Research Article

Capillary zone electrophoresis of proteins applying ionic liquids for dynamic coating and as background electrolyte component

The use of ionic liquids in capillary electrophoresis, either as coating material or as components of the background electrolyte needs systematic standardization to set up optimal conditions. Excellent separation of the proteins was achieved using 1-ethyl-3-methylimidazolium tetrafluoroborate ([emim][BF₄]) or 1-butyl-3-methylimidazolium tetrafluoroborate ([bmim][BF₄]) ionic liquids using the properly made ionic-liquid–water binary mixtures for the experiments. The binary mixture has a distinctly stable and well perceptible low pH, which depends on the concentration of the ionic liquid, and on the preparation time of the mixture. Optimal conditions for the electrophoretic separation were obtained upon a multivariate analysis of the experimental parameters (applied voltage, migration time, concentration, and type of the ionic liquid). The standardized condition provides a low electroendosmotic flow toward the anode, which, however, did not hinder the proteins to migrate toward the cathode. The migration of cytochrome c, lysozyme, myoglobin, trypsin, and apo-transferrin at a pH around 2, far below the isoelectric points of the proteins, showed RSD values of the migration times less than 7.5% and less than 6.5% when using [emim][BF₄] or [bmim][BF₄], respectively, either in run-to-run or day-to-day experiments. The determination of the extent of the EOF is not possible with the commonly used EOF markers, due to interaction with the ionic-liquid constituents. The interaction of the ionic liquids with the proteins influences the migration order in zone electrophoresis. This method has been applied successfully for the analyses of real biological samples such as proteins from egg whites and human tears.

Keywords:
Capillary zone electrophoresis / Chemical interaction / Dynamic coating / Imidazolium-based ionic liquids / Multivariate statistical analysis / Protein separation

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1 Introduction

Room-temperature ionic liquids (RTILs) are generally referred to as molten salts with low melting points, typically below 100°C or near room temperature. These materials have received a great interest, due to their environmental friendly behavior and extensive applicability. One of the most commonly used and studied ionic liquid (IL) families is composed of an asymmetrically substituted N-containing organic cation (e.g., imidazolium, pyridinium, or ammonium) and a weakly coordinated inorganic anion such as tetrafluoroborate or hexafluorophosphate [1,2]. ILS have several fascinating properties (e.g., negligible vapor pressure, good thermal stability, nonflammability, and miscibility with organic and inorganic solvents) and their chemical and physical properties can be finely tuned for special applications by varying the cations or anions. These features together make ILS excellent candidates for many areas of chemical sciences including synthesis [3], catalysis [4], electrochemistry [5], and analytical processes. Regarding the utilization of RTILs in analytical chemistry, recent studies have focused mainly on the development of IL-functionalized stationary phases in the broad field of chromatography. Imidazolium-based ILS have been shown to be successful as stationary phases for GC [6], LC [7,8], and as pseudo-stationary phases for MEKC [9]. Moreover, they have been used as solvents in MALDI-MS [10] and in extraction processes [11], as mobile phase additives in LC [12], as chiral selectors in enantioselective separations [13] or as BGE additives in aqueous and nonaqueous procedures. Vaher et al. have used dialkylimidazolium-based ILS as BGE in nonaqueous CE to separate water-insoluble substances [14], and they also applied a similar IL for separating carboxylic acids and phenolic and polyphenolic...

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Abbreviations: ([bmim][BF₄]), 1-butyl-3-methylimidazolium tetrafluoroborate; ([emim][BF₄]), 1-ethyl-3-methylimidazolium tetrafluoroborate; IL, ionic liquid; MANOVA, multivariate analysis of variance; RTIL, room-temperature ionic liquid
compounds [15,16]. The results indicated that the main mechanism of the separation in acetonitrile containing medium is based on the heterocoujugation between the salt anion and the anlye molecule. Lu et al. separated aromatic acids in a nonaqueous system, and different migration orders were observed depending on the ILs used for separation [17]. Yanes et al. have reported a reproducible method for the identification of polyphenolic compounds isolated from grape seed extracts, using imidazolium-based ILs as running electrolytes [18]. Dynamic coating is becoming more and more popular in the CE techniques to prevent the adsorption of analytes on the silica capillary surface [19]. The application of a silica capillary, coated with 1-alkyl-3-methylimidazolium-based ILs, for the analysis of basic proteins has been described by Jiang et al. [20]. It was found that the migration times of the analytes are attributed to the association between the proteins and the imidazolium groups on the capillary wall or the free imidazolium ions in the solution. Wu et al. used the same IL for coating and found that it was stable for at least 120 h without the necessity for any regeneration between runs [21]. Recently, Guo et al. has successfully employed N-methyl-2-pyrrolidionium methyl sulfonate IL as a coating material for the separation of basic proteins in CE [22]. Recent reviews highlighted experimental and theoretical studies concerning the ionic-liquid–protein [23,24] and water–ionic-liquid–protein interactions [25–27]. One of the crucial aspects is the stability and activity of proteins in the IL solutions. Several factors can influence these protein features, for example, the nature of the IL anion [28], the length of alkyl chains in the IL cations [29], and other physical chemical properties of ILs [30]. Although, IL anions have stronger effect on protein stability than cations [31,32], simulations have shown that the concentration of cations might transcend that of anions at the protein surface, regardless of protein charge [33,34]. This can be explained by the hydrogen bonded network of the anions and water in the bulk phase and the cationic mobility at the nonpolar and polar protein surface. The presence of water can also affect the stability and activity of proteins. Several reports demonstrate lower protein stability with higher water content [35,36]. Furthermore, structural changes of the proteins caused by addition of water in IL solution have been reported by Takekiyo et al. [37]. The computational studies to examine the water–IL–protein systems consider proteins with various characters (hydrophobic, hydrophilic, and amphiphilic), where interactions with IL anions are dominated by Coulomb forces and hydrogen bonding, the action of IL cations comprise Coulomb forces and dispersion interactions, and the water molecules are forming hydrogen bonding interactions [33,34].

In this article, 1-ethyl-3-methylimidazolium tetrafluoroborate and 1-butyl-3-methylimidazolium tetrafluoroborate ILs were used as coating agent and as BGE component in CZE for the separation of proteins. The main goal was to optimize the separation conditions and to study the effect of the alkyl group of the ILs by evaluating the results with a multivariate mathematical approach.

2 Materials and methods

2.1 Chemicals

The 1-ethyl-3-methylimidazolium tetrafluoroborate ([emim][BF₄]) IL (≥98%) was obtained from Alfa Aesar (Kandel, Germany). The 1-butyl-3-methylimidazolium tetrafluoroborate ([bmim][BF₄]) IL (≥97%), lysozyme (chicken egg white; ≥90%; 14.3 kDa), lysozyme (human; 17 kDa), albumin (chicken egg white; ≥98%; 44.3 kDa), cytochrome c (equine heart; ≥95%; 12.4 kDa), myoglobin (equine heart; ≥90%; 17.0 kDa), trypsin (bovine pancreas; 23.8 kDa), apotransferrin (human; ≥98%; 76–81 kDa), acetone (99.9% for HPLC), nitromethane (≥96% for HPLC), sodium hydroxide and phosphoric acid (85%) were purchased from Sigma Aldrich (Steinheim, Germany, and Saint Louis, MO, USA). Stock solutions (10 mg/mL) of the proteins were dissolved in distilled water. The mixtures of proteins were prepared from the stock solutions with a 1 mg/mL final concentration of each protein. Calibration to determine the LOD values was made with protein solutions down to 10 μg/mL of protein concentrations. Lyophilized chicken egg white (stored at 4°C) was dissolved in distilled water (4 mg/mL) and was centrifuged before use. Human tear samples were collected from the same person and stored at 4°C. Mixtures of ILs and water (between 25 and 125 mM IL) were prepared and incubated for 5 days at room temperature to obtain clear and stable solutions (binary mixture) [38]. After the incubation, a stable pH was measurable with a pH electrode between 1.5 and 2.7, that is, decreasing the ionic-liquid concentration (from 400 to 25 mM) resulted in an increase of the pH. The viscosity and the conductivity parameters of these mixtures were obtained from literature data [39–42], and care was taken to maintain the experimental conditions during the experiments. The IL–water mixtures were used for the pretreatment of the capillaries (coating of the capillary surface), and as BGEs in CE experiments. A 40 mM-phosphate buffer (pH 2.3) was also used as BGE. For the EOF measurements, acetone and nitromethane were used as neutral markers and were dissolved in distilled water in 5- and 50-fold dilutions, respectively.

2.2 CE experiments

CE experiments were carried out on the Agilent 7100 Capillary Electrophoresis System (Agilent Technologies, Waldbronn, Germany) equipped with a UV detector. Separations were performed in fused-silica capillaries (50 μm i.d.) purchased from Polymicro Technologies (Phoenix, AZ, USA). The total length of the capillaries varied between 51 and 96.5 cm (the effective length is 8.5 cm shorter than the total length). The capillaries were conditioned at the beginning of the day, by rinsing with distilled water (5 min), with 0.1 M NaOH (20 min), and with distilled water (10 min).
In the case of experiments using capillaries without coating the capillary was rinsed with the desired BGE. When coating was made, the capillary was rinsed with IL–water mixtures (between 25 and 125 mM) for 30 min. Before injection, a 5 min rinsing with the appropriate IL–water mixture was made to fill the capillary with the respective BGE. Samples were injected by applying 50 mbar for 5 s. After the electrophoretic run, the capillary was rinsed with distilled water for 5 min. The applied voltage was varied between +10 kV and +20 kV (injection end is at the anode), except when EOF measurements were performed, when +18 kV or −18 kV voltage was applied. The electropherograms at 200 nm were recorded and processed by the ChemStation software (B. 04.00. and Version 7.01, Agilent Technologies). The temperature of the capillary cartridge was maintained at 20°C.

2.3 Statistical analysis

Migration times, time corrected areas, and resolution values of the proteins were processed using the SPSS version 25.0 statistics software. Comparison of the means was achieved by multivariate analysis of variance (MANOVA) to determine the significant parameters (p < 0.05) that could differentiate the types of the IL. Migration time, time corrected area, and resolution were taken as dependent variables, while applied voltage, ionic-liquid type, and ionic-liquid concentration were taken as independent variables. Wilk’s Lambda index was computed to determine a possible significant effect of the separation conditions on migration time, time corrected area and resolution. The independent samples t-test was used for the comparison of the groups (ionic-liquid type; ionic-liquid concentration). The p-values less than 0.05 were considered to be significant. All data were expressed as means. Resolutions were calculated between pairs of peaks using the common equation $R = 2Δt/\left(w_1 + w_2\right)$, where $Δt$ is the difference between migration times, and $w_1$ and $w_2$ are the corresponding widths of adjacent peaks. The LODs of the lysozyme samples were calculated on the basis of a signal-to-noise ratio of 3, whereas this ratio was 10 for the calculation of LOQ.

3 Results

Samples containing five proteins, cytochrome c (pI: 10.2), myoglobin (pI: 6.97), lysozyme (pI: 11.35) from chicken egg white, trypsin (pI: 10.5), and apo-transferrin (pI: 6.1) were analyzed in CZE in uncoated and coated capillaries. The capillaries were coated by using binary mixtures of water and different concentrations of 1,3-dialkylimidazolium-based ILs, [emim][BF_4] or [bmim][BF_4]. Phosphate buffer or the IL–water mixture was applied as BGE in zone electrophoresis. The effect of various experimental parameters on separation was examined.

3.1 CE of proteins in the presence of IL

The initial experiments were made with uncoated capillaries using a 40 mM phosphate buffer, pH 2.2. The proteins were not baseline-separated in the experiments (Fig. 1A). To test the effect of the presence of ionic-liquid constituents IL–water mixtures were prepared with different ionic-liquid content. The mixtures exhibited a stable pH, which depended on the ionic-liquid concentration: pH = 2.7 ± 0.2 in 25 mM IL, pH = 2.5 ± 0.2 in 50 mM IL, pH = 2.3 ± 0.2 in 75 mM IL, pH = 2.2 ± 0.2 in 100 mM IL, pH = 2.1 ± 0.2 in 125 mM IL, and pH = 1.5 ± 0.2 in 400 mM IL aqueous solution for both IL types. Purging the capillary with such mixtures a stable coating was obtained on the capillary surface. Figure 1B shows the separation in a capillary coated with 100 mM [bmim][BF_4] applying the 40 mM phosphate buffer, pH 2.2, as BGE. The separation of the proteins showed a different migration order, and a slightly better resolution. Figure 1C and D shows the electropherograms of the proteins obtained with the use of capillaries coated with IL–water mixtures (100 mM [bmim][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. The RSD values of the migration times of the proteins were between 2.5−7.5% (run to run), and 3.7−7.2% (day to day), and 1.0−9.1% (month to month) in case of the [emim][BF_4] IL considering all concentrations. The respective values for [bmim][BF_4] were 2.6−8.9% (run to run), 3.5−6.5% (day to day), and 1.1−9.2% (month to month).

Figure 2 shows the dependence of the migration times of the proteins on applied voltage at two concentrations (50 and 100 mM) of the two ILs. Figure 3 shows that by increasing the ionic-liquid concentration in the BGE the migration velocities of the proteins decrease, and the presence of [bmim][BF_4] resulted in a slower migration at every concentration compared to the presence of the [emim][BF_4] IL. The influence of the capillary length on the separation was also studied, and applying the same voltage (18 kV), that is, by decreasing the field strength by a factor of ca. 2 (the length of the capillary was changed from 51 to 96 cm), the migration times increased by a factor of ca. 5 (Fig. 4).

3.2 CE of acetone

Acetone was injected applying different BGEs in CE experiments. While no apparent migration was found in an uncoated capillary with a phosphate buffer at pH 2.2, a high mobility of the acetone zone was observed in capillaries, coated with IL–water mixtures. The apparent mobility of the acetone zone (15.9 cm²/Vs at 18 kV) fell between those of lysozyme and trypsin in case of the capillary (effective length 42.5 cm, 51 cm total length) coated with 100 mM [emim][BF_4], but was higher (15.9 cm²/Vs) than those of the proteins when
Figure 1. Separation of standard protein mixtures using (A), (B) phosphate buffer, and (C) [emim][BF₄], or (D) [bmim][BF₄] containing ionic-liquid–water mixtures as background electrolytes in zone electrophoresis. The capillaries were used in uncoated state (A) and after coating with [emim][BF₄] (C) or [bmim][BF₄] (B), (D) containing ionic-liquid–water mixtures. Peak numbers represent (1) cytochrome c, (2) lysozyme, (3) myoglobin, (4) trypsin, and (5) apo-transferrin. Resolutions of adjacent peaks: (C) R₁–₃ = 15.29, R₃–₂ = 3.43, R₂–₄ = 16.78, R₄–₅ = 3.1; (D) R₁–₃ = 12.59, R₃–₂ = 3.82, R₂–₄ = 12.53, R₄–₅ = 2.31. Experimental conditions: concentration of the ionic liquids: 100 mM; concentration of the phosphate buffer: 40 mM, pH = 2.2; concentration of proteins: 1 mg/mL; uncoated capillary: 51 cm (42.5 cm to the detector) × 50 μm i.d.; injection of samples: 50 mbar for 5 s; applied voltage +18 kV; temperature: 20°C; UV detection at 200 nm.

3.3 Statistical analysis

A large dataset was built including the experimental parameters, and the values obtained in the experiments. Different “analysis of variance” (ANOVA) procedures were performed to discover the relationship between the data. Table 1 shows a summary of the data for the five proteins. MANOVA was performed to determine a possibly significant effect of the fixed factors on the dependent variables. MANOVA, including ionic-liquid types ([bmim][BF₄], [emim][BF₄]), applied voltages (10, 15, 18, 20 kV) and ionic-liquid concentrations (50, 100 mM) as fixed factors showed statistically significant differences between the ionic-liquid types: F(3, 8) = 387.198, p < 0.001, Wilks’ Λ = 0.007, partial η² = 0.993. Three-way ANOVA and two-way ANOVA were performed with several combinations of independent variables. Statistically significant interactions on migration time (F(3) = 5.712; p = 0.015; partial η² = 0.631) and on peak-area (F(3) = 1476.28; p < 0.001; partial η² = 0.998) were obtained for the ionic-liquid concentration × ionic-liquid type × applied voltage variables, but no significant interaction was obtained on the resolution (F(3) = 0.162; p = 0.919; partial η² = 0.046). The two-way ANOVA procedures showed that the ionic-liquid type × applied voltage variables have statistically significant interaction on peak-area, but no significant interaction on migration time and resolution. Similarly, the ionic-liquid type × ionic-liquid concentration variables have statistically significant interaction on migration time and peak-area, but no significant interaction on resolution. The applied voltage × ionic-liquid concentration variables have statistically significant interaction on migration time and peak-area, but no significant interaction on resolution. The one-way ANOVA showed that the ionic-liquid type has statistically significant effect on migration time and peak-area, the applied voltage has statistically significant effect on migration time and peak-area, and the ionic-liquid concentration has statistically significant effect on migration time, on peak-area and on resolution.
Figure 2. Dependence of the migration times of proteins on the applied voltage in capillary zone electrophoresis using [emim][BF₄] (A, B) or [bmim][BF₄] (C, D) ionic-liquid–water mixtures for coating the capillary and for background electrolyte. The concentrations of the ionic liquids were 50 mM (A, C) or 100 mM (B, D). Markers indicate (■) cytochrome c, (●) myoglobin, (▲) lysozyme, (▼) trypsin, and (●) apo-transferrin. Other experimental conditions are indicated in the legend of Fig. 1.

3.4 CZE of real samples

The applicability of the method was tested with real samples containing similar proteins to the previously tested standards. Figure 5 shows electropherograms of chicken egg white and tear samples applying [bmim][BF₄] as coating agent and also as BGE component. The spiking of the samples with respective (chicken egg white or human) lysozyme standards shows the good resolution of the lysozyme and ovalbumin components of the real biological samples. The electrophoretic runs provided LOD values of 21.77 (± 0.15) μg/mL and of 37.27 (± 0.25) μg/mL for the chicken egg white and for the human lysozyme, respectively. Using calibration curves (the calibration data are listed in Table 2), the lysozyme content of the samples was determined and found to be 0.100 (± 0.01) mg/mL lysozyme in the egg white and 3.65 (± 0.39) mg/mL lysozyme in the human tear samples.

4 Discussion

4.1 Effect of coating with ionic-liquid constituents

To prevent the adsorption of proteins on the silica capillary surface, and to eliminate or modify the EOF, various methods can be used. Early procedures have used low pH [43] or high ionic strength buffers [44], but the most common approach is based on the coating of the capillary inner wall to decrease the interaction between the capillary wall and the proteins [45]. The bare fused-silica capillary can be covalently or non-covalently coated to eliminate the wall adsorption of proteins. Several groups reported that ILs were covalently bonded to the capillary wall to modify the surface [46, 47]. Compared to covalent binding, dynamic coating has the advantage of easier operation, whereas, the resulting surface is stable enough [21,22,48]. In this article, the separation of five proteins was
tested in capillaries coated with 1,3-dialkylimidazolium-based ILS.

The pH of the IL-containing BGEs was between 1.5 and 3, depending on the IL concentration. The proteins had high isoelectric points, therefore, at the applied pH, they were positively charged. In an uncoated bare fused silica capillary, the proteins can adsorb on the capillary inner surface depending on the surface charge (pH), but with the use of an appropriate coating, for example, the cationic constituent of IL the adsorption of the proteins can be prevented. One of the most important question, whether EOF will be developed or not, and what will be the direction of the EOF, when using ionic-liquid component in the BGE. In previous studies both, positive [21] and negative [20] polarities (i.e., migration toward the cathode or anode, respectively) were used successfully for separations of proteins in the presence of ILS. Using a low IL concentration together with acetate buffer at pH 4 in the BGE a “co-electroosmotic capillary electrophoresis” showed separation of basic proteins within a short time, but with low resolution [19]. 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

Table 1. Summary of MANOVA analyses

| Fixed factors           | Dependent variables | F value | df | p value | partial η² |
|-------------------------|---------------------|---------|----|---------|------------|
| Ionic liquid type       | Migration time      | 8.703   | 1  | 0.015   | 0.465      |
| (A)                     | Time corr. area     | 1075.088| 1  | <0.001  | 0.991      |
|                         | Resolution          | 4.545   | 1  | 0.059   | 0.312      |
| Applied voltage         | Migration time      | 9853.769| 3  | <0.001  | 1.000      |
| (B)                     | Time corr. area     | 1153.689| 3  | <0.001  | 0.997      |
|                         | Resolution          | 1.359   | 3  | 0.311   | 0.290      |
| Ionic liquid concentration (C) | Migration time  | 10392.125| 1  | <0.001  | 0.999      |
|                         | Time corr. area     | 8307.918| 1  | <0.001  | 0.997      |
|                         | Resolution          | 21.375  | 1  | 0.001   | 0.681      |
| AB                      | Migration time      | 1.55    | 3  | 0.262   | 0.317      |
|                         | Time corr. area     | 1532.245| 3  | <0.001  | 0.998      |
|                         | Resolution          | 0.242   | 3  | 0.865   | 0.068      |
| AC                      | Migration time      | 132.923 | 1  | <0.001  | 0.930      |
|                         | Time corr. area     | 1231.948| 1  | <0.001  | 0.992      |
|                         | Resolution          | 1.193   | 1  | 0.300   | 0.107      |
| BC                      | Migration time      | 700.953 | 3  | <0.001  | 0.995      |
|                         | Time corr. area     | 1093.704| 3  | <0.001  | 0.997      |
|                         | Resolution          | 0.579   | 3  | 0.642   | 0.148      |
| ABC                     | Migration time      | 5.712   | 3  | 0.015   | 0.631      |
|                         | Time corr. area     | 1476.28 | 3  | <0.001  | 0.998      |
|                         | Resolution          | 0.162   | 3  | 0.919   | 0.046      |

Results corresponding to p values shown in bold are statistically significant.
Figure 5. Capillary zone electrophoresis of proteins in egg white and in human tear samples. A 100 mM \([\text{bmim}]\text{[BF}_4\text{]}\)–water mixture was used for coating and as background electrolyte. (A) Electropherograms of lyophilized egg white dissolved in water (solid line) and egg white sample spiked with lysozyme and ovalbumin (dashed line). Final concentration of the added lysozyme was 0.5 mg/mL, and the added ovalbumin was 5.5 mg/mL. (B) Zone electrophoresis of a human tear sample (solid line), and after spiking with human lysozyme (dashed line). Other experimental conditions are described in the legend of Fig. 1.

Table 2. Calibration data for lysozyme content determination in real samples

| Compound | Slope | Intercept (μg/mL) | Correlation Coefficient | LOD (μg/mL) | LOQ (μg/mL) |
|----------|-------|-------------------|-------------------------|-------------|-------------|
| Lys-human | 205.06 | -18.04 | 0.997 | 250–2000 | 37.27 | 124.23 |
| Lys-egg | 372.37 | -13.83 | 0.996 | 125–2000 | 21.77 | 72.57 |

\(n\) (number of data points) for Lys-human is 5, for Lys-egg is 6. SE (standard error) of peak areas for Lys-human is 22.01 and 24.35 for Lys-egg.

EOF. Within a reasonable run-time, high resolution and enhanced peak sharpness were obtained. Besides these observations, however, another effect appeared due to the presence of ILs in the system. While in conventional systems, increasing the buffer concentration might lead to a decrease in EOF, possibly causing a longer separation process, we observed, that with increasing the IL concentration from 25 to 125 mM, the migration times of the protein samples increased. This might be ascribed to the adsorption of the IL constituents onto the wall of the capillaries, which affects the EOF, but more importantly the effect is due to the altered electrophoretic behaviour of the basic proteins by the interaction with the ILs to dissimilar extents. It is also important to recognize that the measured pH increases upon decreasing the IL concentration. The follow-up of such effects needed the application of the statistical analysis.

It is difficult to determine the exact EOF mobility with the commonly used EOF-markers, such as acetone, or, for example, nitromethane, because the ionic-liquid constituents provide a distinct interaction with these markers [49,50]. We could realize that the acetone showed different behaviour in BGEs containing \([\text{emim}]\text{[BF}_4\text{]}\) or \([\text{bmpm}]\text{[BF}_4\text{]}\), but in both cases the migration of the acetone zone was higher than some of the proteins studied. This shows that acetone, which obviously had a strong interaction with the cations of the ILs, cannot be used as an EOF marker, since in this system, where a surface with high positive charge exists, the EOF should be lower than the mobilities of the positively charged substances. This phenomenon was not further investigated.

4.2 Mechanism of separation

Besides the dynamic coating of the capillary wall, the ionic-liquid–protein interaction also contributes to the successful separation of proteins. 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 (Fig. 1C and D), compared to the low resolution obtained with the phosphate buffer (Fig. 1A and B). The effect of phosphate at pH 2.2 has been shown to influence the conformation of proteins (mainly the hydrodynamic radius) [51]. The change in the migration order observed in the experiments with ILs indicates that substantial interaction must exist between the ionic-liquid constituents and proteins. Similar results have been observed by Jiang et al. [20], who studied the effect of the alkyl group of different kinds of ILs and obtained that, although, the migration order of the proteins was not affected, significant differences in the analysis time and peak shape could be observed for ILs with different alkyl chains. They found that \([\text{emim}]\text{[BF}_4\text{]}\) provided short analysis times and sharp peaks for cytochrome c, lysozyme, trypsinogen, and \(\alpha\)-chymotrypsinogen. It should be noted that the mobility of myoglobin in the different experimental
setups is varying somehow interestingly. Such phenomenon can be derived from the structural properties of this protein, which has previously been observed, when crystallization was studied in the presence of ILs [52,53]. Our results, however, showed that no significant differences in separation times (p = 0.874) and no change in the migration order were obtained for the two ILs, although, the [bmim][BF₄] possessed a more sensitive detection with enhanced peak sharpness.

Although, anions might have stronger effect on protein stability than cations [31,32], there are no clear data in the literature about the possible effect of IL anions on the protein separation by CE in aqueous media. Simulations, however, show that the concentration of cations transcends that of anions at the protein surface, independently of the protein charge [33,34]. This can be explained by the hydrogen bonded network of the anions and water in the bulk phase and the cationic mobility at the nonpolar and polar protein surface [34]. The interactions between water and IL ions are predominantly stronger with anions, than cations [54].

Based on these, it is believed that imidazolium cations can have an effect on the separation. Nevertheless, anions interact with the appropriate parts of proteins via Coulomb forces and hydrogen bond interactions. A recent study demonstrated that surface active ILs can modulate the activity, structure and stability of lysozyme, and the IL-lysozyme interaction was found to depend on the composition and concentration of the SAILs [55].

### 4.3 Applicability of the method

The applicability of the method, using ionic-liquid-containing electrolytes, was proven by analyzing chicken egg white and human tear. The excellent separation of the lysozyme from other proteins allows the determination of the lysozyme content in the samples. It was, however, not possible to make quantitative evaluation of the other major protein component, ovalbumin, in the egg white, since, in fact, this protein is a complex mixture of several glycoforms, as it has been shown previously [56]. Although, the many ovalbumin components are also separated here, the identification and quantitation is not possible due to the overlapping with other proteins in egg white.

### 5 Concluding remarks

Coating the capillary with IL constituents enhances resolution, peak efficiency, and peak symmetry, so their potential use in protein separation is increasing. In this article, we present an effective method for the separation of basic proteins by CZE using 1,3-dialkylimidazolium-based ILs for dynamic coating and also as BGE components. Imidazolium-based ILs with ethyl or butyl groups in the structures were used, and the concentration of the [emim][BF₄] or the [bmim][BF₄] ILs, the applied voltage, and the capillary length were studied and evaluated by multivariate statistical analysis for the separation of cytochrome c, lysozyme, myoglobin, trypsin, and apo-transferrin. Excellent separation of the proteins is obtained with 100 mM ionic-liquid solutions applying 350 V/cm field strength for the separation. 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. Based on our results, we assume that the imidazolium cations can have high effect on the separation, although, anions might interact with the appropriate parts of proteins via Coulomb forces and hydrogen bond interactions. In our article, we show that the use of ILs in the separation of proteins can be reproduced with high certainty and also the experiments were statistically confirmed by the MANOVA procedure. We could prove that a significant contradiction exists in some articles, which show that the coating with IL may provide both, anodic or cathodic endosmotic flow. This contradiction was resolved in our experiments, since we showed the strict preparation process of the IL-water mixtures. In this way, we systematically standardized the capillary zone electrophoretic separation to set up optimal conditions. The interaction of the ILs with the proteins influences the migration order in zone electrophoresis, which provides a good basis to investigate the interaction between proteins and ionic-liquid components. Such approaches have not been demonstrated in the cases of the application of other coatings currently available. It is also important that this method has been applied successfully for the analyses of real biological samples such as proteins from egg whites and human tears.

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