylation, Hcy acquires a methyl group from N5-methyltetrahydrofolate or from betaine to form methionine. The reaction with N5-methyltetrahydrofolate occurs in all tissues and is vitamin B12-dependent, whereas the reaction with betaine is confined mainly to the liver and is vitamin B12-independent. A considerable proportion of methionine is then activated by ATP to form S-adenosylmethionine [1].

Increased concentration levels of Hcy in body fluids are considered to be an important risk factor or marker of various diseases, particularly cardiovascular ones [2]. In review papers [3–5], individual methods of determining Hcy in biological samples are summarized. Analytical methods using high-performance liquid chromatography (HPLC) to separate Hcy are some of the most commonly used. HPLC methods for the quantification of Hcy utilize derivatization procedures as well as procedures with non-derivatized Hcy by electrochemical detection (ECD). The growing interest in clinical analyses has generated increased attention due to the rapid determination of Hcy using automated methods. For this reason, the immunoassay of Hcy in plasma has become a preferred analytical approach [5].

Recently several procedures have been published for the determination of Hcy by capillary electrophoresis (CE). CE compared to HPLC is more advantageous in terms of the need for a very small sample volume, good resolution, short analysis time, simplicity of automation and elimination of various (toxic) solvents. This is evidenced by many of the works that have dealt with the determination of Hcy in body fluids, e.g. in plasma [6–18], serum [13] or urine [16, 19]. Most of them dealt with the use of laser-induced fluorescence (LIF) as a detection technique, however, UV detection was also used.

A miniaturized form of CE, microchip electrophoresis (MCE), was used for the separation and detection of Hcy and glutathione. The analysis time on the glass microchip using amperometric detection was less than 80 s [6]. A polydimethylsiloxane (PDMS) microchip produced by a simple photolithographic technique facilitated the rapid separation of HCy and cysteine [20]. MCE analyses are more favorable than CE because they are considerably shorter, achieve higher degrees of separation efficiency and, in particular, reduce overall costs associated with chemical consumption and waste production.

This paper deals with the development of a new method for the determination of Hcy in various body fluids on a microchip with coupled-channels (CC). The

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