ELSEVIER

Journal of Chromatography A, 849 (1999) 175–189

JOURNAL OF CHROMATOGRAPHY A

Evaluation of volatile eluents and electrolytes for high-performance liquid chromatography–electrospray ionization mass spectrometry and capillary electrophoresis–electrospray ionization mass spectrometry of proteins
II. Capillary electrophoresis

Christian G. Huber*, Andreas Premstaller, Gerald Kleindienst

Institute of Analytical Chemistry and Radiochemistry, Leopold-Franzens-University, Innrain 52a, 6020 Innsbruck, Austria

Received 19 February 1999; accepted 19 April 1999

Abstract

Peptides and proteins were separated by capillary electrophoresis (CE) in fused-silica capillaries coated with an irreversibly adsorbed monolayer of derivatized polystyrene nanoparticles. Whereas phosphate buffer, pH 3.10, enabled the highly efficient separation of basic proteins with plate counts up to 1 400 000 m^{-1}, volatile buffer components such as formic acid or acetic acid titrated with ammonia to the desired pH had to be used for the direct coupling of CE with electrospray ionization mass spectrometry (ESI-MS). Compared to 40 m*M* phosphoric acid–sodium hydroxide, pH 3.10, a background electrolyte containing 125 m*M* formic acid–ammonia, pH 4.00, was shown to yield equivalent separation efficiency. Investigation of the influence of buffered electrolytes on the ESI-MS signal of lysozyme at pH 2.70–4.00 showed that the charge state distribution shifted to lower charge states at higher pH with a concomitant five-fold decrease in signal intensity of the most abundant signal. The presence of trifluoroacetic acid in the background electrolyte greatly increased the level of baseline noise and completely inhibited the observation of any mass signals related to proteins. Full scan spectra could be acquired from 50–500 fmol amounts of proteins during coupled CE–ESI-MS utilizing 100–125 m*M* formic acid–ammonia, pH 3.10. However, compared to UV detection, considerable band broadening is observed with ESI-MS detection which is mainly attributed to column overloading, band spreading in the interface, and scanning data acquisition. Finally, the major whey proteins β-lactoglobulin A, β-lactoglobulin B, and α-lactalbumin were identified in a whey drink by comparison of molecular masses determined by CE–ESI-MS to molecular masses calculated from the amino acid sequence.
© 1999 Elsevier Science B.V. All rights reserved.

Keywords: Buffer composition; Capillary electrophoresis–mass spectrometry; Electrospray ionization; Polystyrene coating; Coating; Capillary columns; Peptides; Proteins

1. Introduction

Capillary electrophoresis (CE) and mass spectrometry (MS) are among the most powerful instrumental techniques for protein analysis known

*Corresponding author. Tel.: +43-512-5075-176; fax: +43-512-5072-767.

E-mail address: christian.huber@uibk.ac.at (C.G. Huber)

0021-9673/99/$ – see front matter © 1999 Elsevier Science B.V. All rights reserved.
PII: S0021-9673(99)00533-6

today. CE is able to separate biopolymers with high efficiency [1,2], and MS provides the sensitivity and detectability required for the small volumes characteristic of CE, particularly in biochemical applications where sample amount can be limited [3]. Moreover, MS greatly enhances the utility of CE with information about the structure and identity of the separated analytes. The multiple charging observed in electrospray ionization (ESI) permits the mass spectrometric analysis even of large biomolecules such as proteins with molecular masses up to several hundreds of thousands on instruments with significantly lower mass range [4]. Furthermore, because ESI produces ions from flowing liquid streams it is an ideal interface between liquid phase separation techniques such as chromatography or electrophoresis and mass spectrometry [5–11].

CE was initially coupled with ESI-MS by Smith and co-workers using an interface in which the column effluent was introduced directly into the ion source of the mass spectrometer [12,13]. Subsequent interfaces have either employed make-up flow provided by a coaxial flow delivery system or nanospray ion sources in order to improve the stability of the electrospray [14–17]. Although the coupling of CE and MS presents physical, electrical and chemical challenges, the technique has been successfully applied to the separation and analysis of proteins and peptides [18–30]. The success of coupling a CE separation system to ESI-MS depends on many factors, including the design and operating parameters of the electrospray interface [31], the composition and pH of the electrosprayed solution [16,21], and the chemical properties of the analyte [32]. The proper choice of a suitable electrolyte system is, therefore, essential in achieving both a successful CE separation and good-quality ESI mass spectra. Generally, the best detector response is produced by volatile electrolyte systems at the lowest practical concentration and ionic strength. On the other hand, low electrolyte concentrations cause solute zone broadening during the electrophoretic separation process due to conductivity differences and adsorption. Consequently, a balance must be achieved between high buffer concentration, which diminishes solute detectability, and low buffer concentration, which degrades the separation efficiency. Particularly capillary electrophoretic separation of proteins in plain fused-silica capillaries is impaired by the adsorption of such analytes at the capillary inner wall due to the electrostatic interaction between the positively charged residues of the protein and the negatively charged silanols of the fused-silica surface [33,34]. Adsorption of analytes is undesirable in capillary electrophoresis because it results in peak dispersion and peak asymmetry [35,36].

Recently, we demonstrated the applicability of capillaries coated with derivatized polystyrene nanoparticles for high-efficiency capillary electrophoretic separations of peptides and proteins [2]. The irreversibly adsorbed monolayer of polymer particles allowed the efficient suppression of solute–wall interactions resulting in column efficiencies in excess of one million theoretical plates per meter for basic proteins. In this communication, we report on the conjugation of CE in particle coated capillaries with ESI-MS. Different volatile electrolyte systems are compared for the separation and detection of proteins by CE–ESI-MS. The influence of solution chemistry on separation efficiency, electrospray stability, detection sensitivity, and charge state distribution is investigated in order to better understand the most important factors that determine the performance of the coupled CE–ESI-MS system.

2. Experimental

2.1. Chemicals and standards

Acetonitrile (HPLC gradient grade), methanol (analytical-reagent grade), phosphoric acid (analytical-reagent grade), sodium hydroxide (analytical-reagent grade), hydrofluoric acid (48%, analytical-reagent grade) and 2,3-epoxy-1-propanol (analytical-reagent grade) were obtained from Merck (Darmstadt, Germany). Acetic acid (AcOH, analytical-reagent grade), formic acid (HCOOH, 90%, analytical-reagent grade), trifluoroacetic acid (TFA, for UV spectroscopy), ammonia solution (25%, analytical-reagent grade), and ammonium acetate (analytical-reagent grade) were from Fluka (Buchs, Switzerland). Ethylenediamine (>99%) was purchased from Aldrich (Milwaukee, WI, USA). Chloromethylated polystyrene nanoparticles were kindly provided by Sarasep (Santa Clara, CA, USA). For preparation of

Table 1
Standard proteins and peptides used in this study

Protein/peptide	Abbreviation	Source	Isoelectric point (p*I*)
α-Chymotrypsinogen A	CHG	Bovine pancreas	9.5
Cytochrome *c*	CYT	Horse heart	10.7
α-Lactalbumin	LALB	Bovine milk	4.8
β-Lactoglobulin A	LAC A	Bovine milk	5.1
β-Lactoglobulin B	LAC B	Bovine milk	5.2
Lysozyme	LYS	Chicken egg white	11.0
Myoglobin	MYO	Horse heart	6.9
Ribonuclease A	RIB	Bovine pancreas	8.8
Trypsin	TRY	Bovine pancreas	–
Trypsin inhibitor	STI	Soybean	4.5
[Sar^{1},Ile^{8}]-Angiotensin II	A1	Synthetic	
[Asn^{1},Val^{5}]-Angiotensin II	A2	Synthetic	
Angiotensin II[a]	A3	Synthetic	

[a] Sequence: Asp–Arg–Val–Tyr–Ile–His–Pro–Phe.

the electrolytes, high-purity water (Epure, Barnstead, Newton, MA, USA) was used. All standard proteins and peptides (Table 1) were obtained from Sigma (St. Louis, MO, USA). The whey drink was purchased in a local grocery (Lattella Molkegetränk Natur from Tirol Milch, Austria). Phosphate buffer was prepared by diluting a 1.0 *M* stock solution of phosphoric acid and adjusting the pH with 4.0 *M* sodium hydroxide solution. Electrolytes containing ammonium formate and ammonium acetate were prepared by dissolving the appropriate amount of acid in water and adjusting the pH with 10% ammonia solution. The ammonium acetate–trifluoroacetic acid electrolyte was prepared from the salt with subsequent adjustment of the pH with trifluoroacetic acid. All electrolyte solutions were degassed and filtered through 0.20-μm syringe filters (Nalge, Rochester, NY, USA).

2.2. Capillary electrophoresis

Derivatization of chloromethylated polystyrene nanoparticles (Sarasep, San Jose, CA, USA) of 50–100 nm size with ethylenediamine, coating of a fused-silica capillary with the derivatized particles, and subsequent derivatization of the coating with 2,3-epoxy-1-propanol was carried out as described previously [2]. Two commercial CE systems were used in this work. Evaluation of electrolyte systems with UV detection at 215 nm was performed with a Perkin-Elmer Applied Biosystems (Foster City, CA, USA) Model 270A capillary electrophoresis system. Samples were injected hydrodynamically for 1.0 s at 17.0 kPa. Between the runs, the capillary was rinsed with running electrolyte for 3.0 min at 17.0 kPa. Data was recorded on a personal computer-based data system (GynkoSoft, Version 5.22, Gynkotek, Germering, Germany). For coupling electrophoresis with mass spectrometry, a HP ^{3D}CE capillary electrophoresis system (Hewlett-Packard, Waldbronn, Germany) with HP ChemStation software (Version A 05.04) was utilized. Samples were injected hydrodynamically for 1.0–10 s at 5.0 kPa.

2.3. Electrospray ionization mass spectrometry and coupling to capillary electrophoresis

ESI-MS was performed on a Finnigan MAT TSQ 7000 triple quadrupole mass spectrometer (Finnigan MAT, San Jose, CA, USA) equipped with the electrospray ion source. Protein mass spectra were recorded by scanning the third quadrupole Q3, scan range and scan time are given in the figure captions. For CE–MS analysis with ESI and coaxial sheath flow a spray voltage of 4.0–4.5 kV was employed. The temperature of the heated transfer capillary was set to 200–210°C. The mass spectrometer was calibrated by direct infusion of a solution of L-methionyl–arginyl–phenylalanyl–alanine (20 pmol/μl, Finnigan) and apomyoglobin from horse skeletal muscle (5.0 pmol/μl, Sigma) in methanol–water (50:50, v/v) containing 1% acetic acid to give the

average molecular masses of the observed signals. The ion source parameters were tuned for CE–ESI-MS operation using a 0.20 mg/ml solution of cytochrome *c* in 75 m*M* aqueous formic acid infused through the separation capillary at a voltage of 25 kV. The sheath liquid comprising 26 m*M* formic acid in acetonitrile–water (50:50, v/v) was added at a flow-rate of 3.0 μl/min by means of a syringe pump (Model 980-532, Harvard Apparatus, South Natick, MA, USA). Mass electropherograms and mass spectra were recorded on a DEC-Alpha workstation with the ICIS software, version 7.01 (Finnigan).

For coupling with ESI-MS the square-cut outlet end of the coated capillary had to be modified in order to increase the stability of the electrospray. The wall thickness at the end of a 375 μm O.D.×50 μm I.D. coated capillary was decreased by removing 10 mm of the polyimide coating with hot, concentrated sulfuric acid and subsequent etching of a 5 mm section of the outer capillary surface with concentrated hydrofluoric acid until the outer diameter was not more than 150 μm. Alternatively, a tapered tip was ground at the end to an angle of ca. 30° using a ceramic polishing plate (Quadrex, New Haven, CT, USA). No significant difference between the two tip designs was observed.

Fig. 1 schematically represents the set-up for liquid and electrical connections between the CE system (1) and the electrospray ion source of the mass spectrometer (6). The outlet of the separation capillary was fitted into the electrospray needle of the ion source and a continuous flow of conductive sheath liquid (9) established electrical contact between capillary effluent and electrospray needle. Typically, a potential of 20–25 kV was applied to the inlet electrolyte reservoir (3) of the CE system and of 5 kV to the electrospray needle resulting in an effective separation voltage of 15–20 kV. CE, ESI and MS referred to a common ground potential (14) in order to maintain a continuous electrical circuit. The electrospray high-voltage power supply and, hence, the capillary outlet were connected electrically to the ground of the CE high-voltage power supply through a 100 MΩ resistor (13). This con-

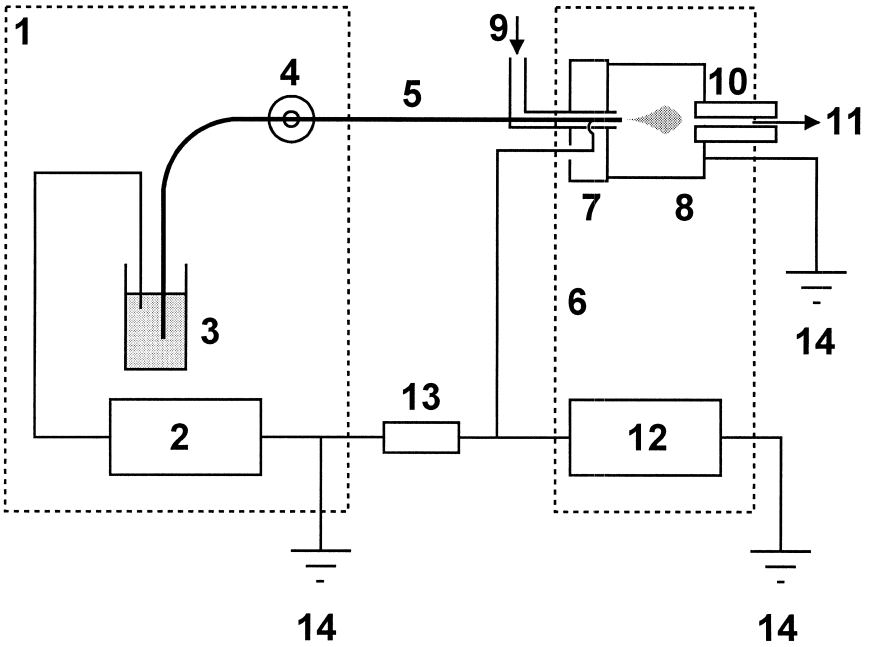

Fig. 1. Schematic diagram of the liquid and electrical connections for CE–ESI-MS coupling. 1=CE system; 2=CE high-voltage power supply, 0–30 kV; 3=inlet electrolyte vial; 4=UV detector; 5=nanoparticle-coated 50 μm I.D. fused-silica capillary; 6=electrospray ion source; 7=ESI probe assembly; 8=spray shield; 9=inlet for sheath liquid provided by a syringe pump; 10=heated capillary to mass analyzer; 11=mass analyzer; 12=ESI high-voltage power supply, 0–8 kV; 13=100 MΩ resistor; 14=ground.

nection reduced the current from the capillary tip to the spray shield (8) and eliminated the occurrence of arcing [27]. Moreover, this connection was necessary to maintain the capability of regulating the ESI voltage independently of separation voltage, especially at CE currents higher than 30 μA.

3. Results and discussion

3.1. Performance of phosphate buffer as background electrolyte for separation of proteins in nanoparticle coated capillaries

The different functional groups of proteins exposed to the electrolyte solution employed in capillary electrophoresis may interact with a variety of active sites on the inner surface of bare fused-silica capillaries giving rise to peak broadening and asymmetry, non reproducible migration times, and low mass recovery. Our approach to overcome this problem is the coating of the inner wall of fused-silica capillaries with polystyrene nanoparticles which have been derivatized with ethylenediamine and 2,3-epoxy-1-propanol (PS–EDA–diol) (Fig. 2) [2]. In particle-coated capillaries, electrostatic and solvophobic interactions are believed to be efficiently suppressed by the approximately 100 nm thick layer of PS–EDA–diol particles having a positively charged, hydrophilic surface. The separation efficiency attainable with particle-coated capillaries is illustrated in Fig. 3 by the optimized capillary electrophoretic separation of seven proteins in a 50 μm I.D. PS–EDA–diol-coated fused-silica capillary. Using 40 m*M* phosphoric acid titrated to pH 3.1 with sodium hydroxide, the numbers of theoretical plates ranged from 1 372 500 m^{-1} for lysozyme to 684 900 m^{-1} for β-lactoglobulin A. The high resolving power of the separation system is also reflected in the baseline resolution of β-lactoglobulin A and B, the sequences of which differ only in 2 out of 162 amino acids resulting in a difference of their isoelectric points of 0.11 units [37]. Moreover, peaks of the basic proteins were symmetrical with asymmetry factors ranging from 0.76–1.27. An exception is only the peak of ribonuclease A with an asymmetry factor of 2.7. The peaks of the two acidic proteins β-lactoglobulin A and B showed considerable tailing with asymmetry factors of 3.2 and 2.7, respectively. This observed tailing is most probably due to interaction of deprotonated acidic side chains with the positively charged surface of the particle coating

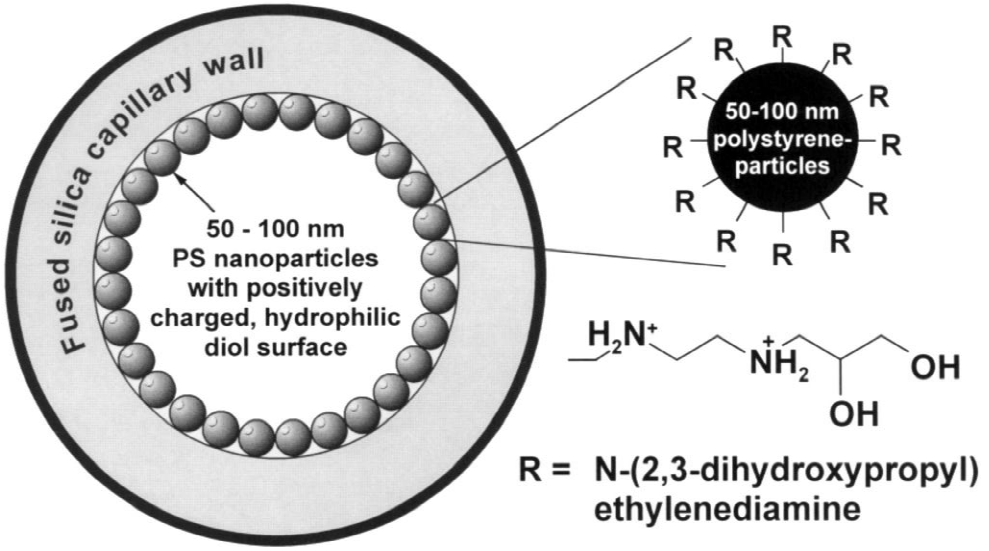

Fig. 2. Schematic illustration of the PS–EDA–diol coated capillary and the structure of the surface modified particles (drawing is not to scale).

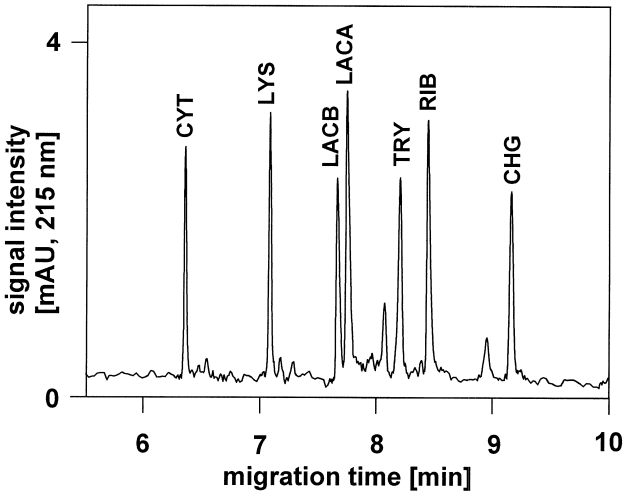

Fig. 3. High-efficiency separation of seven proteins in a PS–EDA–diol coated capillary using phosphate buffer, pH 3.10, as background electrolyte. Capillary, PS–EDA–diol coated, 60 cm (effective 40 cm)×50 μm I.D.; temperature, 25°C; electrolyte, 40 m*M* phosphoric acid–sodium hydroxide, pH 3.10; voltage, 24 kV; current, 22 μA; injection volume, 5.0 nl; sample, CYT, LYS, LACB, LACA, TRY, RIB, CHG, 50–100 μg/ml each.

and results in comparatively low numbers of theoretical plates (874 000 m^{-1} for β-lactoglobulin B and 684 900 m^{-1} for β-lactoglobulin A).

In capillary electrophoresis of proteins, the ionic strength of the background electrolyte exerts a significant impact on separation efficiency [38]. Generally, higher ionic strength entails higher efficiency up to a point where convectional mixing due to Joule heating begins to effect band broadening. In particle-coated capillaries, the optimal phosphate buffer concentration with respect to separation efficiency and resolution was 40–50 m*M* at an electrophoretic current of 70–90 μA [pH 3.1, 60 cm (effective 40 cm)×75 μm I.D. capillary, 20 kV]. The influence of pH on separation efficiency in particle-coated capillaries was studied in the pH range of 2.7–3.6. A mixture of nine standard proteins (cytochrome *c*, lysozyme, β-lactoglobulin B, myoglobin, trypsin, ribonuclease A, α-lactalbumin, α-chymotrypsinogen A, and trypsin inhibitor, approximately 1.5–3.0 ng each) was injected and separated at 20 kV in a 50 cm (effective 30 cm)×75 μm I.D. coated capillary using 40 m*M* phosphate buffer adjusted to the desired pH with sodium hydroxide. At pH 2.7, the difference in migration times between the most rapidly and most slowly migrating protein (cytochrome *c* and trypsin inhibitor) was only 0.94 min resulting in incomplete separation of the protein mixture. At pH 3.6, cytochrome *c* and trypsin inhibitor passed the detector at 3.8 min and 7.0 min, respectively, corresponding to a time window of 3.2 min for separation of the nine proteins. However, some of the peaks showed considerable peak broadening at this pH. The optimum pH was reached at pH 3.1, where all proteins present in the mixture were separated to baseline within a time window of 1.7 min with excellent efficiencies.

For the direct coupling of CE to ESI-MS substitution of the non volatile phosphoric acid–sodium hydroxide buffer by volatile buffer components is obligatory. In contrast to high-performance liquid chromatography (HPLC), where unbuffered solutions are applicable as eluents (see Part I of this series), an electrolyte suitable for CE must contain a buffer system in order to avoid excessive changes in pH caused by electrolysis during electrophoretic separation. The choice of buffer components for CE–ESI-MS is limited to those of high volatility so as to improve detectability and to avoid problems of fouling the mass spectrometer ion source with salt deposits. Furthermore, the conductivity of the electrolyte should be low. Formic acid and acetic acid titrated to the desired pH with ammonia were considered as practicable alternatives to phosphoric

acid–sodium hydroxide as buffering compounds for the following reasons: (1) Formic acid and acetic acid were found to enable ESI-MS detection of proteins with excellent detectability (see Part I of this series). (2) The buffering ranges of the two acids with pK_a values of 3.75 and 4.76 at 25°C cover the optimal pH range for protein separations in particle-coated capillaries (pH 3.00–4.25). (3) The conductivity of formic acid–ammonia or acetic acid–ammonia solutions is considerably lower than that of phosphoric acid–sodium hydroxide solutions of the same concentration resulting in lower CE currents which is desirable especially for the coupling of CE to ESI-MS.

3.2. Optimization of electrolyte composition employing volatile buffer components

To establish the optimum conditions for the capillary electrophoretic separation of proteins in particle-coated capillaries using volatile buffer systems the influence of acetic acid and formic acid concentration as well as electrolyte pH on protein migration and separation efficiency was investigated. UV absorbance detection was used for these investigations to expedite the evaluation process and to avoid the complexities of MS detection. Fig. 4a illustrates the dependence of separation efficiency on acetic acid concentration which was characterized by separation of a mixture of five proteins (cytochrome *c*, lysozyme, myoglobin, ribonuclease A, α-chymotrypsinogen A). It can be seen that a significant increase in the number of theoretical plates is observed at higher concentration of acetic acid. The maximum separation efficiency was achieved with 125 m*M* acetic acid–ammonia, pH 4.25, yielding a number of theoretical plates of 1 170 000 m^{-1} for the peak of cytochrome *c*. Compared to 40 m*M* phosphate buffer, the resultant electrophoretic current of 58 μA with 125 m*M* acetic acid was notably lower due to only partial dissociation of acetic acid and the low equivalent conductivity of acetate. Myoglobin exhibited an anomalous electrophoretic behavior in acetic acid buffer solutions. Although myoglobin is a relatively small, basic protein, considerable band broadening and/or poor resolution from ribonuclease A at acetic acid concentrations higher than 75 m*M* made the determination of peak

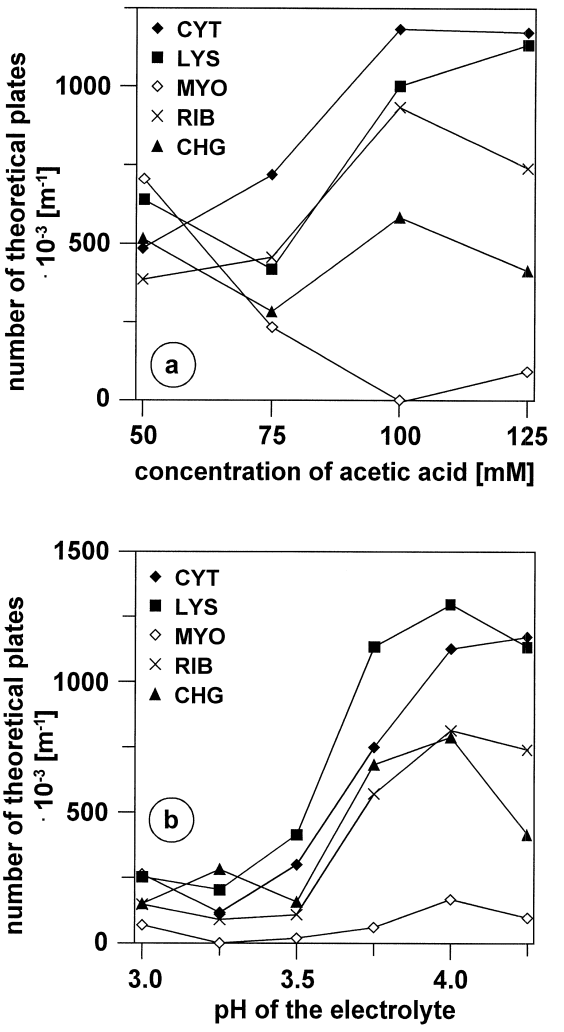

Fig. 4. Influence of acetic acid concentration and pH on separation efficiency. Capillary, PS–EDA–diol coated, (a) 50 cm (effective 30 cm), (b) 60 cm (effective 40 cm)×75 μm I.D.; temperature, 25°C; electrolyte, (a) 50–125 m*M* acetic acid–ammonia, pH 4.25, (b) 125 m*M* acetic acid–ammonia, pH 3.00–4.25; voltage, 20 kV; current, (a) 23–58 μA, (b) 9.5–85 μA; injection volume, (a) 30 nl, (b) 25 nl; sample, CYT, LYS, MYO, RIB, CHG, 25–50 μg/ml each.

parameters for myoglobin difficult. One possible explanation for this behavior is an acetic acid-induced change in the three-dimensional folding of the polypeptide chain resulting in a more heterogeneous structure of the protein. The optimum pH for protein separations with 125 m*M* acetic acid was 4.00 (Fig. 4b) which is 0.90 units higher than the optimum pH

with phosphate buffer. In this series of experiments, a maximum number of theoretical plates of 1 296 000 m^{-1} was observed for the peak of lysozyme at pH 4.00. The electrophoretic current varied from 9.5–85 μA in the pH range of 3.00–4.25. This strong dependence of conductivity on pH results from titration of the acid with ammonia which not only introduces a new ion species (ammonium ions) but also increases the concentration of dissociated acetic acid molecules at higher pH.

The concentration dependence of separation efficiency with formic acid was similar to that of acetic acid with a maximum separation efficiency of 1531000 theoretical plates per meter for lysozyme at 150 m*M* formic acid–ammonia, pH 4.25 (Fig. 5a). Because of the higher conductivity of formate the separation had to be carried out with a separation voltage of 15 kV in order not to exceed a maximum current of 100 μA. The influence of pH on separation efficiency was studied with a 50 m*M* formic acid buffer titrated to pH 3.00–4.00 with ammonia. As already observed with acetic acid buffer, protein separations were more efficient at higher pH with formic acid–ammonia buffer compared to phosphate buffer (Fig. 5b). The number of theoretical plates obtained for lysozyme with 50 m*M* formic acid–ammonia, pH 4.0, was 1 118 000 m^{-1}.

The performance of phosphoric acid–sodium hydroxide, acetic acid–ammonia and formic acid–ammonia as buffer systems for separation of proteins under optimum conditions is compared in Fig. 6. All separations were performed using a 50 cm (effective 30 cm)×50 μm I.D. PS–EDA–diol-coated capillary at a separation voltage of 20 kV. Migration times of proteins gradually increased with the different buffers in the order of phosphoric acid–sodium hydroxide<acetic acid–ammonia<formic acid–ammonia. Under counterelectroosmotic separation conditions, this increase in migration times is primarily caused by a decrease in electroosmotic flow velocity at higher ionic strength. Migration times with formic acid are longer than with acetic acid because more ammonia is required to titrate formic acid to a given pH. The abnormal electrophoretic behavior of myoglobin in acetic acid buffer is evident from the strongly tailing peak observed in Fig. 6b. Although the peaks of myoglobin with phosphoric acid–sodium hydroxide (Fig. 6a) and formic acid–ammonia

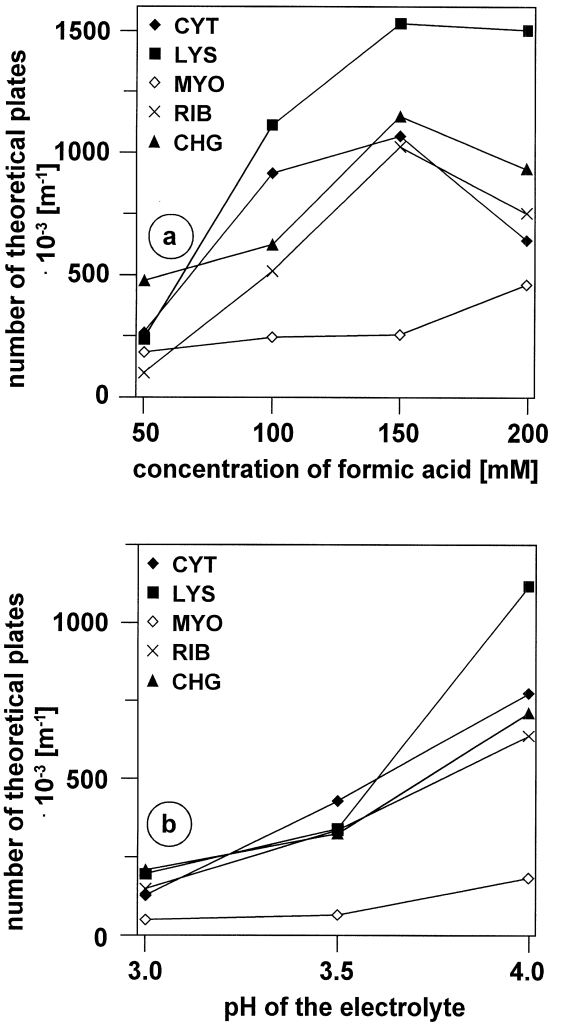

Fig. 5. Influence of formic acid concentration and pH on separation efficiency. Capillary, PS–EDA–diol coated, (a) 50 cm (effective 30 cm), (b) 60 cm (effective 40 cm)×75 μm I.D.; temperature, 25°C; electrolyte, (a) 50–200 m*M* formic acid–ammonia, pH 4.25, (b) 50 m*M* formic acid–ammonia, pH 3.00–4.00; voltage, (a) 15 kV, (b) 20 kV; current, (a) 21–95 μA, (b) 21–69 μA; injection volume, (a) 30 nl, (b) 25 nl; sample, CYT, LYS, MYO, RIB, CHG, 50–105 μg/ml each.

(Fig. 6c) were relatively broad compared to the peak widths of the other proteins, none of the latter two buffer systems showed such a strong tailing. The comparison of the efficiencies with phosphoric acid–sodium hydroxide and formic acid–ammonia depicted in Fig. 7 reveals that both buffer systems are equivalent with respect to separation efficiency for

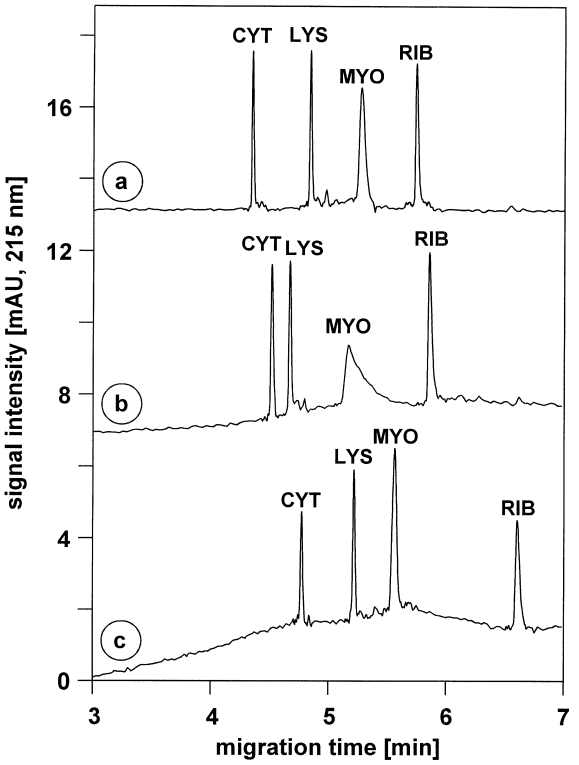

Fig. 6. Optimized separations of proteins in a particle-coated capillary with different electrolyte systems. Capillary, PS–EDA–diol coated, 50 cm (effective 30 cm)×50 μm I.D.; temperature, 25°C; electrolyte, (a) 40 m*M* phosphoric acid–sodium hydroxide, pH 3.1, (b) 125 m*M* acetic acid–ammonia, pH 4.0, (c) 125 m*M* formic acid–ammonia, pH 4.0; voltage, 20 kV; current, (a) 23 μA, (b) 21 μA, (c) 88 μA; injection volume, 25 nl; sample, CYT, LYS, MYO, RIB, 50–100 μg/ml each.

basic proteins whereas protein separations in acetic acid–ammonia buffer were significantly less efficient. The numbers of theoretical plates with formic acid–ammonia, pH 4.00 ranged from 316 000 m^{-1} for myoglobin to 1 215 000 m^{-1} for lysozyme. Therefore, an electrolyte containing 125 m*M* formic acid–ammonia, pH 4.00, was regarded as optimum for basic proteins with respect to maximum separation efficiency in combination with minimum analysis time.

3.3. Influence of buffered electrolytes on ESI-MS signal

There is general agreement regarding the importance of protonation processes that yield multiply positively charged species in ESI-MS of proteins. The number of basic sites in a protein sequence is believed to determine the maximum number of protons which may be attached to a protein. In aqueous solution the exact distribution of charges on a protein is highly pH dependent [39]. However, several reports have demonstrated that there exists a discrepancy between the solution-phase equilibria and the gas-phase charge states observed in ESI-MS leading to the conclusion that the ESI mass spectra are not a direct reflection of the charge state pattern present in solution [32,40,41]. These phenomena have been rationalized by considering the formation of multiply charged ions in the very thin outer layer of the droplets, where the acidity of the solution may be significantly different from that in the bulk solution [42].

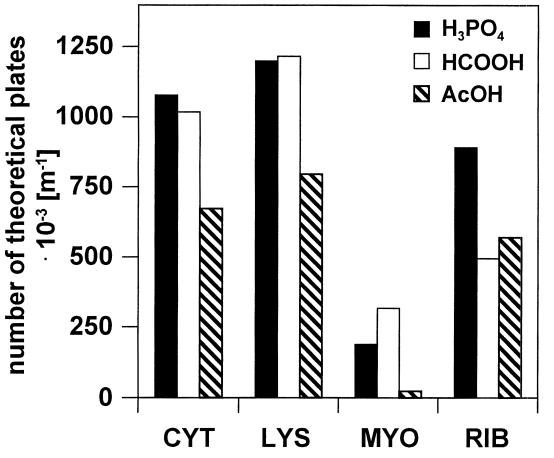

Fig. 7. Comparison of efficiencies obtained with different electrolyte systems in a particle-coated capillary. Electrophoretic conditions as in Fig. 6.

We studied the effect of electrolyte pH on charge state distribution and signal intensity by direct infusion of a 10 mg/ml solution of lysozyme in 26 m*M* aqueous formic acid, pH 2.70 and 26 m*M* aqueous formic acid–ammonia, pH 3.10 and 4.00, respectively. To imitate the conditions during a CE–ESI-MS run as closely as possible the sample solution was infused through a 150 μm O.D.×50 μm I.D. fused-silica capillary at a flow-rate of 200 nl/min with a coaxial sheath flow of 3 μl/min 26 m*M* formic acid in acetonitrile–water (50:50). Fig.

8a depicts the mass spectrum of lysozyme obtained in unbuffered 26 m*M* formic acid solution, pH 2.70. The most abundant charge state was 10+ which was observed with a signal-to-noise ratio of 52:1. Upon titration of the formic acid solution to pH 3.10 with ammonia, the maximum charge state shifted to 9+ with a concomitant decrease in signal-to-noise ratio to 20:1 (Fig. 8b). At pH 4.00, 9+ was still the predominant charge state, however, a decrease in the intensity of the 10+ and an increase in the intensity of the 8+ charge state indicated a further shift to lower charge states at higher pH (Fig. 8c). Moreover, signal intensity of the most abundant charge state additionally decreased to a signal-to-noise ratio of 10:1. This represents a total decrease in signal-to-noise ratio of more than five compared to the unbuffered formic acid solution. The decrease in signal intensity is mainly caused by the increase in conductivity of the buffered electrolytes resulting in competitive ESI of positively charged species. The pronounced effect of electrolyte pH on charge state distribution is remarkable especially because the composition and pH of the sheath liquid was held constant in Fig. 8a–c and the ratio of sheath liquid flow-rate to sample flow-rate was 15:1. Although the charge state distribution of lysozyme has been shown to be relatively insensitive to changes in pH in direct infusion experiments [41], Fig. 8a–c clearly demonstrate that under CE–ESI-MS conditions charge state distribution and signal intensity do vary significantly even in a narrow pH range.

3.4. Coupling of CE with ESI-MS

The strong signal suppressing effect of trifluoroacetic acid on ESI-MS signal intensity of proteins has already been discussed in Part I of this series. In CE–ESI-MS of proteins this effect was so profound that it was impossible to get any protein signal during electrophoretic separations of proteins with background electrolytes containing trifluoroacetic acid. Hence, the strong effect of trifluoroacetic acid is exemplified by the separation of three octapeptides in an electrolyte containing 15 m*M* ammonium acetate which has been titrated to pH 4.00 with trifluoroacetic acid. Fig. 9 demonstrates that a concentration of 13 m*M* trifluoroacetic acid in the background electrolyte entailed a dramatic deteriora-

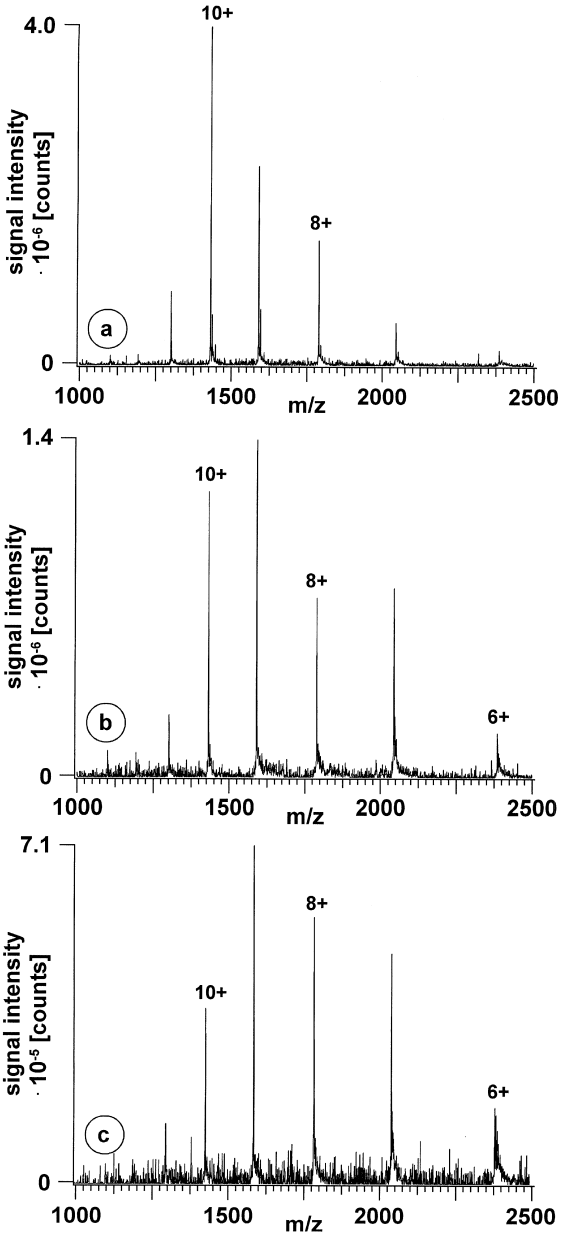

Fig. 8. Influence of buffered electrolyte on signal intensity and charge state distribution. Capillary, fused-silica, 50 μm I.D.; electrolyte, (a) 26 m*M* formic acid, pH 2.70, (b) 26 m*M* formic acid–ammonia, pH 3.10, (c) 26 m*M* formic acid–ammonia, pH 4.00; flow-rate, 200 nl/min; spray voltage, 4.5 kV; sheath liquid, 26 m*M* formic acid in acetonitrile–water (50:50), 3 μl/min; scan, 1000–2500 u in 10 s; concentration of LYS in the electrolyte, 10.0 mg/ml.

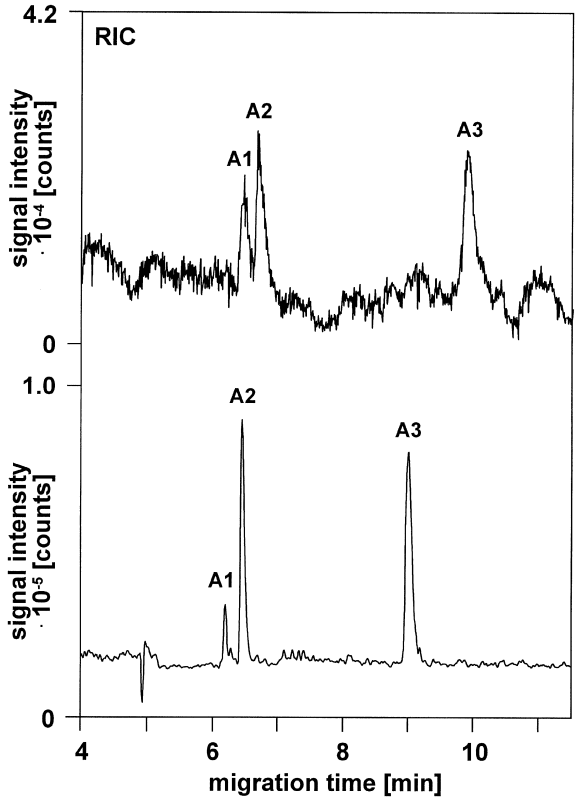

Fig. 9. Effect of trifluoroacetic acid on ionization efficiency and baseline noise. Capillary, PS–EDA–diol coated, 50.7 cm×50 μm I.D.; temperature, 25°C; electrolyte, (a) 15 m*M* ammonium acetate–TFA, pH 3.5, (b) 50 m*M* formic acid–ammonia, pH 3.5; separation voltage, 21 kV; injection volume, 3.5 nl; spray voltage, 4 kV; sheath liquid, 175 m*M* acetic acid in methanol–water (50:50), 1.5 μl/min; SIM *m*/*z* values, masses 484.8, 516.5, 524.4; scan time, 1.0 s; sample, A1, A2, A3 (for abbreviations see Table 1), 160 μg/ml each.

tion of the ESI-MS signal (Fig. 9a) as compared to the separation in 50 m*M* formic acid–ammonia, pH 3.5 (Fig. 9b). The signal intensity for [Asn^1,Val^5]-angiotensin II (A2) was 75 900 counts without and 14 700 counts with trifluoroacetic acid in the background electrolyte. Moreover, the baseline noise was more than doubled when the electrolyte contained trifluoroacetic acid.

From the data discussed in Sections 3.2 and 3.3 it can be deduced that highest capillary electrophoretic separation efficiency for basic proteins in particle-coated capillaries with volatile buffer components is obtained at concentrations between 100 and 125 m*M* and at a pH around 4.00, whereas highest mass spectrometric sensitivity is observed with electrolytes of low ionic strength and low pH. A 125 m*M* formic acid–ammonia buffer, pH 3.1, was therefore regarded as a compromise for a background electrolyte applicable to the CE–ESI-MS analysis of a cytochrome *c* sample with full-scan data acquisition (Fig. 10). The peak width at half height for the cytochrome *c* peak was 11.6 s corresponding to a number of theoretical plates of 8980 m^{-1}. This dramatic reduction in separation efficiency compared to CE separations with UV detection is the result mainly of three different effects, namely overloading of the separation capillary, band broadening by the CE–MS interface, and scanning data acquisition. The effect of scanning data acquisition on band broadening has already been discussed in Part I of this series. Although the injected amount of 0.96 ng (78 fmol) cytochrome *c* was relatively low in terms of ESI-MS detectability (see Part I of this series), it was approximately 10 times the maximum loadability for proteins in a 50 μm I.D. capillary without compromising separation efficiency. For mass determination of cytochrome *c* six scans were extracted from the electropherogram in Fig. 10a and averaged to give the mass spectrum depicted in Fig. 10b. Deconvolution of the series of multiply charged ions yielded a molecular mass of 12 366 which correlates well with the theoretical mass of 12 259.4 for cytochrome *c*.

3.5. CE–ESI-MS analysis of proteins in a whey drink

A whey drink was chosen to test the applicability of CE–ESI-MS to the separation and identification of proteins in real samples. De Jong et al. have successfully separated milk proteins by CE in a hydrophilically coated capillary [43]. While they were able to separate a variety of milk proteins with efficiencies ranging from 300 000 to 700 000 theoretical plates the identification of the different proteins had to rely on comparison of migration times with those of known standard compounds. Consequently, on-line acquisition of ESI mass spectra during capillary electrophoretic separation would greatly enhance the capability of CE to identify whey proteins in differ-

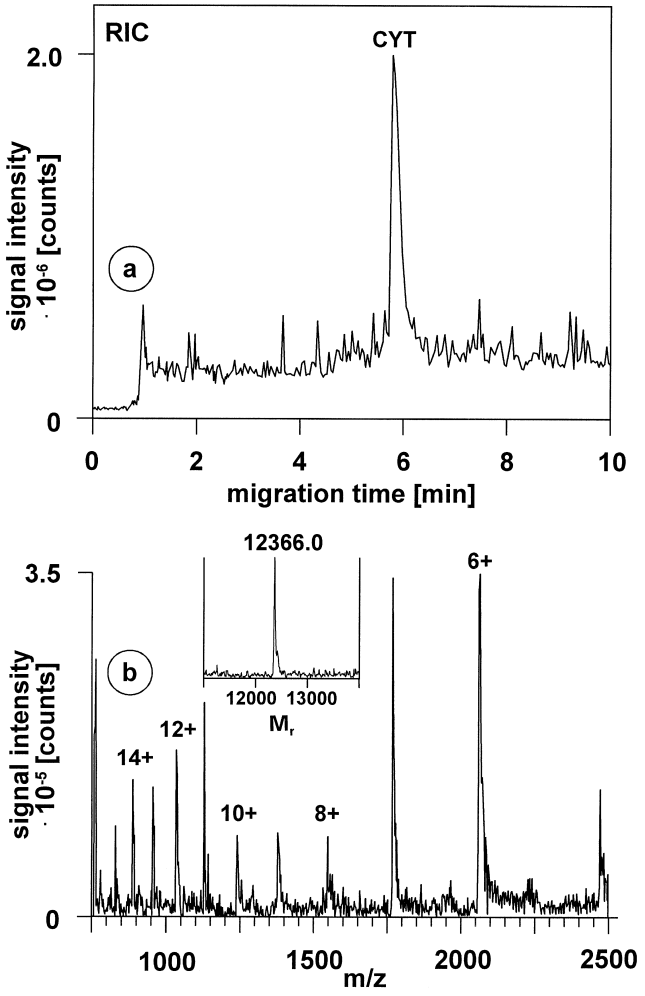

Fig. 10. Electropherogram with full scan ESI-MS detection (a) and extracted mass spectrum of cytochrome *c* (b). Capillary, PS–EDA–diol coated, 54 cm×50 μm I.D.; temperature, 25°C; electrolyte, 125 m*M* formic acid–ammonia, pH 3.1; separation voltage, 20 kV; current, 28 μA; injection volume, 4.8 nl; electrospray voltage, 4.5 kV; sheath liquid, 72 m*M* formic acid in acetonitrile–water (50:50), 3 μl/min; scan, 500–2500 u in 3.0 s; sample, CYT, 0.20 mg/ml.

ent milk products directly on the basis of their molecular masses.

Before injection onto the CE–ESI-MS system, a 1.5-ml aliquot of the whey drink was centrifuged at 13 000 rpm for 5 min and the supernatant was diluted 1:3 with background electrolyte. 10.8 nl of this sample (500–1000 fmol of each whey protein) were injected into the particle-coated capillary for separation by CE–ESI-MS in full scan data acquisition mode. The three major whey proteins β-lactoglobulin B, β-lactoglobulin A and α-lactalbumin were readily identified in the whey drink by their molecular masses of 18 363.0, 18 278.0 and 14 187.0, respectively, which have been calculated by deconvolution of the extracted mass spectra illustrated in Fig. 11a and b (theoretical masses, 18 363.4, 18 277.3 and 14 177.0, respectively). The charge state distributions observed for whey proteins in CE–ESI-MS with 100 m*M* formic acid–ammonia, pH 3.10, differed significantly from the charge state distribution in HPLC–ESI-MS with eluents containing 0.50% (130 m*M*) formic acid. For example, a

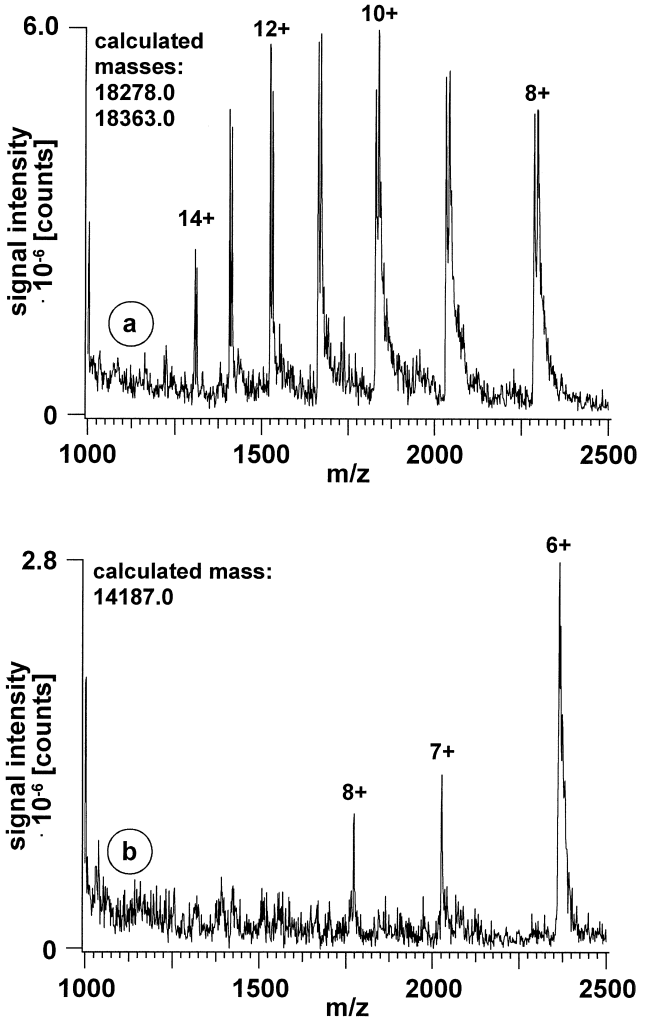

Fig. 11. Full scan mass spectra of proteins detected in a whey drink. Capillary, PS–EDA–diol coated, 80 cm×50 μm I.D.; temperature, 25°C; electrolyte, 100 m*M* formic acid–ammonia, pH 3.1; separation voltage, 25.5 kV; injection, 10.8 nl; electrospray voltage, 4.5 kV; sheath liquid, 64 m*M* formic acid in acetonitrile–water (50:50), 3 μl/min; scan, 1000–2500 u in 2.0 s; sample, whey drink, diluted 1:3.

comparison of Fig. 11b with Fig. 9b in Part I of this series reveals that the most abundant charge state of α-lactalbumin is 8+ in 130 m*M* formic acid and at least 6+ (or less, lower charge states were not observed because of the 2500 u upper mass limit) in 100 m*M* formic acid–ammonia, pH 3.10. These observations correlate well to the charge shift found with buffered electrolytes during direct infusion experiments described in Section 3.3.

A volume of 7.4 nl of diluted whey drink, corresponding to 350–750 fmol of each protein, was injected to obtain the reconstructed ion electropherogram with selected ion monitoring presented in Fig. 12. The m/z values of the selected ions used to acquire this electropherogram were: 1307.0, 1407.5, 1525.0, 1663.1, 1829.3 for β-lactoglobulin B, 1313.5, 1413.4, 1531.7, 1671.2, 1838.0 for β-lactoglobulin A, and 1774.1, 2026.8, 2362.7 for α-lactalbumin. The migration times at 25.7 kV separation voltage in a 58 cm long coated capillary were 7.30, 7.42 and 8.82 min for β-lactoglobulin B, β-lactoglobulin A and α-lactalbumin, respectively. Due

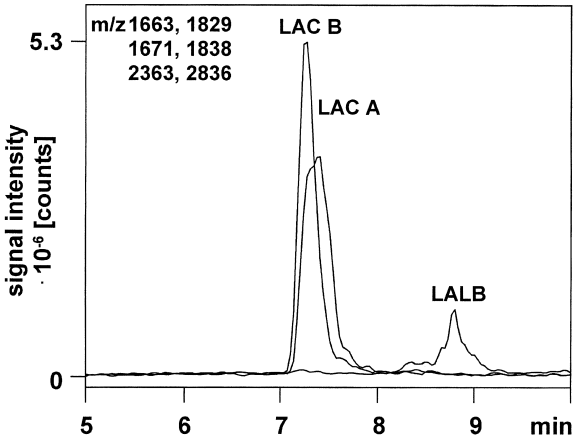

Fig. 12. Analysis of proteins in a whey drink with selected ion monitoring. Capillary, PS–EDA–diol coated, 58 cm×50 μm I.D.; temperature, 25°C; electrolyte, 100 m*M* formic acid–ammonia, pH 3.1; separation voltage, 25.7 kV; injection, 7.4 nl; electrospray voltage, 4.3 kV; sheath liquid, 64 m*M* formic acid acetonitrile–water (50:50), 3 μl/min; SIM *m/z* values, see text; the traces are extracted ion electropherograms at *m/z* 1663.1, 1671.2, 1829.3, 1838.0, 2362.7 and 2836.2; scan time, 1.0 s; sample, whey drink, diluted 1:3.

to peak widths at half height of 11.4 and 18.0 s the peaks of β-lactoglobulin B and β-lactoglobulin overlapped although the selectivity of the electrophoretic system was able to separate the two proteins with a time difference of 7.2 s. However, the superimposed traces demonstrate that all three proteins can be determined through selected ion monitoring without interferences related to the other proteins (Fig. 12).

4. Conclusions

Substitution of non-volatile phosphate buffer by volatile buffers such as formic acid–ammonia and acetic acid–ammonia allowed the capillary electrophoretic separation of proteins with UV and ESI-MS detection. Equivalent separation efficiencies were obtained with the volatile buffers at higher concentration and higher pH. The charge state distribution and ionization efficiency depended significantly on the pH of the background electrolyte. At higher pH lower charge states were favored while signal intensity decreased notably. The optimized CE–ESI-MS system utilizing particle-coated capillaries allowed the separation and identification of proteins on the basis of their molecular masses in real samples in the mid-femtomole range by on-line acquisition of full scan spectra.

Acknowledgements

The authors are indebted to D.T. Gjerde (Sarasep Inc., Santa Clara, CA, USA) for providing the chloromethylated polystyrene nanoparticles. Part of this work was supported by a grant from the Austrian Science Fund (P13442).

References

[1] M. Gilges, M.H. Kleemiss, G. Schomburg, Anal. Chem. 66 (1994) 2038.
[2] G. Kleindienst, C.G. Huber, D. Gjerde, L. Yengoyan, G.K. Bonn, Electrophoresis 19 (1998) 262.
[3] P.E. Andren, M.R. Emmett, R.M. Caprioli, J. Am. Soc. Mass Spectrom. 5 (1994) 867.
[4] C.M. Whitehouse, R.N. Dreyer, M. Yamashita, J.B. Fenn, Anal. Chem. 57 (1985) 675.
[5] K.B. Tomer, L.J. Deterding, C.E. Parker, Adv. Chromatogr. 35 (1995) 53.
[6] J. Cai, J.D. Henion, J. Chromatogr. A 703 (1995) 667.
[7] W.M.A. Niessen, U.R. Tjarden, J. Van der Greef, J. Chromatogr. 636 (1993) 3.
[8] R.D. Smith, H.R. Udseth, in: S.M. Lunte, D.M. Radzik (Eds.), Pharmaceutical and Biomedical Applications of Capillary Electrophoresis, Elsevier, Oxford, 1996, p. 229.
[9] R.D. Smith, H.R. Udseth, J.H. Wahl, D.R. Goodlett, S.A. Hofstadler, Methods Enzymol. 271 (1996) 448.
[10] R.D. Smith, J.H. Wahl, D.R. Goodlett, S.A. Hofstadler, Anal. Chem. 65 (1993) 574A.
[11] J.F. Banks, Electrophoresis 18 (1997) 2255.
[12] J.A. Olivares, N.T. Nguyen, C.R. Yonker, R.D. Smith, Anal. Chem. 59 (1987) 1230.
[13] R.D. Smith, J.A. Olivares, N.T. Nguyen, H.R. Udseth, Anal. Chem. 60 (1988) 436.
[14] R.D. Smith, C.J. Barinaga, H.R. Udseth, Anal. Chem. 60 (1988) 1948.
[15] R.D. Smith, J.A. Loo, C.J. Barinaga, C.G. Edmonds, H.R. Udseth, J. Chromatogr. 480 (1989) 211.
[16] F. Foret, T.J. Thompson, B.L. Karger, P. Gebauer, P. Bocek, Anal. Chem. 66 (1994) 4450.
[17] J.H. Wahl, D.C. Gale, R.D. Smith, J. Chromatogr. A 659 (1994) 217.
[18] I.M. Johansson, E.C. Huang, J.D. Henion, J. Zweigenbaum, J. Chromatogr. 553 (1991) 311.

[19] L.J. Deterding, C.E. Parker, J.R. Perkins, M.A. Moseley, J.W. Jorgenson, K.B. Tomer, J. Chromatogr. 554 (1991) 329.
[20] R.D. Smith, H.R. Udseth, C.J. Barinaga, C.G. Edmonds, J. Chromatogr. 559 (1991) 197.
[21] M.A. Moseley, J.W. Jorgenson, J. Shabanowitz, D.F. Hunt, K.B. Tomer, J. Am. Soc. Mass Spectrom. 3 (1992) 281.
[22] J.H. Wahl, D.R. Goodlett, H.R. Udseth, R.D. Smith, Anal. Chem. 64 (1992) 3194.
[23] J.R. Perkins, C.E. Parker, K.B. Tomer, Electrophoresis 14 (1993) 458.
[24] R. Kostiainen, E.J.F. Franssen, A.P. Bruins, J. Chromatogr. 647 (1993) 361.
[25] T.J. Thompson, F. Foret, P. Vouros, B.L. Karger, Anal. Chem. 65 (1993) 900.
[26] R.B. Cole, J. Varghese, R.M. McCormick, D. Kadlecek, J. Chromatogr. A 680 (1994) 363.
[27] D. Figeys, I. van Oostveen, A. Ducret, R. Aebersold, Anal. Chem. 68 (1996) 1822.
[28] B. Yeung, T.J. Porter, J.E. Vath, Anal. Chem. 69 (1997) 2510.
[29] K.P. Bateman, R.L. White, M. Yaguchi, P. Thibault, J. Chromatogr. A 794 (1998) 327.
[30] R. Zand, M.X. Li, X.Y. Jin, D. Lubman, Biochemistry 37 (1998) 2441.
[31] J.H. Wahl, R.D. Smith, J. Cap. Electrophoresis 1 (1994) 62.
[32] G. Wang, R.B. Cole, Org. Mass Spectrom. 29 (1994) 419.
[33] J.S. Green, W. Jorgenson, Science 222 (1983) 266.
[34] H.H. Lauer, D. McManigill, Anal. Chem. 58 (1986) 166.
[35] B. Gas, M. Stedrý, A. Rizzi, E. Kenndler, Electrophoresis 16 (1995) 958.
[36] M.R. Schure, A.M. Lenhoff, Anal. Chem. 65 (1993) 3024.
[37] P.G. Righetti, T. Caravaggio, J. Chromatogr. 127 (1976) 1.
[38] J.S. Green, J.W. Jorgenson, J. Chromatogr. 478 (1989) 63.
[39] S.K. Chowdhury, V. Katta, B.T. Chait, J. Am. Chem. Soc. 112 (1990) 9012.
[40] J.A. Loo, H.R. Udseth, R.F. Smith, Rapid Commun. Mass Spectrom. 2 (1988) 207.
[41] M.A. Kelly, M.M. Vestling, C.C. Fenselau, P.B. Smith, Org. Mass Spectrom. 27 (1992) 1143.
[42] R.B. Cole, Electrospray Mass Spectrometry – Fundamentals, Instrumentation and Applications, Wiley, New York, 1997.
[43] N. De Jong, S. Visser, C. Olieman, J. Chromatogr. A 652 (1993) 207.