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Influence of electrolyte composition on the electroosmotic flow and electrophoretic mobility of proteins and peptides

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Abstract

The influence of electrolyte composition on the electroosmotic flow and peptide/protein migration behavior in capillary zone electrophoresis, either with bare fused-silica or polyacrylamide-coated capillaries, has been investigated. The examined electrolyte solutions consist of buffers tailored for controlling the protonic equilibrium over a wide pH range and effective at masking the active adsorption sites of the capillaries for proteins and peptides. Such buffers are composed of the aliphatic oligoamine triethylentetramine (TETA), in combination with either a monoprotic or a polyprotic acid. The drastic variations in the electroosmotic flow and the inhibition of untoward interactions of basic proteins with the capillary wall observed over a wide pH range were associated with the specific adsorption of TETA ions at the interface between the capillary wall and the electrolyte solution. Modifications of the migration behavior of basic proteins and closely related peptides observed using different buffer anions, such as perchlorate, phosphate and citrate, in combination with TETA may be the result of selective interactions of these counter-ions with the analytes.

Keywords: Buffer composition; Electroosmotic flow; Electrophoretic mobility; Proteins; Peptides

1. Introduction

Proteins and most peptides are ionogenic substances which also bear other functionalities such as hydrogen-bonding regions, hydrophobic patches and biospecific interaction sites that may interact to different extents with the components of the electrolyte solutions used for their separation by capillary zone electrophoresis (CZE). Depending on the chemical nature of either the protein or the peptide and the ionic species in solution, such interactions can lead to significant variations in the electrophoretic mobility of these analytes that can greatly affect their selective separation by CZE [1–4]. In addition, the different functionalities of proteins exposed to the aqueous solution may interact with a variety of active sites on the inner surface of the fused-silica capillaries, which comprise inert siloxane bridges, hydrogen-bonding sites and different types of ionizable silanol groups (vicinal, geminal, and isolated) [5]. These interactions may give rise to peak broadening and asymmetry, irreproducible migration times, low mass recovery, and, in some cases, irreversible adsorption.

The composition of the electrolyte solution employed in capillary electrophoresis influences both the mobility of the analytes by a variety of mechanisms involving secondary equilibrium in solution [6,7] and the distribution of ions in the electric double layer at the interface between the capillary wall and the electrolyte solution [8]. The result is

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that the composition of the electrolyte solution can be tailored not only for controlling the protonic equilibrium within the desired pH range, but also for preventing untoward interactions of peptides and proteins with the capillary wall, modulating selectivity and adjusting the electroosmotic flow (EOF) to meet the requirements for a given peptide and/or protein separation [9,10].

Our research has centered on the effect of the composition of the electrolyte solution on the electroosmotic flow and on the migration behavior of peptides and proteins, with the purpose of modulating resolution and selectivity while controlling protonic equilibrium and untoward interactions of these analytes with the capillary wall. Selectivity in CZE is based on differences in the electrophoretic mobility of the analytes, which depends on their effective charge-to-hydrodynamic radius ratios. This implies that selectivity is strongly affected by the pH of the electrolyte solution and by any interaction of the analyte with the components of the electrolyte solution affecting its charge and/or hydrodynamic radius.

Additives in the electrolyte solution can improve selectivity by interacting specifically, or to different extents, with the components of the sample. Most of the additives employed in peptide and protein CZE are amino-modifiers, zwitterions, anionic or cationic ion-pairing agents, organic solvents, and denaturing agents [11].

We have already reported a variety of amino compounds, including amines [12], amino sugars [13], and oligoamines [14,15], that can meet the multitasking characteristics requested of the components of electrolyte solutions employed in peptide and protein capillary electrophoresis. In this study we have further investigated the influence of the acidic component of buffers consisting of the oligoamine triethylentetramine (TETA) in combination with either a monoprotic or a polyprotic acid on the mobility of peptides and proteins either in bare fused-silica or polyacrylamide-coated capillaries. Triethylentetramine is an aliphatic tetramine with pK_a values of 3.25, 6.56, 9.08, and 9.74 [16]. Therefore, TETA exhibits an effective buffering capacity both in acidic and basic pH domains and, when combined with a polyprotic acidic buffering agent, such as phosphoric or citric acid, the capability of the resulting buffer at controlling the protonic equilibrium can span a wide pH range. This study examined the dependence of the electroosmotic flow and electrophoretic behavior of selected basic model proteins and closely related decapeptides on the anionic component of buffers consisting of an aqueous solution of triethylentetramine in combination with either a monoprotico or a polyprotic acid.

2. Experimental

2.1. Chemicals and samples

Triethylentetramine (TETA) was obtained from Fluka (Milan, Italy) and was used without further purification. Mesityl oxide and phenyltrimethylammonium iodide were purchased from Aldrich (Milan, Italy). Cytochrome *c* (from horse heart), lysozyme (from chicken egg white), ribonuclease A (from bovine pancreas), α-chymotrypsinogen A (from bovine pancreas), and synthetic decapeptides with the amino acid sequence of human (DRVYIHPFHL), salmon (DRVYVHPFNL), and bullfrog (NRVYVHPFNL) angiotensin I were obtained from Sigma (Milan, Italy). Reagent-grade perchloric acid, phosphoric acid, citric acid, hydrochloric acid, acetic acid, potassium hydroxide, sodium acetate, and HPLC-grade water were supplied by Carlo Erba (Milan, Italy). Acrylamide, potassium persulfate, *N*,*N*,*N*′*N*′-tetramethylethylendiamine (TEMED), all electrophoresis grade, were purchased from Bio-Rad Labs. (Milan, Italy).

2.2. Capillaries

Bare fused-silica capillary tubes of 0.050 mm I.D.×0.375 mm O.D., and total length either 415 or 405 mm were purchased from Quadrex (New Haven, CT, USA). The distance from the detection window to the end of the capillary was 85 mm. Prior to use, the new fused-silica capillary was flushed successively with 0.5 *M* sodium hydroxide (30 min), water (10 min), and 0.5 *M* hydrochloric acid (30 min), followed by a second treatment with water (10 min), 0.5 *M* sodium hydroxide (30 min), and water (10 min). Chemical coating of the inner surface of the capillary tube with non-crosslinked polyacrylamide

was performed according to a method reported previously [17].

2.3. Electrophoresis

The experiments were performed using an HP ^{3D}CE capillary electrophoresis system, equipped with a diode-array UV–Vis detector, and an air-cooling device for temperature control of the capillary cartridge (Agilent, Waldbronn, Germany). The instrument was interfaced to an HP Vectra XM 5 166 MHz personal computer utilizing HP ^{3D}CE ChemStation software for system control and data acquisition and evaluation. Samples were injected in hydrodynamic mode at 50 mbar for 2.0 s and were detected at 214 nm. Buffers were prepared daily and conserved in brown bottles at room temperature. The volume of amine required for the desired concentration was mixed in a beaker with at least 90% (v/v) of the total water volume and the solution was titrated to the desired pH with the appropriate acid under stirring at 25 °C. The solution was transferred to a volumetric flask and the final volume adjusted with water. Thereafter, the pH of the final solution was measured again. The running electrolyte solutions containing TETA and one of the investigated acids at a fixed concentration were prepared in a similar manner by titrating the requested amount of acid to the desired pH value with an aqueous solution of TETA of adequate concentration. The pH was measured with a Model HI 1131 glass electrode and a Model HI 9017 microprocessor pH meter, both from Hanna Instruments (Woonsocked, RI, USA). All solutions were filtered through a type HA 0.22 μm membrane filter (Millipore, Vimodrome, Italy) and degassed by sonication before use. Before each run the running electrolyte in the electrode compartments was renewed and the capillary was rinsed with the running electrolyte for 3 min. Each time a running electrolyte solution of different pH was employed, it was flushed through the capillary for 30 min. When the new solution also differed in composition, a new acrylamide-coated capillary was employed, whereas the bare fused-silica capillary was flushed successively with water (10 min), 0.5 *M* sodium hydroxide (30 min), water (10 min), and the new electrolyte solution (30 min). All experiments were carried out at a constant applied voltage with the temperature of the capillary cartridge set at 25 °C.

2.4. Measurement of the electroosmotic flow

The electroosmotic flow was determined by measuring the migration time of mesityl oxide, used as a neutral tracer. Depending on the composition and pH of the electrolyte solution, the electroosmotic flow was either cathodic or anodic. In the first case, mesityl oxide was detected at the cathodic end of the capillary. In the case of anodic electroosmotic flow the electrode polarity was reversed so that sample introduction and detection took place at the cathodic and anodic end, respectively. The electroosmotic flow was considered to be virtually eliminated when the neutral marker did not appear at the detector after 300 min of sample injection either at the cathodic or the anodic end. All measurements of the electroosmotic flow were made in quintuplicate.

3. Results and discussion

3.1. Effect of triethylentetramine

Both pH and ionic strength influence the protonic equilibrium of ionogenic substances in the electrolyte solution and that of fixed charged groups on the surface of the capillary wall, affecting the charge density in the electric double layer and, consequently, the zeta potential. In addition, the ionic strength influences the thickness of the electric double layer (κ^{-1}) at the interface between the capillary wall and the electrolyte solution, with the result that, when the ionic strength increases, the zeta potential and, consequently, the EOF decrease [18]. Accordingly, we performed a comparative study on the influence of the composition of the electrolyte solution on the EOF and protein mobility maintaining both the pH and ionic strength constant.

Fig. 1 shows the separation of four standard basic proteins carried out in a bare fused-silica capillary with either sodium acetate or TETA phosphate buffer, both at pH 4.0 and the ionic strength adjusted to the common value of 138 m*M* by incorporating an appropriate amount of sodium chloride in the electrolyte solution. The first peak in each electrophero-

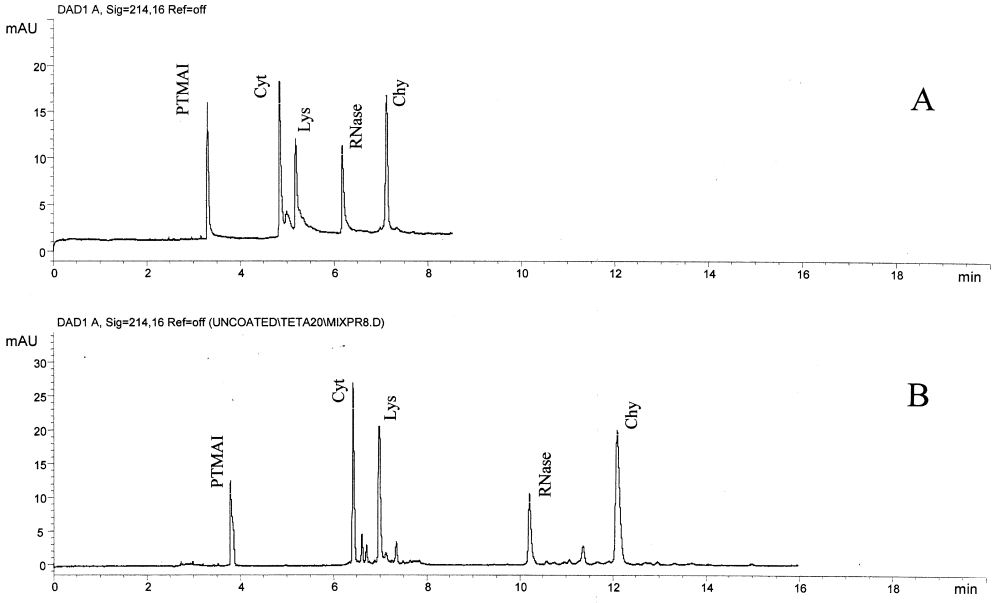

Fig. 1. Separation of basic proteins in a bare fused-silica capillary with 100 m*M* sodium acetate buffer (A) and with 20 m*M* TETA phosphate buffer (B), both at pH 4.0 and the ionic strength adjusted to the common value of 136 m*M* by adding an appropriate amount of sodium chloride (see Experimental). Capillary, bare fused-silica 405 mm (320 mm to the detector)×0.050 mm I.D.×0.375 mm O.D.; applied voltage, 10 kV [current, (A) 49.1 μA and (B) 24.0 μA]; detection wavelength, 214 nm at the cathodic end; temperature of the capillary cassette, 25 °C; samples, phenyltrimethylammonium iodide (PTMAI), cytochrome *c* (Cyt), lysozyme (Lys), ribonuclease A (RNase), α-chymotrypsinogen A (Chy).

gram corresponds to the detection of the alkyl quaternary ammonium salt phenyltrimethylammonium iodide (PTMAI), which was included in the protein mixture to obtain an approximate estimation of the contribution of the electroosmotic flow to the apparent mobility of the proteins [19]. Being a quaternary ammonium salt, PTMAI is fully ionized over the whole pH range and its electrophoretic mobility is consequently unaltered by varying the pH of the electrolyte solution. In addition, the electrophoretic mobility of PTMAI has been shown to be unaffected by the composition of the electrolyte solutions employed in this study. Therefore, its apparent mobility is indicative of changes in the electroosmotic flow. The electrophoretic mobility of PTMAI measured at different pH values within the range from pH 3.0 to 8.0 and with various electrolyte solutions of different composition was found to be $3.31(\pm 0.09)\cdot 10^{-8}$ m^2 V^{-1} s^{-1}.

From the electropherograms displayed in Fig. 1 the results show that with TETA phosphate buffer the apparent mobility of PTMAI and, hence, the EOF, is significantly lower than with acetate buffer at the same value of pH and ionic strength. This results in the longer migration times displayed by all proteins. In effect, with the electrolyte solution consisting of 20 m*M* TETA phosphate buffer at pH 4.0 the EOF was anodic, as was assumed by measuring the migration time of the neutral marker mesityl oxide injected at the cathodic end of the capillary tube. The coefficient of the anodic electroosmotic flow measured with TETA phosphate buffer was $1.96\cdot 10^{-9}$ m^2 V^{-1} s^{-1}. The reversal of the direction of the EOF from cathodic to anodic is indicative of the specific adsorption of the positively charged TETA ions at the interface between the capillary wall and the electrolyte solution [13]. The adsorption of the aliphatic oligoamine results in a drastic variation of the positive charge density in the immobilized region of the electric double layer, which exceeds the negative charge density on the capillary wall resulting from the ionization of silanol

Table 1
Observed current at different values of the applied voltage with TETA phosphate and sodium acetate buffer. Capillary and experimental conditions as in Fig. 1

Applied voltage (kV)	Current (μA)	
	20 m*M* TETA phosphate	100 m*M* sodium acetate
5	11.4	22.8
10	24.0	49.1
15	38.4	82.9
20	56.5	133.6
25	79.9	
30	111.9	

groups. The result is that the zeta potential becomes positive and the concomitant electroosmotic flow is reversed from cathodic to anodic.

Due to the low mobility of both TETA and phosphate ions, TETA phosphate buffer exhibits much lower conductivity than an acetate buffer of identical ionic strength and pH, as can be seen in Table 1. This permits us to carry out protein separation at an electric field 2.5 times higher than that applied with the acetate buffer, consisting of ions of higher mobility, with the result of drastically reducing the separation time, while maintaining unaffected the resolution and selectivity, as depicted by the electropherogram shown in Fig. 2.

3.2. Influence of pH

Besides allowing the application of higher values of the electric field in comparison with traditional buffers, TETA phosphate buffer is effective at masking the silanol adsorption sites for proteins in bare fused-silica capillaries also in the alkaline pH domain, as shown by the electropherogram displayed in Fig. 3, showing the separation of the above protein test mixture at pH 8.0. Comparing this electropherogram with that displayed in Fig. 1(A) it can

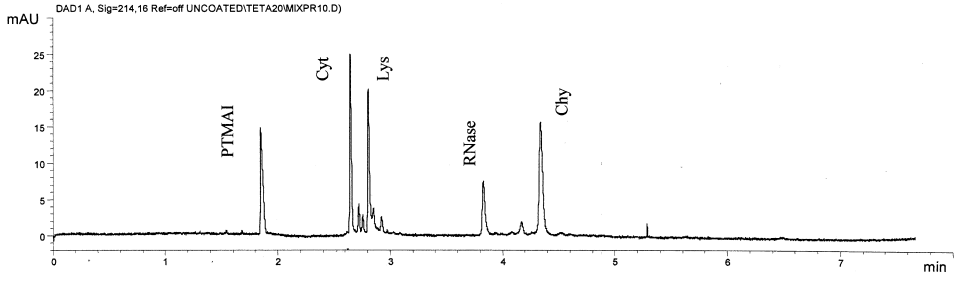

Fig. 2. Separation of basic proteins in a bare fused-silica capillary with 20 m*M* TETA phosphate buffer at pH 4.0 (ionic strength, 136 m*M*) and an applied voltage of 25.0 kV (current, 79.9 μA). Capillary, samples and other experimental conditions as in Fig. 1.

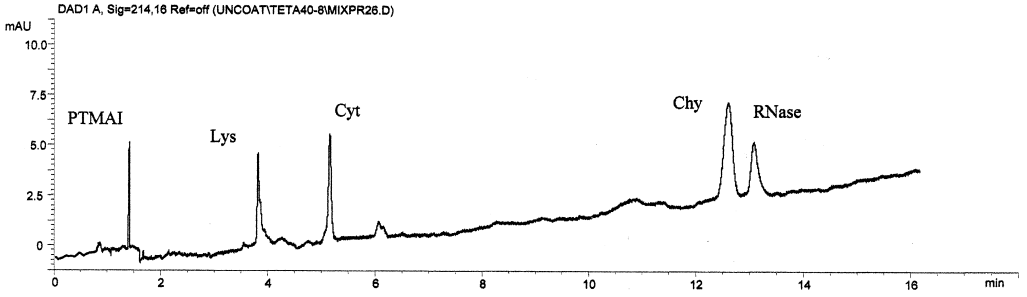

Fig. 3. Separation of basic proteins in a bare fused-silica capillary with 40 m*M* TETA phosphate buffer at pH 8.0. Applied voltage, 25.0 kV (current, 76.2 μA); capillary, samples and other experimental conditions as in Fig. 1.

be seen that, at pH 8.0, the migration order of the protein pair cytochrome *c* and lysozyme and that of the protein pair ribonuclease A and α-chymotrypsinogen A is reversed with respect to that observed at pH 4.0.

In order to explain the different migration order of the two protein pairs we investigated the influence of pH on the EOF and protein migration behavior of the four proteins within the pH range from 3.0 to 8.0. The study was conducted with 40 m*M* TETA phosphate buffer at different pH values spanning the range from pH 3.0 to 8.0. These buffers were prepared by titrating an aqueous solution of 40 m*M* triethylentetramine to the desired pH value with phosphoric acid.

Table 2 reports the electrophoretic mobility (μ_e) of the proteins and the coefficient of the electroosmotic flow (μ_{eof}), calculated from the migration time of the neutral tracer mesityl oxide, measured at each pH value. A negative value of μ_{eof} indicates that the electroosmotic flow is directed towards the anode, whereas a positive value of μ_{eof} indicates the cathodic direction of the EOF. Analysis of the data displayed in Table 2 shows a strong impact of pH on the EOF, which is very low and cathodic at pH 8.0, whereas it is anodic at pH values <8.0. The values of the coefficient of the electroosmotic flow reported in Table 2 indicate that the anodic EOF progressively increases with increasing pH from 3.0 to 5.0 and gradually decreases with increasing pH from 5.0 to 7.0.

The reversal of the direction of the EOF from cathodic to anodic indicates the specific adsorption of TETA on the capillary wall. In such a case the positively charged TETA ions are believed to be firmly held in the compact region of the electric double layer by forces additional to those of simple Coulombic origin. Increasing the pH of the electrolyte solution has an opposite effect on the protonation of the amino groups of TETA ions, which decreases with increasing pH, and on the ionization of the silanol groups on the capillary wall, which increases with increasing pH. The progressive increase of the pH from 3.0 to 8.0 has the effect of progressively lowering the number of protonated amino groups, with the result of reducing the net positive charge of TETA ions while increasing the number of neutral amino groups available for establishing interactions additional to those of simple Coulombic origin and the number of negatively charged silanol groups attracting other TETA ions into the Stern layer.

The values of the coefficient of the electroosmotic flow reported in Table 2 indicate that, up to pH 5.0, the reduction in the number of protonated amino groups is largely balanced by the increment in adsorbed TETA ions, resulting in a more positive zeta potential and consequent higher anodic EOF. It can also be seen that the decreasing anodic EOF observed above pH 5.0 is a result of the increase of the superficial density of ionized silanol groups, which progressively reduces the positive charge density in the Stern layer due to the adsorption of TETA ions, whose net positive charge decreases with increasing pH. Moreover, from the cathodic EOF measured at pH 8.0 it can be inferred that, at this pH, the net negative charge in the Stern region of the electric double layer, due to silanol ionization, exceeds the positive net charge of adsorbed TETA ions, which are still positively charged, as can be deduced from the pK_a values of TETA (3.25, 6.56, 9.08, and 9.74) [16].

Table 2
Coefficients of the electroosmotic flow (μ_{eof}) and values of the electrophoretic mobility of proteins (μ_e) obtained with 40 m*M* TETA phosphate buffer at pH values ranging from 3.0 to 8.0

pH	μ_{eof} ($m^2\ V^{-1}\ s^{-1}$)	Electrophoretic mobility of proteins ($m^2\ V^{-1}\ s^{-1}$)			
		Cytochrome *c*	Lysozyme	Ribonuclease A	α-Chymotrypsinogen A
3.0	$-1.74\cdot10^{-9}$	$2.55\cdot10^{-8}$	$2.22\cdot10^{-8}$	$1.82\cdot10^{-8}$	$1.67\cdot10^{-8}$
4.0	$-2.96\cdot10^{-9}$	$2.01\cdot10^{-8}$	$1.90\cdot10^{-8}$	$1.38\cdot10^{-8}$	$1.21\cdot10^{-8}$
5.0	$-8.33\cdot10^{-9}$	$1.52\cdot10^{-8}$	$1.62\cdot10^{-8}$	$1.00\cdot10^{-8}$	$9.40\cdot10^{-9}$
6.5	$-4.57\cdot10^{-9}$	$1.09\cdot10^{-8}$	$1.39\cdot10^{-8}$	$5.60\cdot10^{-9}$	$4.57\cdot10^{-9}$
7.0	$-2.57\cdot10^{-9}$	$1.13\cdot10^{-8}$	$1.44\cdot10^{-8}$	$5.80\cdot10^{-9}$	$6.00\cdot10^{-9}$
8.0	$9.65\cdot10^{-10}$	$7.60\cdot10^{-9}$	$1.02\cdot10^{-8}$	$2.10\cdot10^{-9}$	$2.40\cdot10^{-9}$

The electrophoretic mobilities (μ_e), calculated for each protein at the different pH values from the corresponding values of the apparent mobility and coefficient of electroosmotic flow, are plotted as a function of the electrolyte pH in Fig. 4. As expected, for all proteins the electrophoretic mobility decreases with increasing electrolyte pH in accordance with the corresponding lower degree of protonation of the proteins. It can also be seen that the reversed order of the migration times of the protein pairs lysozyme/cytochrome *c* and ribonuclease A/α-chymotrypsinogen A above pH 4.0 arises from the different slopes of the curves describing the dependence of the electrophoretic mobility of the proteins on pH.

3.3. Dependence of selectivity on the buffer anion

Several authors have shown that the buffer anion may influence the electrophoretic behavior of a variety of analytes, as well as the extent of the electroosmotic flow in bare fused-silica capillaries [20–22]. Therefore, we investigated the dependence of the electrophoretic mobility of peptides and proteins on the chemical composition of the acid employed in combination with TETA as the buffer at pH 5.8. This pH value was selected on the basis of its equidistance from the pH values at which the inversion of the migration order of the two protein pairs lysozyme/cytochrome *c* and ribonuclease A/α-chymotrypsinogen takes place. The study was conducted with three different electrolyte solutions, each containing 50 m*M* of either a monoprotic or a triprotic acid, which were titrated to pH 5.8 with an aqueous solution of TETA. The triprotic phosphoric and citric acid and the monoprotic perchloric acid were selected on the basis of their hydrogen-bonding properties [23].

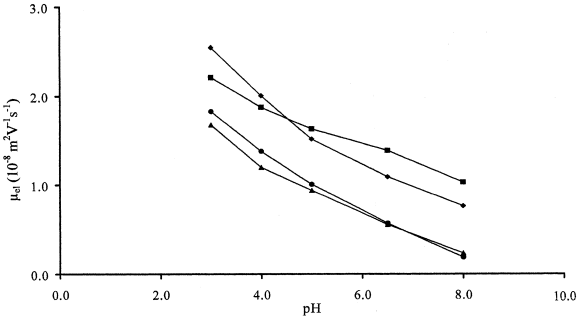

Fig. 4. Plots of the electrophoretic mobility of the proteins as a function of pH with electrolyte solutions consisting of 40 m*M* TETA adjusted to the desired pH by adding an appropriate amount of phosphoric acid. Proteins, cytochrome *c* (♦), lysozyme (■), ribonuclease A (●), α-chymotrypsinogen A (▲); applied voltage, 12.0 kV; capillary and other experimental conditions as in Fig. 1.

A polyacrylamide-coated capillary exhibiting an absence of EOF with any of the above buffers was preferred to a bare fused-silica capillary to make the observations independent of the EOF, which can be influenced by the different acids comprising the buffer system [20]. Three TETA-based buffers were employed to separate the above four basic proteins and a peptide test mixture comprising three synthetic decapeptides, having the sequence of human, salmon, and bullfrog angiotensin I, whose primary structure varies from one to three amino acids [24].

The electropherograms displayed in Figs. 5 and 6 show that both the electrophoretic mobility and the migration order of the peptides and proteins are strongly influenced by the composition of the electrolyte solution, whereas the migration time of phenyltrimethylammonium iodide is nearly the same with any buffer system, indicating that the absence of electroosmotic flow with the polyacrylamide-coated capillary is basically maintained with either electrolyte solution and that the electrophoretic mobility of the quaternary ammonium salt is practically unaffected by the composition of the investigated buffers.

Perchlorate, phosphate and citrate ions are believed to interact with the amino functions of peptides and proteins via ion-pair formation [25,26]. Selective ion-pair formation is expected to increase the differences in the effective charge-to-hydrodynamic radius ratio of peptides and proteins, leading to enhanced differences in their electrophoretic mobilities with the result of improved selectivity and resolution [6,27]. Here, the occurrence of selective interactions between the analytes and the buffer anions is inferred by the inversion of the migration order of ribonuclease A and α-chymotrypsinogen A observed with TETA citrate buffer and by the significantly different migration behavior exhibited by human and salmon angiotensin I with the three buffer systems. The differences in selectivity of these two peptides are evidenced by their diverse migra-

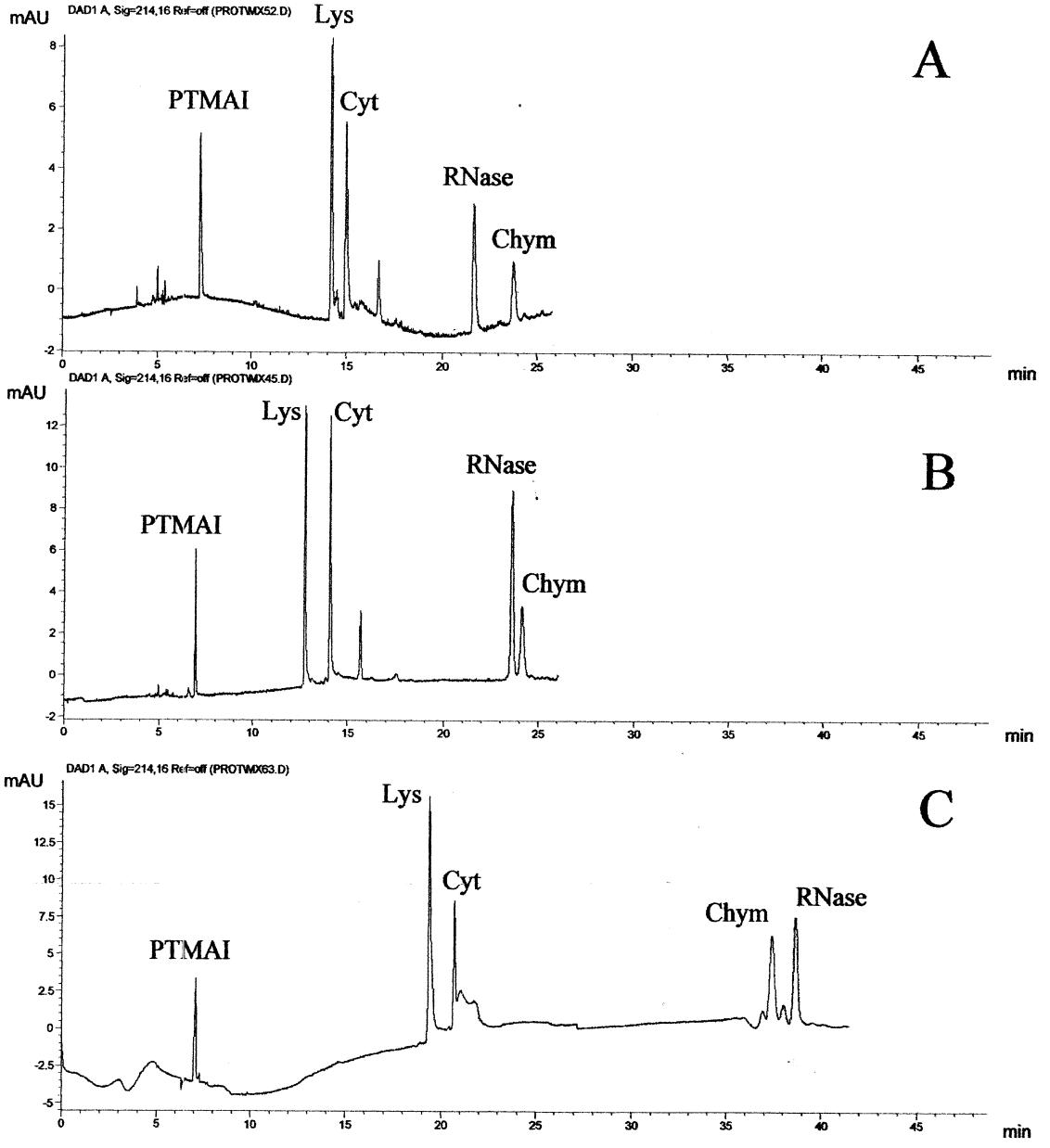

Fig. 5. Separation of basic proteins in a polyacrylamide-coated fused-silica capillary with electrolyte solutions consisting of 50 m*M* perchloric acid (A), 50 m*M* phosphoric acid (B), and 50 m*M* citric acid (C), all titrated to pH 5.8 with TETA aqueous solution. Capillary, polyacrylamide-coated fused-silica capillary 415 mm (330 mm to the detector)×0.050 mm I.D.×0.375 mm O.D.; applied voltage, 10 kV [current, (A) 24.3 μA, (B) 11.8 μA and (C) 10.7 μA]; detection wavelength, 214 nm at the cathodic end; temperature of the capillary cassette, 25 °C; samples as in Fig. 1.

tion order with TETA perchlorate and TETA citrate buffer and their co-migration with TETA phosphate.

The presence of either a monoprotic or a triprotic acid and the different pK_a values of citric and phosphoric acid required different amounts of TETA to adjust to pH 5.8 the equal concentration of acid

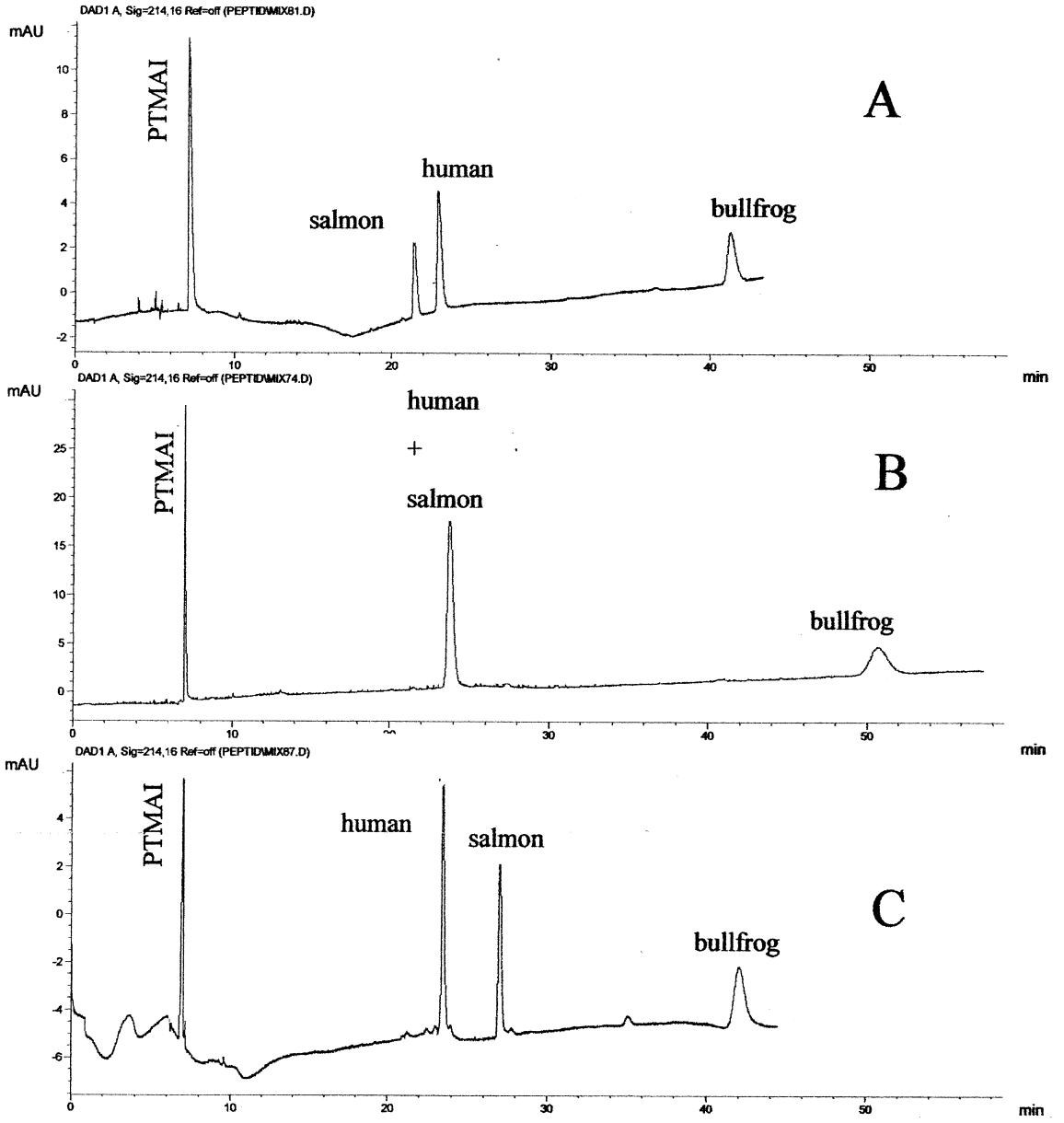

Fig. 6. Separation of closely related peptides in a polyacrylamide-coated fused-silica capillary with electrolyte solutions consisting of 50 m*M* perchloric acid (A), 50 m*M* phosphoric acid (B), and 50 m*M* citric acid (C), all titrated to pH 5.8 with TETA aqueous solution. Peptides, angiotensin I from salmon, human, and bullfrog. Capillary and experimental conditions as in Fig. 5.

incorporated in each buffer system, which, consequently, exhibited different concentrations of the aliphatic oligoamine. The influence of the different concentrations of TETA on the migration behavior of peptides and proteins was investigated by performing a series of experiments with electrolyte solutions containing a fixed concentration of citric acid (50 m*M*) and different concentrations of TETA ranging from 4.0 to 35.0 m*M*, while adjusting the electrolyte solution to pH 5.8 by adding the required amount of

sodium hydroxide. The concentration range of TETA was selected on the basis of the amounts of the aliphatic oligoamine required to titrate a 50 m*M* solution of either acid at pH 5.8. With these buffers, both peptides and proteins exhibited almost unvaried migration times, indicating a negligible effect of the concentration of TETA on the migration behavior and selectivity of the examined peptides and proteins (data not shown). The absence of a significant variation of the migration behavior of peptides and proteins on varying the concentration of TETA was also observed using buffers containing a constant concentration of phosphate (80 m*M*) and different concentration of TETA ions at pH 5.8, adjusted to this value by adding the required amount of sodium hydroxide. However, with TETA phosphate buffer prepared from 80 m*M* phosphoric acid, human and salmon angiotensin I, co-migrating with the TETA buffer containing 50 m*M* phosphate (see Fig. 6B), were separated regardless of the concentration of TETA ions (see Fig. 7). This result further supports the occurrence of selective interactions of the buffer anion with the analytes, as shown by the electropherograms displayed in Fig. 7, showing the separation of the decapeptides angiotensin I from three diverse biological sources obtained with TETA phosphate buffer prepared from phosphoric acid at three different concentrations, i.e. 20, 50, and 80 m*M*.

As expected, all peptides exhibit an increased migration time with increasing buffer concentration and, consequently, ionic strength. Increasing the ionic strength induces several effects, including temperature increases, viscosity changes and a lowering of the zeta potential around the molecules of charged analytes, which results in a decrease in the electrophoretic mobility [21,28,29]. Also, the occurrence of ion-pair formation results in a decrease of the electrophoretic mobility with increasing concentration of ion-pairing agent incorporated into the electrolyte solution, which, however, depends on to what extent each analyte forms an ion-pair complex with the ion-pairing agent [8,30].

The different migration times exhibited by salmon and human angiotensin I with 80 m*M* phosphate TETA indicate a selective effect of the buffer at this concentration of phosphate ion which brings about their separation. This selective effect can be attributed to ion-pair associations between the two decapeptides and the phosphate ions in the electrolyte solution, likewise the ion-pairing effect exhibited by these peptides in reversed-phase HPLC with mobile phases containing phosphoric acid as the acidic additive [31]. The electropherograms displayed in Fig. 7 show that using buffers with higher concentrations of phosphate ions results in a decrease of the electrophoretic mobility of the peptides to different extents, which may be related to the diverse degree of ion-pair formation with the phosphate ions present in the electrolyte solution. At pH 5.8, phosphoric acid is predominately present as the dihydrogenphosphate ion carrying one negative charge and may establish electrostatic interactions with positively charged analytes. In addition, phosphate ions exhibit strong hydrogen-bonding properties, acting both as donors and acceptors [32], which might contribute to establishing selective interactions with peptides and proteins with the result of affecting their electrophoretic mobility and, hence, selectivity. Such an hypothesis is in accordance with the property of phosphate ions of exhibiting specific interactions with peptides and proteins, which has been observed both in chromatography [33,34] and in capillary electrophoresis [35].

4. Conclusions

Triethylentetramine has been examined as a promising buffering agent for controlling protonic equilibria and protein–capillary wall interactions over a wide pH range. The results indicate that TETA ions strongly interact with the silanol groups on bare fused-silica capillaries, with the result of inhibiting untoward interactions of basic proteins with the capillary wall over a wide pH range. The experimental data show that the adsorption of TETA ions at the interface between the capillary wall and the electrolyte solution results in a drastic variation of the positive charge density in the compact region of the electric double layer, which influences the zeta potential and, hence, the electroosmotic flow.

The study also illustrates the dependence of the migration behavior of selected basic proteins and closely related peptides on the buffer anions perchlorate, phosphate and citrate, which were em-

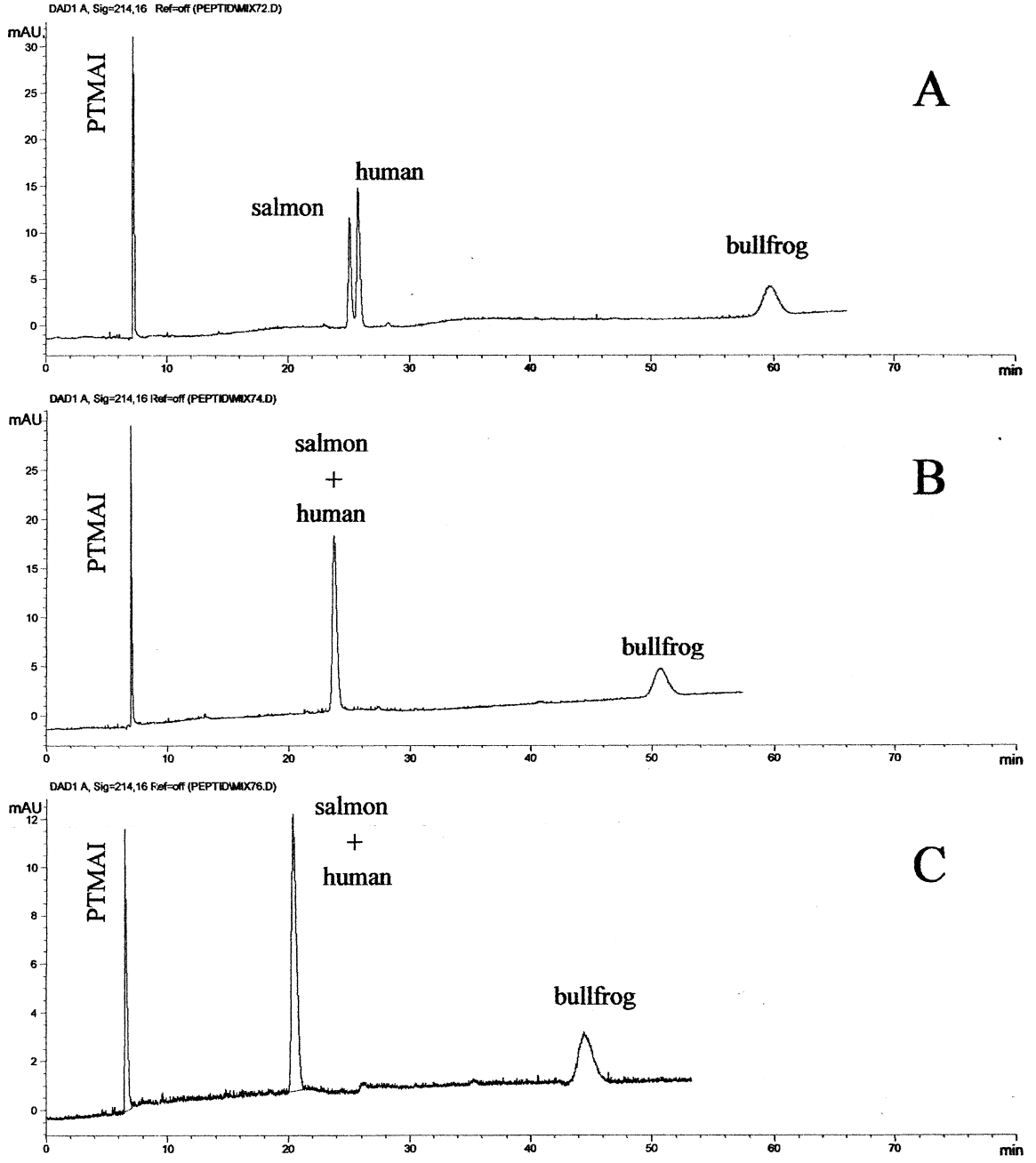

Fig. 7. Separation of closely related peptides in a polyacrylamide coated fused-silica capillary with electrolyte solutions consisting of 80 m*M* (A), 50 m*M* (B), and 20 m*M* (C) phosphoric acid, titrated to pH 5.8 with TETA aqueous solution. Capillary, polyacrylamide-coated fused-silica capillary 415 mm (330 mm to the detector)×0.050 mm I.D.×0.375 mm O.D.; applied voltage, 10 kV [current, (A) 16.6 μA, (B) 11.8 μA, and (C) 6.6 μA]. Samples and other experimental conditions as in Fig. 6.

ployed in combination with TETA as the buffering electrolyte solution. The specific variations of the selectivity with the type and concentration of buffer anion indicate that the action of counter-ions is not limited to surrounding the protein or peptide molecule with the result of decreasing the net charge and, consequently, decreasing the electrophoretic mobility with increasing ionic strength of the electrolyte solution. In addition, counter-ions may specifically interact with peptides and proteins via ion-pair formation, affecting the selectivity.

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