UNIVERSITY OF PÉCS

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The Role of Imidazolium-based Ionic Liquids in the Efficiency of Analytical Separations

PhD Thesis

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1 Introduction

One of the cardinal challenges of modern analytical chemistry is the efficient and reliable separation of complex biological samples. Over the past few decades, ionic liquids (ILs) have garnered increasing attention for their applications in analytical chemistry due to their numerous advantageous properties, which facilitate more effective separation of certain compounds. These ionic substances, which are typically liquid at room temperature, possess highly diverse structural units. This diversity enables them to interact with molecules of varying nature, including polar, apolar, amphiphilic, or ionic substances. They typically consist of an organic cation (such as ammonium, imidazolium, pyridinium, piperidinium, or pyrrolidinium) and a halide or other inorganic (possibly fluorinated) or organic anion.

Currently, one of the most commonly used and studied ionic liquid families is imidazolium-based ionic liquids. Their unique properties – such as low melting point, negligible vapor pressure, high thermal stability and good solubility – make them attractive reagents in many fields of chemistry.

The purpose of my dissertation is to demonstrate the application of imidazolium-based ionic liquids in two important separation techniques: capillary zone electrophoresis (CZE) for the separation of proteins and gas chromatography-mass spectrometry (GC-MS) analyzes for the study of fatty acids of bacterial origin. In our work, we place great emphasis on demonstrating the efficiency improvements that can be achieved by using ionic liquids in these techniques. The use of ionic liquids offers several advantages. On the one hand, when used as a buffer component, the dynamic coating of the capillary can prevent the components from adsorbing to the inner surface of the capillary and increase the efficiency of electrophoretic separations. On the other hand, as a gas chromatography stationary phase, they can enable the control of the polarity and efficiency of the columns. Based on our hypothesis, the specific interactions with ionic liquids play a key role in improving the efficiency of analytical chemistry procedures. Throughout our research, we attempted to gain a deeper understanding of these processes.

2 Objectives

The primary objective of the PhD work is the use of imidazolium-based ionic liquids in the development of capillary electrophoretic and gas chromatographic methods, which can be used to determine the quality and quantity of proteins and fatty acids from various biological samples (human tears, egg whites, bacterial cell membranes). Accordingly, the following goals have been set:

1. The application of aqueous mixtures of imidazolium-based ionic liquids as background electrolyte and for coating the capillary wall in the separation of basic proteins (cytochrome c, lysozyme, myoglobin, trypsin, apo-transferrin) using capillary zone electrophoresis. Optimization of the separation parameters, such as applied voltage, type of ionic liquid, buffer concentration, and capillary length. Statistical evaluation of the developed method and examination of its applicability in determining the protein composition of biological samples (egg white and human tear).

2. Investigation of the separation efficiency of gas chromatographic columns with different polarities (non-polar HP-5MS and medium/high-polarity DB-225MS polysiloxane-based columns, as well as extremely polar ionic liquid-based SLB-IL111 column) for the separation of fatty acid methyl esters with various carbon numbers and saturation levels. Development of the optimal separation parameters for a standard mixture of saturated, polyunsaturated (containing 2-6 double bonds), *cis/trans-*, omega-3, 6-fatty acid methyl esters with carbon numbers ranging from C4 to C24. Demonstration of the reliability and accuracy of the developed method through statistical analysis and validation procedure.

3. Considering the excellent separation properties of the extremely polar ionic liquid-based SLB-IL111 column in the determination of fatty acid methyl esters (point 2), the utilization of this column to monitor the changes in the fatty acid composition of bacteria and bacterial endotoxins induced by external influences. Investigation of the effect of different growth temperatures (25, 37, 42 °C), culture media (agar, blood agar), and incubation times (1, 3, 5 days) on the fatty acid composition of bacteria and bacterial endotoxins of the *Pseudomonas* genus using GC-MS. Development of optimal separation parameters, validation of the method, and statistical evaluation. Demonstration of the impact of changes in fatty acid composition on the properties of bacterial cell membranes.

3 Materials and methods

3.1 Capillary zone electrophoresis method development

3.1.1 Capillary zone electrophoresis experiments

The ionic liquids 1-ethyl-3-methylimidazolium tetrafluoroborate ([emim][BF_4]) and 1-butyl-3-methylimidazolium tetrafluoroborate ([bmim][BF_4]); and cytochrome c, lysozyme, myoglobin, trypsin and apo-transferrin protein standards were used for the measurements.

Stock solutions with a concentration of 10 mg/ml were prepared from the protein standards using distilled water. The samples used for the measurements were prepared from the protein stock solutions that the final concentration of each protein in the solution was 1 mg/ml. The chicken egg white was lyophilized, then a solution with a concentration of 4 mg/ml was prepared with distilled water and centrifuged before use. Human tear samples were collected from the same person.

Stock solutions with a concentration of 400 mM were prepared from the ionic liquids using distilled water. Solutions in the concentration range of 25 and 125 mM were prepared by diluting the stock solutions. The IL-water mixtures were used to coat the inner surface of the capillaries and as a background electrolyte in the CE experiments. For comparison, a phosphate buffer with a concentration of 40 mM (pH 2.2) was also used as BGE. Acetone was used as a sample for the EOF measurements, which was dissolved in distilled water in a 5-fold dilution.

CE experiments were performed using an Agilent 7100 capillary electrophoresis apparatus (Agilent Technologies, Waldbronn, Germany) equipped with a UV detector. The separations were carried out in quartz capillaries (fused silica, 50 µm i.d.). The total length of the capillaries varied between 51 and 96.5 cm, where the effective length was in all cases 8.5 cm shorter than the total length.

Capillaries were conditioned at the beginning of the day by flushing with distilled water (5 min), 0.1 M NaOH (20 min), and again with distilled water (10 min). In experiments with uncoated capillaries, the capillary was then flushed with the desired buffer solution. When preparing the coating, the capillary was flushed with IL-water mixture of the appropriate concentration (between 25 and 125 mM) for 30 minutes. Before injection, a 5-minute wash was performed with IL-water mixture of the appropriate concentration in order to fill the capillary with the appropriate buffer solution.

The samples were injected with a pressure of 50 mbar for 5 seconds. Between measurements, the capillary was only flushed with distilled water for 5 minutes. The applied voltage was varied between +10 and +20 kV with anodic injection, except for the EOF measurements, when +18 or -18 kV was applied. The electropherograms were recorded at 200 nm, the temperature of the capillary catridge was kept at 20 °C during the measurements.

3.1.2 Statistical analysis

A multivariate analysis of variance (MANOVA) is performed to determine the possible significant effect of the independent variables on the dependent variables. The decision level was determined at $\alpha = 0.05$, and the results were considered significant if $p < 0.05$ was met. The migration time, the time-corrected peak area and the resolution were considered as dependent variables, while the applied voltage, the type of ionic liquid and the ionic liquid concentration were considered as independent variables. Wilks's lambda index was calculated to determine potential significant effects of separation parameters on migration time, time-corrected peak area, and resolution. An independent sample t-test was used to compare the groups (ionic liquid type; ionic liquid concentration). Data were expressed as mean for the analyses. The analysis was performed with the SPSS 25.0 statistical software. The LOD values of the lysozyme samples were calculated based on a signal-to-noise ratio of 3, while a signal-to-noise ratio of 10 was used to calculate the LOQ values.

3.2 Gas chromatography–mass spectrometry method development

3.2.1 Chemicals and GC columns

The GLC-674 Reference Standard Mixture, containing 52 fatty acid methyl ester components with chain lengths from C4 to C24 and the CRM47791, containing 4 linoleic acid methyl ester components were used. A stock solution with a total concentration of 1 mg/ml for the GLC-674 reference standard and 0.08 mg/ml for the CRM47791 standard was prepared with n-hexane.

The separations were performed on three commercially available GC columns of different polarities, the HP-5MS with a non-polar 5% phenyl-95% methylpolysiloxane phase composition (length: 25 m; inner diameter: 0.20 mm; film thickness: 0.33 µm); the DB-225MS with medium/high polarity 50% cyanopropylphenyl-50% methylpolysiloxane phase composition (length: 30 m; internal diameter: 0.25 mm; film thickness: 0.25 µm); and the SLB-IL111 with extremely high polarity 1,5-di-(2,3-dimethylimidazolium)pentane bis-((trifluoromethyl)sulfonyl)imide phase composition (length: 30 m; inner diameter: 0.25 mm; film thickness: 0.20 µm). Before the first use, the columns were conditioned according to the factory recommendations.

3.2.2 GC-MS analysis

An Agilent Technologies 6890N gas chromatograph with a 5975 mass selective detector (Agilent, Waldbronn, Germany) was used for the analysis of FAMEs. The chromatograph and the detector conditions were as follows: flow rate of the helium carrier gas, 1.5 ml/min; injection

mode, splitless; the temperature of the injector, ion source, and quadrupole mass analyzer, 250 °C, 230 °C and 150 °C, respectively. The injection volume was 1 μL, applied with an autosampler. The mass spectrometer was operated at 70 eV in the electron impact (EI) mode.

The following settings were found to be suitable for the separation of FAMEs:

For the HP-5MS column, the column temperature was initially held at 50 °C for 2 min, raised to 170 °C at the rate of 10 °C/min, held at 170 °C for 2 min, then raised to 190 °C at the rate of 4 °C/min, held at 190 °C for 5 min, then raised to 290 °C at the rate of 5 °C/min, held at 290 °C for 2 min, then raised to 320 °C at the rate of 20 °C/min, held at final temperature for 2 min. The temperature of the transfer line was set to 280 °C.

For the DB-225MS column, the column temperature was initially held at 50 °C for 2 min, raised to 170 °C at the rate of 10 °C/min, held at 170 °C for 2 min, then raised to 190 °C at the rate of 2.5 °C/min, held at 190 °C for 5 min, then raised to 220 °C at the rate of 5 °C/min, held at 220 °C for 2 min, then raised to 230 °C at the rate of 10 °C/min, held at final temperature for 8 min. The temperature of the transfer line was set to 230 °C.

For the SLB-IL111 column, the column temperature was initially held at 40 °C for 4 min, raised to 220 °C at the rate of 4.5 °C/min, then raised to 260 °C at the rate of 20 °C/min, held at final temperature for 1 min. The temperature of the transfer line was set to 260 °C.

The fatty acids were identified with the help of the MS library.

3.2.3 Validation procedure

The two FAME mixtures were studied in different experimental setups by performing at least three analyses with each. Solution series of five (total) concentrations of the GLC-674 mixture (0.1, 0.25, 0.5, 0.75 and 1 mg/ml concentrations prepared in n-hexane) and the CRM47791 mixture (0.008, 0.02, 0.04, 0.06 and 0.08 mg/nl concentrations prepared in n-hexane) were analyzed. Calibration curves were established from three (n=3) complete analyses under the same conditions. System suitability was expressed by the relative standard deviation (RSD) values of the retention time and the concentration obtained from seven (n=7) complete analyses of each sample under the same conditions within one day. The general criteria for the system suitability were that the RSD values should be less than 2% for the retention time and less than 10% for the concentration. The precision of the methods was checked by intraday and interday experiments, as well. The intraday repeatability was obtained from three (n=3) complete analyses of each sample under the same conditions within one day, and the interday repeatability was obtained from three (n=3) complete analyses of each sample repeated on three consecutive days (n=9). The general criteria for the intraday and interday repeatability were that the RSD values should be less than 10%. The mean values of repeatability were expressed by the RSD values. The general criteria for the average accuracy were that the RSD values should be between 80 and 120%.

3.3 Effect of culture conditions on fatty acid profiles of bacteria and bacterial endotoxins by GC-MS

3.3.1 Chemicals and GC column

The Bacterial Acid Methyl Esters (BAME) CP Mixture, a 26-component BAME mixture with chain-lengths from C11 to C20, containing saturated, unsaturated, hydroxy-, branched and cyclic-fatty acids, was used to validate a GC-MS analysis. The 10 mg/ml stock solution in methyl caproate was diluted ten times with n-hexane.

The separations were acquired by using the extremely polar column, SLB-IL111 (with phase composition, 1,5-di(2,3-dimethylimidazolium)pentane bis(trifluoromethylsulfonyl)imide; length, 60 m; inner diameter, 0.25 mm; film thickness, 0.20 µm). Prior to the first use, the conditioning of the columns was carried out in full accordance to the factory recommendations.

3.3.2 Bacterial strains

Pseudomonas aeruginosa PAO1 (PSAE PAO1), *Pseudomonas aeruginosa* ATCC 27853 (PSAE ATCC 27853), *Pseudomonas putida* (*P. Putida*) and *Pseudomonas aeruginosa* polyresistant (PSAE PR) bacteria were used in the experiments. The bacteria are available from our own strain collection at the Department of Medical Microbiology and Immunology, Medical School, University of Pécs.

The strains were plated on Müller-Hinton agar and 5% sheep blood agar media and incubated for at least 1 day at 25 °C, 37 °C and 42 °C. Fatty acid composition of bacterial strains was determined following 1, 3 and 5 days of the incubation.

3.3.3 Preparation of lipopolysaccharides

Endotoxins extracted from *P. aeruginosa* PAO1 bacteria cultivated at 25 °C, 37 °C and 42 °C were analyzed. Following cultivation, the bacteria were centrifuged by 5000 g for 10 min at 4 °C. The sediments were washed twice at 4 °C in physiological saline solution (500 mL) and dried in acetone. The LPSs were isolated by the hot phenol-water extraction method. The lipopolysaccharides were purified by dialysis and ultracentrifugation at 4 °C three times by 100,000 g for four hours and lyophilized.

3.3.4 Derivatization procedure

The bacteria and LPSs were saponified with 1 mL of 3.75 M NaOH in 50% aqueous methanol at 100 °C for 30 min and rapidly cooled to room temperature. Free fatty acids were derivatized to obtain methyl ester derivatives with 2.0 mL of 3.25 M HCl in a methanol solution at 80 °C for 10 min, and rapidly cooled to room temperature. The fatty acid methyl esters were

extracted from the aqueous phase with 1.25 ml of hexane- methyl tert-butyl ether (l:l v:v) in the shaker-thermostat for 10 min, and the acidified lower phase was discarded; and the extract was washed and neutralized with 3.0 ml of a 0.3 M NaOH solution in shaker-thermostat for 5 min, centrifuge 2000 × g revolution for 5 min. The organic extract in the upper phase was the final FAME extract.

To ensure the detectability of hydroxyl-fatty acids on the ionic liquid-based column, acetylation was performed. The hexane phase was evaporated with a nitrogen stream and a mixture of pyridine (100 μL) and acetic anhydride (100 μL) was used at 100 °C (1 h) for acetylation. After evaporating the reaction mixture, a 1 mg/ml final solution was prepared using acetone.

3.3.5 GC-MS analysis

The chromatograph and the detector conditions are the same as described in *Chapter 3.2.2*. To find the appropriate method for the separations, the method used in our previous work was modified and the following settings were found to be suitable. The column temperature was initially held at 50 °C for 0 min, then raised to 220 °C at the rate of 4.5 °C/min, held at 220 °C for 1 min, and raised to 260 °C at 50 °C/min, held at final temperature for 2 min. The fatty acids were identified with the help of the MS library.

3.3.6 Validation procedure

The procedure is the same as described in *Chapter 3.2.3* with the difference that in this work the solution series of five (total) concentrations of the acetylated BAME CP Mix standard mixture (0.1, 0.25, 0.5, 0.75 and 1 mg/ml concentrations prepared in acetone) were analyzed.

3.3.7 Statistical analysis

The analysis is the same as described in *Chapter 3.1.2*, with the difference that in this work the independent variables were culture temperature (25; 37; 42 °C), culture medium type (agar, blood agar) and incubation time (1, 3, 5 days), while the dependent variables were the retention time, the time-corrected area and the area percentages of the fatty acid components.

4 Results and discussion

4.1 Capillary zone electrophoresis of proteins applying imidazolium ionic liquids

Samples containing five proteins, cytochrome c, myoglobin, lysozyme, trypsin, and apo-transferrin were analyzed in CZE in uncoated and coated capillaries. The capillaries were coated by using binary mixtures of water and different concentrations of [emim][BF_4] or [bmim][BF_4]. Phosphate buffer or the IL–water mixture was applied as BGE in zone electrophoresis. The initial experiments were made with uncoated capillaries using a 40 mM phosphate buffer, pH 2.2. In these experiments the proteins were not baseline-separated. To test the effect of the presence of ionic liquid constituents, the separation was firstly carried out in a capillary coated with 100 mM [bmim][BF_4], applying the 40 mM phosphate buffer, pH 2.2, as BGE. In this case, the separation of the proteins showed a different migration order, and a slightly better resolution. Finally, when the capillaries were coated with IL–water mixtures (100 mM [emim][BF_4] or 100 mM [bmim][BF_4], respectively) and applying the same IL–water mixtures as BGEs, significantly higher resolution and enhanced peak sharpness were obtained, and a change in the migration order can be observed in these experimental circumstances.

As it is obvious from the results obtained by using the same pH, but different BGEs, the resolution of the proteins increases dramatically, when ILs are present. The separation mechanism includes the effect of the ionic-liquid constituents in two ways, both in the change of the capillary surface (i.e., by the coating), but also by the interaction of the ILs with the proteins.

It is important to recognize that the pH of the IL-containing mixtures changed depending on the concentration of the ionic liquid, and on the preparation time of the mixture, which also affected protein separations. When the freshly prepared ionic liquid-containing stock solution was diluted to the appropriate concentration, the separation could not be achieved with the resulting buffer solution. Effective separations were achieved by diluting the stock solution after a 10 day of storage at room temperature. These experiences can be traced back to the hydrolysis of the tetrafluoroborate anion.

The effect of various experimental parameters (applied voltage, migration time, concentration, and type of the ionic liquid) on separation was examined. Optimal conditions for the electrophoretic separation were obtained upon a multivariate analysis of the experimental parameters. Our results showed that there was no significant difference in the separation times when using the two ionic liquids ($p = 0.874$), and during the method development, the 100 mM ionic liquid concentration, +18 kV voltage and 51 cm capillary length were found to be optimal for the separation of proteins.

In our experiments, an anodic EOF was formed in the dynamically coated capillaries and applying the different concentrations of the IL–water mixtures, but the migration of the basic

proteins was cathodic, which means that the mobilities of the proteins suppressed the effect of the anodic EOF.

In intra-day (run to run) and inter-day (day to day and month to month) repeated measurements, the RSD values of the migration times of the proteins were below 10% for both ionic liquids.

This method has been applied successfully for the analyses of real biological samples such as proteins from egg whites and human tears. The electropherograms of real samples showed good resolution of lysozyme and ovalbumin components.

4.2 The role of ionic liquid interaction in the separation of fatty acid methyl esters – separation of polyunsaturated geometric isomers in GC–MS

The retention properties of two FAME mixtures (the 52-component GLC-674 and the 4-component CRM47791, including C4-C24 chain length compounds with high structural diversity) on three commercial capillary columns of different polarities (a non-polar HP-5MS, and a mid/high polarity DB-225MS polysiloxane-based, and an extremely polar ionic liquid-based SLB-IL111 column) were studied. Significant differences in the FAME separation properties were observed between the two polysiloxane-based columns and between the polysiloxane- and the IL-based columns. Although the polysiloxane-based columns were efficient in the separation of saturated and monounsaturated fatty acids, the extreme polar ionic liquid-based column showed the least coelution for both standard mixtures. Additionally, baseline separation of the geometric isomers of linoleic acid methyl ester was obtained by the SLB-IL111 column, which was the first direct GC–MS separation of *cis*,*cis*-9,12; *cis*,*trans*-9,12; *trans*,*cis*-9,12 and *trans*,*trans*-9,12 C18:2 geometric isomers using a 30 m long ionic liquid-based column.

The methods developed for the three columns were validated, *i.e.* the response linearity, limit of detection, limit of quantification, system suitability, intraday and interday repeatability and accuracy were determined. Although all three methods corresponded to the validation criteria, the method developed for the SLB-IL111 column proves to be the most appropriate for the separation of *cis* and *trans* fatty acids. Results showed the separation power of the ionic liquid interaction in the analyses by using short (25–30 m long) columns.

4.3 Effect of culture conditions on fatty acid profiles of bacteria and bacterial lipopolysaccharides by GC-MS

The effect of the culture conditions on the fatty acid components of *P. aeruginosa* PAO1, *P. aeruginosa* ATCC 27853, *P. aeruginosa* polyresistant, and *P. putida* bacteria, and *P. aeruginosa* PAO1 lipopolysaccharides were examined by GC-MS. The fatty acids were analyzed in their methyl ester form, and the separations were carried out on the extremely polar ionic liquid-based SLB-IL111 column, which had previously been successfully used for the separation of fatty acid methyl esters with various carbon atom numbers and saturations. The chromatographic method was validated by applying the 26-component bacterial fatty acid methyl ester mixture by determining the response linearity, limit of detection, limit of quantification, system suitability, intraday and interday repeatability and accuracy. Results showed the high separation efficiency of the ionic liquid-based column in the analyses.

The effects of the cultivation conditions were followed by using agar and blood agar media at the characteristic temperatures, 25 °C, 37 °C and 42 °C, respectively, and an analysis was made during the 1st, 3rd and 5th day following inoculation. Under **optimal conditions** (37 °C growth temperature, agar cultivation media and one-day incubation time), the greatest proportions of the fatty acids in the investigated *Pseudomonas* species were the saturated (SAFAs - 60.6%), and the monounsaturated (MUFAs - 24.4%) fatty acids. Hydroxy- (9.0%), and cyclopropane (8.0%) fatty acids were also detected in the isolates. Furthermore, mainly long chain fatty acids (LCFAs), and to a small extent medium chain fatty acids (MCFAs) were present in the isolates under the ideal conditions. Generally, one of the main mechanisms of *P. aeruginosa* resistance to antibiotics is the maintenance of low permeability of the outer membrane. Given that SAFAs were present generally in high proportions in the cultures indicates a rigid and less permeable membrane structure under optimal conditions. This effect is further enhanced by the presence of a large amount (90%) of LCFAs. Comparing the *P. aeruginosa* PAO1 bacteria and its LPS, significant differences were found mainly in the distribution of the components. Results show that while the bacterium is dominated by MUFAs, LPSs are dominated by hydroxy fatty acids.

Bacteria can evolve including a number of mechanisms which allow them to survive under adverse conditions. These processes are mainly based on the alteration of the membrane composition. The comparison of the major fatty acid classes revealed that the increased **growth temperature** from 25 to 42 °C resulted in an uncresing abundance of saturated fatty acids in all tested isolates. It implies an increasing rigidity and a decreasing permeability of the cell membrane, preventing antibiotics/pollutants to enter the cell. In contrast, a decrease in the proportion of *cis*-MUFAs and an increase in the ratio of cyclopropane fatty acids relative to their MUFA precursors were observed at 37 °C. Since these changes reduce the permeability of the cell membranes, it can be stated, that in our case, the membrane permeability and fluidity

decreases to 37 °C. Additional reactions to the changes in cultivation temperature were found to be the appearance or even the disappearance of fatty acids. Several branched fatty acids could be detected only at 42 °C, and numerous fatty acids, mainly SAFAs, disappeared when the temperature was elevated to 37 or 42 °C. Finally, we found that 2-OH and 3-OH-C12:0 could be identified in PAO1 LPS, of which, the proportion changed in response to the increasing temperature. Changes in the proportion of hydroxy fatty acids can be linked to changes in antibiotic resistance of the bacterium.

Both qualitative and quantitative differences were observed when comparing the fatty acid profiles obtained on agar and blood agar **cultivation media**. In spite of blood agar are enriched medium, in our experiments the conspicuous difference was the disappearance of fatty acids compared to the agar medium. In general, more fatty acids could be identified, and in many cases more intense peaks were observed on agar medium. In only one case, specifically, a new fatty acid formation, C18:1c, occurred in blood agar medium, which is a consequence of uptake from the 18:1c-containing blood agar growth medium.

Incubation time also is found to have a notable effect on the concentration of fatty acids, using agar or blood agar medium. Distinctively, we experienced the most intense fatty acid peaks on the 3rd day of incubation. Since the incubation time increased from day 1 to day 5, we noted mainly distributional differences in the fatty acid profiles, yet in some cases, new fatty acids appeared on days 3 and 5 compared to day 1. *Iso*-branched fatty acids appeared on 3rd and 5th days indicating an increasing membrane fluidity which facilitates compounds to enter the cell membrane. The 42 °C and the 5-day incubation time showed as large as a deviation from the ideal conditions which caused an overall decrease in fatty acid abundances, except hydroxy fatty acids. Additionally, they showed an increasing tendency as the days progressed in agar and blood agar medium. This can be explained by the large amount of accumulation of hydroxy fatty acid containing compounds in bacteria when nutrients are limited. Similarly, a significant decrease in the percentage composition of LCFAs was observed on the 5th day at 42 °C. On agar media, the proportions of MUFAs are decreased, the amounts of cyclopropane fatty acids are increased (e.g., at 25 °C between 1 and 3 days, and at 37 and 42 °C between 3 and 5 days), which confirm the fact that Gram-negative bacteria can respond to limited nutrients by converting MUFAs to cyclopropane fatty acids.

The application of ionic liquid-based column unveils new possibilities for the analyses of fatty acids in GC-MS experiments for bacterial fatty acid profiling.

5 Thesis points

1. Using imidazolium-based ionic liquids, we have developed efficient capillary zone electrophoresis and gas chromatography methods for the determination of proteins and fatty acids in various biological samples.

2. Our results confirm that the use of imidazolium-based ionic liquids in capillary zone electrophoresis and gas chromatography for the examination of biological samples significantly improves the selectivity of separations.

3. We have presented in detail the processes underlying the efficiency increase achieved by using ionic liquids in the case of individual analytical procedures.

4. The validation results confirm that all developed methods are accurate, authentic and suitable for the analysis of the tested materials.

5. Excellent separation of basic proteins was achieved – compared to the experiments carried out in uncoated capillary – by using [bmim][BF_4] and [emim][BF_4] ionic liquids as a buffer component and to modify the inner surface of the capillaries in capillary zone electrophoresis. We have demonstrated that the use of ILs in the separation of proteins can be repeated with high certainty. We showed that the preparation process of ionic liquid solutions affects the pH of the solution and thus the separation of proteins, which relationship has not been previously studied. The developed method was successfully applied to the analysis of proteins in real biological samples, such as egg whites and human tear samples.

6. The use of ionic liquids in capillary electrophoresis offers several advantages. Used as a buffer component and by the dynamic coating of the capillary, they can prevent the components from sticking to the inner surface of the capillary, and the interactions with ionic liquids can increase the efficiency of electrophoretic separations.

7. We found that the highly polar, ionic liquid-based SLB-IL111 column provides exceptional efficiency towars *cis/trans* isomers, most notably for the four geometric isomers of linoleic acid methyl ester, which was the first direct GC–MS separation of *cis,cis*-9,12; *cis,trans*-9,12; *trans,cis*-9,12; and *trans,trans*-9,12 C18:2 geometric isomers using the ionic liquid-based column.

8. The variety of intermolecular interactions (hydrogen bonding, dipole-dipole, dispersion interactions) provided by the ionic liquid stationary phase enables the successful separation, qualitative and quantitative determination of fatty acids with various carbon atom numbers and saturations from biological matrices, such as bacterial isolates. We found that differences in cultural conditions (growth temperature, culture media, incubation time) can

cause changes in the ratio of fatty acids, or even the disappearance of fatty acids or appearance of new fatty acids. This kind of use of the SLB-IL111 column has not been described before.

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7 Publications

Publications related to this thesis

1. **Emerencia Mező**, Csilla Páger, Lilla Makszin, Ferenc Kilár: *Capillary zone electrophoresis of proteins applying ionic liquids for dynamic coating and as background electrolyte component.* ELECTROPHORESIS: (**2020**) 41, 2083-2091. **IF: 3,535**
2. **Emerencia Mező**, Anita Bufa, Csilla Páger, Viktória Poór, Tamás Marosvölgyi, Ferenc Kilár and Lilla Makszin: *The Role of Ionic Liquid Interaction in the Separation of Fatty Acid Methyl Esters—Polyunsaturated Geometric Isomers in GC–MS.* SEPARATIONS: (**2021**) 8, 38-54. **IF: 2,6**
3. **Emerencia Mező**, Fruzsina Hartmann-Balogh, Ibolya Madarászné Horváth, Anita Bufa, Tamás Marosvölgyi, Béla Kocsis and Lilla Makszin: *Effect of Culture Conditions on Fatty Acid Profiles of Bacteria and Lipopolysaccharides of the Genus Pseudomonas—GC-MS Analysis on Ionic Liquid-Based Column.* MOLECULES: (**2022**) 27, 6930-6951. **IF:4,6**

Publications not related to this thesis

1. Buzády Andrea, Tóth György, Unferdorben Márta, Hebling János, Oláh Laura, Hajdara Ivett, Kovács László, **Mező Emerencia**, Lemli Beáta, Kunsági-Máté Sándor, Pálfalvi László: *Dielektromos jellemzők meghatározása a THz-es frekvenciatartományban.* FIZIKAI SZEMLE: (**2016**) 66, 413-417.
2. Lilla Makszin, Péter Kustán Balázs Szirmay, Csilla Páger, **Emerencia Mező**, Krisztina Ildikó Kalács, Vera Pászthy, Erzsébet Györgyi, Ferenc Kilár, Andrea Ludány, Tamás Kőszegi: *Microchip gel electrophoretic analysis of perchloric acid-soluble serum proteins in systemic inflammatory disorders.* ELECTROPHORESIS: (**2019**) 40, 447-454. **IF: 3,535**

Presentations related to this thesis:

1. **Mező Emerencia**, Páger Csilla, Makszin Lilla, Kilár Ferenc: *Application of Imidazolium-based Ionic Liquids in the Separation of Proteins by Capillary Zone Electrophoresis.* 24th International Conference on Chemistry (Szovátafürdő, Románia 2018.10.24-27.): p. 37.

Posters related to this thesis

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