ELSEVIER

Journal of Chromatography B, 699 (1997) 221–256

JOURNAL OF CHROMATOGRAPHY B

Review

Buffer additives other than the surfactant sodium dodecyl sulfate for protein separations by capillary electrophoresis

Danilo Corradini

Istituto di Cromatografia del CNR Area della Ricerca di Roma, P.O. Box 10, I-00016 Monterotondo Stazione, Rome, Italy

Abstract

The different compounds utilized as additives to the electrolyte solutions employed in protein capillary zone electrophoresis (CZE) for minimizing protein–capillary wall interactions, for improving selectivity and resolution and for controlling the electroosmotic flow are reviewed. The dependence of the electroosmotic flow on the different variables that can be affected by the incorporation of an additive into the electrolytic solution is discussed. A list of the most effective additives employed for protein separations by CZE is reported in Appendix A. © 1997 Elsevier Science B.V.

Keywords: Reviews; Proteins

Contents

1. Introduction

The growth of interest in life sciences and the purity requirements of both natural and biotechnological proteins have stimulated formidable developments in advanced chromatographic and elec-

0378-4347/97/$17.00
PII S0378-4347(97)00301-0

trophoretic techniques for protein analysis in the last 15 years. The most recent impact has been the development of capillary zone electrophoresis (CZE) which, like high-performance liquid chromatography (HPLC), combines the advantages of high efficiency and resolution, accurate quantification, short analysis time and automation. The use of capillary tubes as a differential electromigration microchannel ensures the absence of connective mixing of the separated zone in the electrolyte solution and enables the application of high electric fields with minimal generation of Joule heat, which is efficiently dissipated by transfer through the tube wall as a result of the large surface-to-volume ratio of the capillary. The application of elevated electric fields results in high efficiency as a consequence of the short residing time of the analyte in the capillary which minimize its longitudinal diffusion.

In addition, CZE performed in fused-silica capillaries generally allows the detection of both cationic and anionic samples at the cathodic end of the capillary, as a result of the electroosmotic flow. This is the bulk flow of the liquid in the capillary tube resulting from the effect of the applied electric field across the capillary on the uneven distribution of ions in the electric double layer at the interfacial region between the negatively charged capillary wall, generated by silanol dissociation, and the electrolyte solution.

Proteins are ionogenic substances also bearing other functionalities as hydrogen-bonding regions, hydrophobic patches and biospecific interaction sites that can interact to different extents with the other constituents of the sample mixture and with the components of the electrolyte solutions used for their separation by CZE. Depending on the chemical nature of both the protein and the ionic species in solution these interactions can lead to significant variations in the electrophoretic mobilities of proteins that can greatly affect their selective separation by capillary electrophoresis. 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) [1]. These interactions may give rise to peak broadening and asymmetry, irreproducible migration times, low mass recovery and in some cases irreversible adsorption.

In the first capillary electrophoretic system described by Hjerten [2], the interactions between the proteins and the wall of the quartz tubes (1–3 mm I.D.) used in the apparatus were minimized by coating the inner surface of the capillaries with methyl cellulose. This coating was realized by thermal immobilization of the neutral polymer on the capillary wall, followed by cross-linking reaction with formaldehyde. In a different approach, a variety of polymers have been covalently bonded to the silica capillary wall by silan derivatization [3–6]. However, hydrogen-bonding and hydrophobic interactions may also occur in chemically modified capillaries [7] which often suffer from a gradual loss of surface coverage, particularly at high pH values. Other strategies consist in the use of electrolyte solutions at extreme pH values, whether acidic to suppress the silanol dissociation [8] or higher than the protein p*I* to have both the proteins and the capillary wall negatively charged [9,10]; the use of electrolyte solutions with high concentrations of alkali salts [11] or zwitterions [12], to suppress the electrostatic interactions between the proteins and the capillary wall, and the application of an applied radial voltage to the capillary wall to lower the zeta potential [13,14]. There are several disadvantages of the above strategies. First, pH extremes tend to denature proteins and compromise recovery by reducing solubility. In addition, the current derived from high ionic strength limits the voltage that can be applied, with concomitant decrease in efficiency and increase in the analysis time. Finally, the option of controlling the zeta potential by an external electric field is not available on commercial units.

Another approach to overcome the detrimental effects of protein–capillary wall interactions is to incorporate a suitable additive into the running electrolyte solution. Most of the additives act either as masking or competing agents for the silanol groups on the inner wall of the capillary, so that they are not accessible to protein interactions. Other additives may function as strong ion-pairing or competing agents for the basic amino acid residues of the proteins exposed to the electrolyte solution, in order to subtract their availability to the silanol groups on the capillary wall. Additives may also be

introduced to the electrolyte solution to improve the selectivity by interacting specifically or to different extents with the proteins in the sample [15] and to extend the application of CZE to study specific non-covalent interactions between proteins and organic or inorganic compounds, either charged or neutral [16].

This paper reviews the various classes of additives that have been employed in protein CZE to address biochemical and biomedical separation problems, to probe biochemical mechanisms or to obtain critical information in the form of biophysical parameters. Most of the substances employed as additives of the running buffer may alter the electric double layer at the interface between the capillary wall and the electrolyte solution, resulting in drastic variations of the electroosmotic flow which can be reduced, eliminated or reversed in direction from cathodic to anodic. Consequently, the examination of the most relevant literature describing the use of additives in protein CZE is preceded by a section that discusses the dependence of the electroosmotic flow on the different variables that may be affected by the introduction of an additive to the electrolyte solution.

2. Effect of the additives on the electroosmotic flow

The electroosmotic flow is generated by the effect of the applied electric field across the capillary on the uneven distribution of ions in the electric double layer at the interface between the capillary wall and the electrolyte solution. In fused-silica capillaries, the electric double layer is the result of the excess of cations in the solution in contact with the capillary tube to balance the negative charges on the wall arising from the ionization of the silanol groups on the surface exposed to the electrolyte solution. Part of the excess cations are firmly held in the region of the double layer closer to the capillary wall (the compact or Stern layer, depicted in Fig. 1) and are believed to be less hydrated than those in the diffuse region of the double layer [17]. Certain counterions may be held in the compact region of the double layer by forces additional to those of purely electrostatic origin, resulting in their adsorption in the Stern layer. When an electric field is applied across the capillary, the remain excess cations in the diffuse part of the electric double layer move toward the cathode, dragging their hydration spheres with them. Since the molecules of water associated with the cations are in direct contact with the bulk solvent, all the electrolyte solution moves toward the cathode producing a plug like flow having a flat velocity distribution across the capillary diameter.

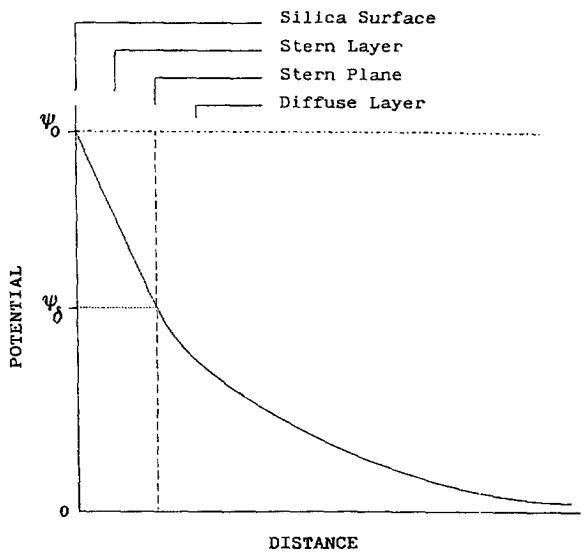

Fig. 1. Representation of the variation of the electric potential in the electric double layer at the interface between the capillary wall and the electrolyte solution.

The variation of the electric potential in the electric double layer with the distance from the capillary wall is depicted in Fig. 1. The potential at the capillary wall (ψ_0), which is proportional to the charge density resulting from the dissociation of the silanol groups, linearly decreases in the Stern layer to the value of the zeta potential (ζ). This is the electric potential at the plane of shear between the Stern layer (plus that part of the double layer occupied by the molecules of solvent associated with the adsorbed ions) and the diffuse part of the double layer. The zeta potential decays exponentially from ζ to zero with the distance from the plane of shear between the Stern layer and the diffuse part of the double layer.

The dependence of the electroosmotic flow (v_{eo}) on the zeta potential is expressed by the Helmholtz–von Smoluchowski equation:

$$v_{eo} = -\frac{\varepsilon_0 \varepsilon \zeta}{\eta} E \quad (1)$$

where E is the applied electric field, ε_0 is the permittivity of vacuum, ε and η are the dielectric constant and the viscosity of the electrolyte solution, respectively. This expression assumes that the dielectric constant and viscosity of the electrolyte solution are the same in the electric double layer as in the bulk solution.

The Helmholtz–von Smoluchowski equation indicates that under constant composition of the electrolyte solution, the electroosmotic flow depends on the magnitude of the zeta potential which is determined by many different factors, the most important being the dissociation of the silanol groups on the capillary wall, the charge density in the Stern layer and the thickness of the diffuse layer. Each of these factors depends on several variables, such as pH, specific adsorption of ionic species in the compact region of the double layer, and ionic strength, that can be affected by the introduction of an additive into the electrolyte solution, which may influence viscosity and dielectric constant of the electrolyte solution as well.

Several authors have tackled the problem of the dependence of the electroosmotic flow on the different variables that may be affected by the introduction of an additive into the electrolyte solution. A brief description of the effects of these variables on the electroosmotic flow is reported below.

2.1. pH

The effect of pH on the electroosmotic flow has been described in several reports [6,8,18–25]. Ionic additives may alter the hydrogen ion concentration of electrolyte solutions having low buffering capacity. A change in the protonic equilibrium directly influences the zeta potential through the variation of the charge density on the capillary wall resulting from the deprotonation of the surface silanol groups, which increases with increasing pH. At acidic pH, the ionization of the surface silanol groups is suppressed and the zeta potential approaches zero, determining the virtual annihilation of the electroosmotic flow. Under alkaline conditions, the silanol groups are fully charged and the zeta potential reaches its maximum value corresponding with a plateau value of the electroosmotic flow. Between these extreme conditions, the zeta potential rapidly increases with increasing pH up to the complete dissociation of the silanol groups, determining the well known sigmoidal pH-dependence of the electroosmotic flow [18,20].

2.2. Ionic strength

Besides the pH, the introduction of an additive to the electrolyte solution may alter the ionic strength. This parameter influences the thickness of the diffuse part of the electric double layer to which the zeta potential is directly proportional [26,27]. Since the thickness of the diffuse part of the electric double layer is proportional to the inverse of the square root of the ionic strength [28], the electroosmotic flow is expected to decrease with increasing the ionic strength of the electrolyte solution. The impact of buffer concentration and ionic strength of the electrolyte solution on the electroosmotic flow has been the subject of several investigations which have produced data showing decreasing electroosmotic flow with increasing buffer concentration or ionic strength [19,27,29–37]. Tsuda et al. [27] and, subsequently, Issaq et al. [33] have reported that the electroosmotic mobility (μ_{eo}) depends on the inverse of the square root of the electrolyte concentration, according to the following relationship:

$$\mu_{eo} \approx \frac{e}{3 \times 10^7 |z| \eta \sqrt{C}} \quad (2)$$

where e, z, η and C are the total charge per unit surface area, the electron valence of the electrolyte, the viscosity, and the concentration of the electrolyte in the bulk solution, respectively.

Another model that accounts for the decrease of the electroosmotic flow with increasing the electrolyte concentration has been developed by Salomon et al. [34]. According to this model, the electroosmotic mobility depends on the concentration of a monovalent counter-ion, introduced with the buffer, through the following parameters: the initial charge per unit area at the surface of the silica capillary wall (Q_0); the thickness of the mobile region of the electric double layer, which is postulated to be composed of a fixed thickness (d_0) and the Debye–Hückel thickness (δ); the constant K_{wall}, defined as

the equilibrium constant between the cations in the buffer solution and adsorption sites on the capillary wall. These parameters are related to the dependence of the electroosmotic mobility on the concentration of the monovalent cation of the buffer $[M^+]$ by the following relationship:

$$\mu_{eo} = \frac{Q_0}{\eta(1 + K_{wall}[M^+])}\left(d_0 + \frac{1}{K'\sqrt{[M^+]}}\right) \quad (3)$$

where η is the viscosity and K' is a constant that for a dilute aqueous solution at 25°C is equal to $3.2 \cdot 10^9$ $m^{-1}(mol\,l^{-1})^{-1/2}$. According to this model, increasing the concentration of the monovalent counter-ion in the bulk solution influences the zeta potential by reducing the Debye–Hückel thickness of the diffuse double layer and by neutralizing the negative charges on the capillary wall, resulting from the ionization of the silanol groups.

Salomon et al. have also reported data on the variations in the electroosmotic flow observed with the 2-(N-morpholino)ethanesulfonic acid (MES) buffers of lithium, sodium, potassium, rubidium and histidine, under otherwise constant conditions. The variation of the electroosmotic flow observed with different types of cations used in the buffer solution has been attributed to a combination of factors [34].

Other authors [33,38–40] have observed that the electroosmotic mobility increases with increasing the crystal radii of the different counter-ions used in buffer solutions containing various alkali metal ions. It has been hypothesized that the larger and more complex the structure of a buffer cation, the greater its Coulombic binding at the interface between the capillary wall and the electrolyte solution which reduces the zeta potential and, consequently, the electroosmotic flow.

2.3. Adsorption of counter-ions

Certain counter-ions, such as polycationic species, cationic surfactants, and several amino compounds, can be firmly held in the compact region of the electric double layer by forces additional to those of simple Coulombic origin. This specific adsorption of counter-ions at the interface between the capillary wall and the electrolyte solution results in a drastic variation of the positive charge density in the Stern layer which reduces the zeta potential and hence the electroosmotic flow. If the positive charge density of the adsorbed counter-ions exceeds the negative charge density on the capillary wall, resulting from the ionization of silanol groups, the zeta potential becomes positive and the concomitant electroosmotic flow is reversed from cathodic to anodic. Reversal of the direction of the electroosmotic flow in CZE has been reported first by Tsuda [41] who investigated the use of cetyltrimethylammonium bromide (CTAB) to control the electroosmotic flow in capillary electrophoresis, as previously reported by Reijenga et al. in capillary isotachophoresis [42]. The dependence of the electroosmotic mobility on the concentration of CTAB and other alkylammonium surfactants [43–45] has been interpreted on the basis of the model proposed by Fuerstenau [46] and Somasundnam et al. [47] to explain the adsorption of alkylammonium salts on quartz. According to this model, the adsorption in the Stern layer as individual ions of surfactant molecules in dilute solution results from the electrostatic attraction between the head groups of the surfactant and the ionized silanol groups at the surface of the capillary wall. As the concentration of the surfactant in the solution is increased, the concentration of the adsorbed alkylammonium ions also increases and reaches a critical concentration at which the Van der Waals attraction forces between the hydrocarbon chains of adsorbed and free surfactant molecules in solution cause their association into hemimicelles, i.e. pairs of surfactant molecules with one cationic group directed toward the capillary wall and the other directed out into the solution.

The beneficial effect of lowering the velocity or reversing the direction of the electroosmotic flow on the resolution of two adjacent peaks was evidenced first by Jorgenson and Lukacs [48] on the basis of the following expression for resolution in electrophoresis elaborated by Giddings [49]

$$R_s = \frac{\sqrt{N}}{4}\left(\frac{\Delta\mu}{\mu_{av} + \mu_{eo}}\right) \quad (4)$$

where N is the number of theoretical plates, $\Delta\mu$ and μ_{av} are the difference and the average value of the electrophoretic mobilities of two adjacent peaks, respectively, and μ_{eo} is the electroosmotic mobility. According to this equation, the highest resolution is obtained when the electroosmotic mobility has the

same value but opposite direction of the average electrophoretic mobility of the two adjacent peaks.

Several other compounds have been found to adsorb at the interface between the capillary wall and the electrolyte solution, determining the reduction or/and the reversal of the direction of the electroosmotic flow [9,50–58]. A theoretical model which correlates the electroosmotic mobility with the charge density in the Stern part of the electric double layer (arising from the adsorption of counter-ions) and the charge density at the capillary wall (resulting from the ionization of silanol groups) has been developed in this laboratory [54]. According to this model, the dependence of the electroosmotic mobility on the concentration of the adsorbing additive (C) in the electrolyte solution is expressed as

$$\mu_{eo} = \frac{4\pi}{\kappa\eta}\left[\frac{zen_0\dfrac{C}{55.6}\exp\left(\dfrac{ze\psi_\delta + \Phi}{kT}\right)}{1+\dfrac{C}{55.6}\exp\left(\dfrac{ze\psi_\delta + \Phi}{kT}\right)} - \left(\frac{\gamma}{1+\dfrac{[H^+]}{K_a}}\right)\right] \quad (5)$$

where κ is the Debye–Hückel thickness of the diffuse double layer, η is the viscosity of the electrolyte solution, e is the elementary charge, z is the valence of the adsorbing ion, k is the Boltzmann constant, T is the absolute temperature, n_0 is the number of accessible sites in the Stern layer, Φ allows for any specific adsorption potential, γ is the sum of the ionized and protonated surface silanol groups, H^+ is the bulk electrolyte hydrogen ion concentration and k_a is the silanol dissociation constant. According to this equation, at constant ionic strength and pH, the electroosmotic mobility depends mainly on the surface density of the adsorbed counter-ions in the Stern region of the electric double layer, which should follow a Langmuirian-type adsorption model. This prediction has been shown to be in good agreement with the experimental electroosmotic mobility trends measured with several additives at different concentrations [54,55,59] and pH values [59] (see Figs. 2 and 3).

2.4. Viscosity and dielectric constant

The incorporation of an additive into the electrolyte solution may alter the viscosity and dielectric constant of the bulk solution resulting in a variation

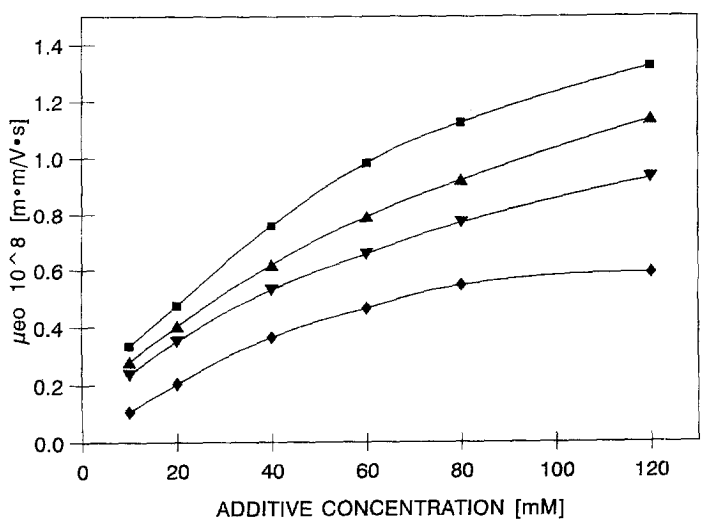

Fig. 2. Dependence of the electroosmotic mobility on the concentration of additive in the running electrolyte at constant ionic strength (123 mM) and pH (2.5). Capillary, fused-silica 37 cm total length (30 cm to the detector)×75 μm I.D.; applied voltage, 10 kV; temperature, 25°C; neutral marker, 5-(hydroxymethyl)-2-furaldehyde; detection wavelength, 280 nm at the anodic end; additives, ■=Triethylamine, ▲=N,N-diethylethanolamine; ▼=N-ethyldiethanolamine, ♦=triethanolamine. Adapted from Ref. [55] with permission.

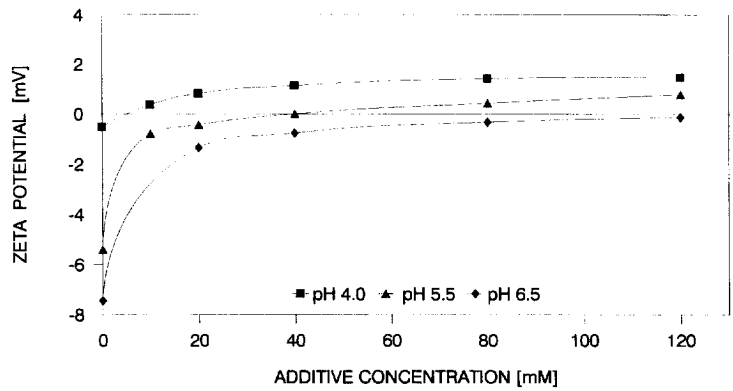

Fig. 3. Dependence of the zeta potential on the concentration of N,N,N′,N′-tetramethyl-1,3-butanediamine (TMBD) at three different pH values. Symbols, ■ = pH 4.0, ▲ = pH 5.5, ♦ = pH 6.5. Capillary, bare fused-silica 37 cm total length (30 cm to the detector) × 75 μm I.D.; applied voltage, 10 kV; temperature, 28°C; neutral marker, acrylamide (3.4 mg/ml in H_2O); detection wavelength, 254 nm either at the cathodic or anodic end upon reversing the polarity. Adapted from Ref. [59] with permission.

of the electroosmotic flow, as it is predicted by the Helmholtz–von Smoluchowski's equation for the electroosmotic flow (see Eq. (1)). The impact of several additives on the viscosity of the electrolyte solution and on the electroosmotic flow is depicted in Fig. 4 [60]. It is observed that, with the exception of acetonitrile, the variation of the electroosmotic flow parallels the changes in the viscosity of the bulk solution. On the other hand, there have been several papers that have reported variations of the electroosmotic flow resulting from the use of neutral polymers and organic solvents [32,60]. that could not be simply explained by the corresponding changes in the values of viscosity and dielectric constant of the bulk solution, as for the case of acetonitrile in Fig. 4.

There have been several studies aimed at explaining the mechanisms involved in the reduction of the electroosmotic flow by the addition of neutral polymers or organic solvents to the electrolyte solution [34,61–64]. Hjertén [2] has speculated that neutral polymers may adsorb onto the Stern layer causing a variation of viscosity in the double layer with distance from the capillary wall, which will affect the electroosmotic mobility according to the following relationship

$$\mu_{eo} = \frac{\varepsilon}{4\pi} \int_0^{\zeta} \frac{1}{\eta} \, d\psi \qquad (6)$$

where ε is the dielectric constant, ζ is the zeta potential, η is the viscosity and ψ is the electric potential. The value of the integral in this expression will approach zero when the viscosity in the double layer approaches infinity. Accordingly, the electroosmotic flow is drastically reduced when the local viscosity of the double layer is increased as a result of the adsorption of a neutral polymer onto the Stern layer. It is worth to note that at constant value of the viscosity in the electric double layer, Eq. (6) is equivalent to the Helmholtz–von Smoluchowski's expression for the electroosmotic flow.

Similarly to the neutral polymers, organic solvents can adsorb at the interface between the capillary wall and the electrolyte solution, through hydrogen bonding or dipole interaction, thus increasing the local viscosity within the electric double layer [32]. Organic solvents may also influence the zeta potential by affecting the ionization of the silanol groups at the capillary surface, whose pK has been found to be shifted toward higher values with increasing the content of organic solvents in the electrolyte solution [61]. Schwer and Kenndler [61] have also related the dependence of the zeta potential on the fraction of an organic solvent incorporated in the electrolyte solution to the consequent variation of both the dielectric constant and the adsorption of counter-ions in the Stern layer. In practice, introducing a neutral polymer or an organic solvent to the electrolyte solution results in multiple changes, generally involving the viscosity and the dielectric constant of the bulk

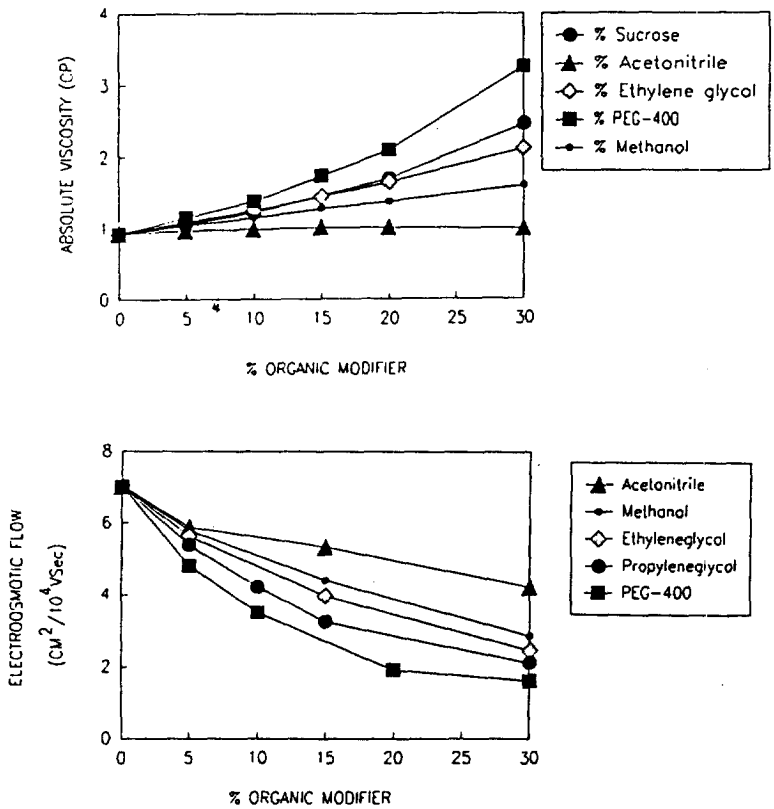

Fig. 4. Effect of the incorporation of neutral additives into the electrolyte solution on absolute viscosity (panel A) and electroosmotic flow (panel B). Capillary, fused-silica 57 cm total length (50 cm to the detector)×75 μm I.D.; applied voltage, 25 kV; background electrolyte, 50 m*M* tricine buffer (pH 8.0); temperature 25°C. Adapted from Ref. [60] with permission.

solution, the ionization of the silanol groups on the capillary wall and the charge density in the Stern layer, as well as the local viscosity and the dielectric constant of the electric double layer.

3. Additives employed in protein CZE

The introduction of a non-buffering additive to the electrolyte solutions for protein CZE is generally aimed at preventing the undesirable interactions between the analyte and the active sites on the inner wall of the fused-silica capillary, consisting of ionizable silanol groups, siloxane bridges, and hydrogen bonding sites. In addition, additives are also employed for enhancing selectivity and resolution, controlling the electroosmotic flow, increasing protein solubility, and probing biochemical mechanisms. This section discusses the relevant literature describing the use of additives in protein CZE, a representative list of which is given in Appendix A.

3.1. Neutral polymers

Hjertén [2,65] was the first to suggest the use of methyl cellulose and other neutral polymers as additives of the electrolyte solution for suppressing convective disturbances in wide bore capillaries (1–3 mm), as well as for controlling electroosmotic flow and minimizing protein–capillary wall interactions. Neutral polymers are believed to adsorb at the interface between the capillary wall and the electrolyte solution with the main consequence of increasing the local viscosity in the electric double layer and masking the silanol groups and the other active sites on the capillary surface. This results in lowering or suppressing the electroosmotic flow (see Section 2.4) and in reducing protein capillary wall

interactions. Since Hjertén's papers, several researchers have reported the successful separation of proteins in bare fused-silica capillaries using electrolyte solutions containing very low concentrations of modified celluloses as dynamic coating agents of the capillary wall to prevent undesirable interactions with the analyte. Examples of this strategy have been reported by Lindner et al. [66,67] who have successfully separated rat liver histones into several fractions [66] and individual histone H1 variants into distinct phosphorylated derivatives [67], using untreated capillaries and electrolyte solutions containing 0.03% (w/v) hydroxypropylmethyl cellulose. The incorporation of modified celluloses into the electrolyte solution has also been employed in conjunction with the use of chemically coated capillaries. Such strategy has been adopted for the analysis of milk serum proteins and caseins by CZE using hydrophilic coated capillaries and electrolyte solutions containing either methylhydroxyethyl cellulose or methylhydroxypropyl cellulose at concentrations as low as 0.05% (w/v) [68]. In another example [69], the analysis of cerebrospinal fluid proteins, of interest for the diagnosis of various disorders of the central nervous system, has been performed using an hydrophilic coated capillary and methyl cellulose at concentration of 0.04% (w/v) as the additive of the electrolyte solution. In these applications employing hydrophilic coated capillaries, the benefit of introducing a modified cellulose to the electrolyte solution has been attributed to the capability of these additives in masking the residual protein adsorption sites on the capillary wall.

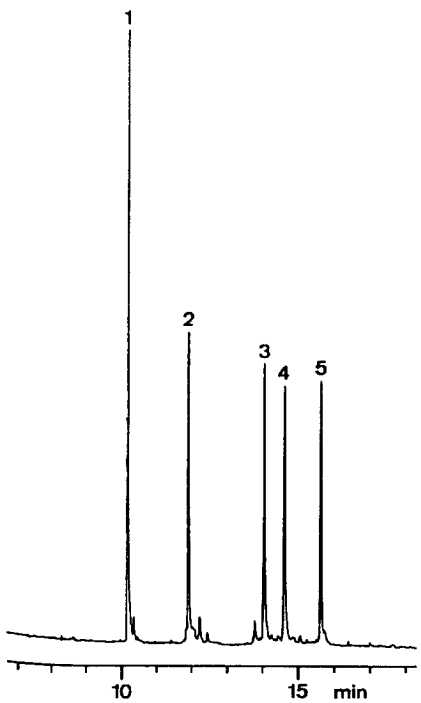

Fig. 5. Separation of basic proteins in a untreated fused-silica capillary using a running electrolyte solution containing polyvinylalcohol (PVA). Capillary, 70 cm total length (57 cm to the detector)×75 μm I.D.; applied voltage, 25 kV; electrolyte solution, 20 m*M* sodium phosphate (pH 3.0) containing 30 m*M* sodium chloride and 0.05% (w/v) PVA 15 000; temperature, 15°C. Proteins, 1=cytochrome *c*, 2=lysozyme, 3=trypsin, 4=trypsinogen, 5=α-chymotrypsinogen A. Adapted from Ref. [70] with permission.

Besides modified celluloses, other linear and branched neutral polymers have been proposed as the additive of the electrolyte solution for the dynamic coating of the capillary wall. For example, Gilges et al. [70,71] have demonstrated that introducing at acidic pH values very low concentrations (0.05%, w/w) of either poly(vinyl alcohol) or dextran to the electrolyte solution allows the successful separation of basic proteins in bare fused-silica capillaries with efficiency in the range of one million of theoretical plates per meter (see Fig. 5). Under these conditions the separations are performed in absence of the electroosmotic flow which is suppressed as a result of the dynamic coating of the capillary wall by the hydroxylic polymeric additives. However, the capability of these additives at masking the protein adsorption sites on the capillary wall is compromised by increasing the pH and results to be not effective at all at pH values higher than pH 5.0 as a consequence of the decreased concentration of protonated silanol groups available for hydrogen bonding.

Recently, Iki and Yeung [72] have reported the use of poly(ethylene oxide) for the dynamic coating of bare fused-silica capillaries which allows the efficient separation of basic proteins in the range of pH 3–7. This polymer is believed to adsorb at the interface between the capillary wall and the electrolyte solution through hydrogen bonding between the ether oxygens of the additive, which act as hydrogen acceptors, and the protonated hydroxyl groups on the capillary wall, acting as hydrogen donors. The most efficient and stable coating is obtained when the capillary is treated with 1.0 *M*

hydrochloric acid for 5 min before use in order to enhance the concentration of protonated superficial hydroxyl groups available for hydrogen bonding. Once the polymer solution is flushed in the capillary, the dynamic coating proves to be very stable and is not desorbed even if electrophoresis is run with the electrolyte solution without the additive, as has been deduced by the highly efficient protein separation obtained under these conditions.

These examples indicate that the use of linear or branched non-ionic polymers as additives for controlling the electroosmotic flow and masking the active protein adsorption sites on the capillary wall is restricted to very low concentrations of the polymer in the electrolyte solution. Above a critical concentration [73–75], the chains of linear or branched polymers begin to overlap and interact with one another through van der Waals forces and hydrogen bonding, giving rise to a polymer network which may act as sieving dynamic matrices for the separation of proteins and other macromolecules. Polymer solutions at concentrations above the threshold value where the network structure begin to be formed are said to be 'entangled'. Zhu et al. [76] were the first to suggest the use of poly(ethylene glycol) (PEG) as a sieving medium for the separation of the monomer, dimer and trimer of albumin by CZE using a capillary coated with a covalently bonded polymer and electrolyte solution consisting of 0.1 *M* phosphate buffer (pH 2.5) containing 5% (w/v) PEG. Electrolyte solutions containing poly(ethylene glycol) have also been employed for monitoring the re-oxidation of reduced bovine trypsinogen [77]. The addition of 5% (w/v) PEG to the electrolyte solution has been reported to facilitate the size-based separation of native and reduced trypsinogen by the sieving effect of the entangled polymer solution. Entangled polymer solutions have also been employed as sieving dynamic matrices for the separation of proteins as their complexes with sodium dodecyl sulfate (SDS) by a size-dependent mechanism, similar to that operating in SDS polyacrylamide gel electrophoresis (SDS-PAGE). This mechanism is based on the assumption that fully denatured proteins bind hydrophobically a constant amount of SDS (1.4 g of SDS per 1 g of protein) [78], resulting in complexes of approximately constant charge-to-mass ratios and, consequently, identical electrophoretic mobilities. Therefore, in a sieving medium protein–SDS complexes migrate proportionally to their effective molecular radius and thus to the protein molecular mass [79].

Deviations from this general rule have been found for highly basic proteins (e.g., histones) which tend to exhibit lower charge-to-mass ratios when complexed with SDS, as a result of the relatively large number of positively charged basic amino acid residues. Higher charge-to-mass ratios may be displayed by phosphorylated or sulfonated proteins because of the additional charges from the phosphate and sulfate groups. On the other hand, hydrophobic membrane proteins may bind a larger amount of SDS molecules than cytosolic proteins, resulting in higher charge-to-mass ratios. Other exceptions are observed with glycoproteins which usually bind a lower amount of SDS, due to the presence of the hydrophilic carbohydrate moieties. However, in spite of the possible exceptions, SDS-PAGE is widely employed in protein analysis for purity assay and molecular mass estimation. The size-dependent separation of protein–SDS complexes in acrylamide gels polymerized in the capillary format has been demonstrated [80,81]. Since then, many other size-dependent separation methods developed for slab gel electrophoresis have been transferred to the capillary format. Specific reviews on the size-dependent separation of protein–SDS complexes by capillary electrophoresis has been published by several authors [75,82–84].

Gel-filled capillaries suffer of several drawbacks which include bubble formation from gel shrinkage during polymerization, deamidation of polyacrylamide at high pH values (producing charged acrylamide residues), and limited stability and column lifetime. Improvements in the separation of protein–SDS complexes have been obtained by using non-crosslinked polyacrylamide [85]. The linear polyacrylamide entangled polymer solutions have proven to be more flexible than the crosslinked gels as a result of the network structure resulting from physical interactions in dynamic equilibrium rather than crosslinking due to covalent bonds between the polymer chains. However, even with linear polyacrylamide several limitations are encountered, such as severe detection limits, resulting from the absorption of amino groups at the same wavelength value where the protein extinction coefficient is larger (214 nm), and limited column lifetime, particularly when

elevated electric fields are employed. These limitations have led investigators to search for UV transparent entangled polymer solutions of ideally low viscosity to facilitate protein detection and ease replacement of the sieving matrix in the capillary.

UV-transparent replaceable polymer networks of dextran and poly(ethylene glycol) for the size-dependent separation of protein–SDS complexes have been described by Gansler et al. [86]. The low to moderate viscosities of these entangled polymer solutions allow the routine replacement of the polymer network into the capillary, leading to increased repeatabilities of the migration times and prolonged column lifetime. In contrast to linear polyacrylamide, protein detection at 214 nm becomes possible resulting in a significative increase in sensitivity. In addition, both dextran and PEG permit the rapid estimation of protein molecular mass and quantitative dosage of the separated proteins using either electrokinetic or pressure injection method. This study demonstrates that the size selectivity of the protein–SDS complexes depends on the molecular mass of dextran. The higher the molecular mass of the polymer the higher the selectivity. However, the use of entangled polymer solutions of dextran of relatively low molecular mass has also been reported [87,88].

Besides being employed as a dynamic coating agent (see above), poly(ethylene oxide) (PEO) has also been used as a sieving matrix for protein separation by SDS capillary electrophoresis, either alone [87,89] or in mixture with PEG [90,91]. The effect of temperature on the migration behavior and band broadening of the protein–SDS complexes separated in entangled polymer solution of dextran and PEO has been investigated by Guttman et al. [87]. They reported that the migration times of the protein–SDS complexes decreased in both sieving matrices whereas peak efficiency increased with increasing temperature in the entangled polymer solution of dextran but decreased with PEO as the sieving matrix.

Size-dependent separation of proteins–SDS complexes with entangled polymer solutions of a branched polysaccharide composed of α-(1-6)-linked maltotriose, namely pullulan, has been described by Nakatani et al. [92–94]. This polymer acts as a sieving matrix at concentrations ranging from 3–10% (w/v), allowing the baseline separation of proteins with molecular mass in the range of 14 000–116 000. The migration times of the protein–SDS complexes decrease with increasing temperature. This effect has been mainly attributed to changes in viscosity of the pullulan solution used as the sieving medium.

Recently, Righetti et al. [95] demonstrated that SDS capillary electrophoresis of proteins can be successfully performed in polymer network of poly(vinyl alcohol) (PVA). This polymer acts as a sieving matrix for protein–SDS complexes at concentration as low as 3% (w/v) allowing entangled polymer solutions of extremely low viscosity with positive effects on the facility of the routinely replacement of the polymer network into the capillary and, hence, on the repeatability of separations. Other advantages of PVA are full transparency in the UV region down to 200 nm and excellent resolution in the 14 000–94 000 protein molecular mass range.

3.2. Ionic salts and zwitterions

The use of electrolyte solutions containing relatively high concentrations of a ionic salt has been one of the earlier strategy for minimizing undesirable interactions between proteins and the surface of fused-silica capillaries [9,11]. This approach is based on the assumption that the main contribution to these interactions is due to the coulombic attraction between the weakly acidic silanol groups on the capillary wall and the positively charged moieties on the basic amino acid residues of the protein [11]. Most of the positively charged moieties on proteins correspond to the guanidinium and ε-NH_2 groups of arginine and lysine residues, respectively, other than the α-NH_2 terminal and histidine residues. These moieties may interact with the acidic silanol groups even with the protein at the isoionic point (zero net charge) as a result of the different distribution of the charge density on the protein molecule. Following this line of reasoning several researchers have viewed the wall of the fused-silica capillary as a cation exchange site which can be suppressed by increasing the ionic strength of the electrolyte solution [9,11,96].

The investigation on the effect of several alkali metal ions on the electrophoretic behavior of model basic proteins in bare fused-silica capillaries has led to the conclusion that the larger the crystal radii of

the metal ion the greater is its effectiveness at minimizing protein capillary wall interactions [11]. This is in agreement with the observation that the larger and more complex is the structure of a cation the greater is its binding at the interface between the capillary wall and the electrolyte solution [33,38–40], resulting in the practical suppression of the cation exchange sites for proteins. However, Cs^+ which among Li^+, K^+ and Na^+ is the most effective at masking the ion exchange sites on the capillary wall suffers from an unacceptably high optical absorbance at low wavelengths. Since the highly hydrated Li^+ has proved to be the less effective masking silanol agent, being the most weakly bound in the Stern layer, the use of the above alkali metal is thus restricted to K^+ and Na^+, which have been found to be equivalent at masking the protein adsorption sites on the capillary wall [11]. Moreover, Green and Jorgenson [11] have reported that different anions of the above alkali metal salts do not have a measurable effect on the capability of these additives at masking the cation exchange sites on the capillary wall. However, sulfates has been demonstrated to be superior to nitrates and halogenides in terms of UV transparency.

The use of electrolyte solutions containing high concentrations of a ionic salt can be accompanied by the employment of zwitterionic buffers of low conductivity to limit the generation of Joule heat. This can be further minimized by reducing the inner diameter of the capillary which has the effect of increasing the surface-to-volume ratio with the consequence of improving the heat dissipation through the capillary wall. On the other hand, this approach results also in shortening the path length of the on-column detection with the consequence of reducing detection sensitivity. In addition, Joule heating can be minimized by reducing the applied voltage which, however, implies a proportional reduction in peak efficiency and an increase in the analysis time.

Recently, the advantages and drawbacks of using electrolyte solutions containing high concentrations of ionic salts have been evaluated in comparison to other additives employed for the separation of model basic proteins and for histone H1 subtypes by Cifuentes et al. [57] and by Lindner et al. [97], respectively. In both cases the use of other additives has been shown to be superior to the employment of ionic salts. An improvement in the strategy of suppressing the positively charged active sites on the capillary wall by increasing the ionic strength has been obtained by incorporating zwitterions rather than ionic salts in the electrolyte solution. Zwitterions have the advantage of non-contribution to the conductivity of the electrolyte solution and of competition with proteins for the acidic silanol groups on the capillary wall. In addition, zwitterions may act as ion-pairing or competing agents for the positively charged moieties on the proteins with the result of reducing their availability to the ion exchange sites on the capillary wall. The ion-pairing formation with proteins is also believed to contribute to break-up of protein–protein interactions.

This strategy was first proposed by Bushey and Jorgenson [12] who demonstrated the benefit of using zwitterions as buffer additives for CZE of basic proteins in bare fused-silica capillary, reporting the highly efficient separation of lysozyme and α-chymotrypsinogen A using phosphate buffer (pH 7.6) containing both 2.0 *M* trimethylglycine (betaine) and 0.1 *M* potassium sulfate (see Fig. 6).

The zwitterion trimethylammonium propylsulfonate, commercialized by Waters under the trade name of AccuPure-Z 1-methyl, has also been suggested to prevent the binding of proteins to fused-silica capillary columns. The additive is effective for this purpose when used in combination with moderate ionic strength buffers. For example, Guzman et al. [98] described the effects of trimethylammonium propylsulfonate on the capillary electrophoretic separation and dosage in a bare fused-silica capillary of a humanized monoclonal antibody in bulk form and in a typical therapeutic formulation. In another example, trimethylammonium propylsulfonate has been successfully used to improve resolution and peak shape of ricin (a heterodimeric glycoprotein phytotoxin) in a crude, acidic extract of the castor bean plant, *Ricinus communis* [99].

The incorporation of high concentrations of either zwitterions or ionic salts in the electrolyte solution causes a reduction in the electroosmotic flow resulting from the increased ionic strength and the consequent reduction in the thickness of the electric double layer (see Section 2.2). In addition, high concentrations of zwitterions increase the viscosity of the electrolyte solution resulting in a further decrease in the electroosmotic flow, as it is discussed in Section 2.4.

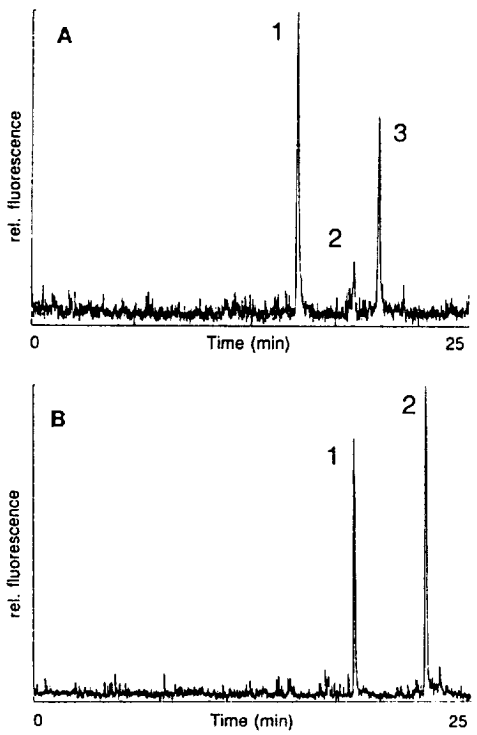

Fig. 6. Electropherograms of (panel A) lysozyme (peak 1), Indole (peak 2) and α-chymotrypsinogen A (peak 3) obtained with 0.04 *M* sodium phosphate buffer (pH 7.0) containing 0.1 *M* potassium sulfate and (panel B) lysozyme (peak 1) and α-chymotrypsinogen A (peak 2) obtained with 0.04 *M* sodium phosphate buffer (pH 7.6) containing 0.1 *M* potassium sulfate and 2.0 *M* betaine. Capillary, bare fused-silica 75 cm total length (65 cm to the detector)×25 μm I.D.; applied voltage, 20 kV. Adapted from Ref. [12].

The ionic strength of the electrolyte solution also influences the electrophoretic mobility of the analytes according to a mechanism similar to that used to describe the impact of this parameter on the electroosmotic flow [33]. According to this model, the electrophoretic mobility is directly proportional to the electrophoretic zeta potential (i.e. the potential at the effective radius of the charged analyte) which, in turn, is inversely proportional to the square root of the ionic strength. The result is that the electrophoretic mobility is expected to decrease with increasing the ionic strength of the electrolyte solution. This prediction is in good agreement with experimental data reported by different authors. Bruin et al. [31] observed that the apparent mobility of lysozyme, trypsin and chymotrypsin decreases linearly with increasing the concentration of K_2HPO_4 in the electrolyte solution. Cole et al. [100] also presented data on the effect of the buffer concentration on protein mobility showing that the migration times of several proteins increase linearly with the logarithim of the buffer concentration, which is consistent with a decreased electrophoretic mobility and a decreased electroosmotic flow.

3.3. Amine modifier

Amino compounds are generally recommended as additives of the mobile phases employed in reversed-phase chromatography of ionogenic substances to suppress the untoward effect of the residual silanol groups at the stationary phase [101]. These additives are believed to act primarily by hydrogen bonding to non-derivatized silanol groups, thereby reducing adsorption and ion-exchange effects [102].

The strategy of masking the silanol groups by amino compounds has also been adopted to improve protein separation by CZE in bare fused-silica capillaries, where the weakly acidic silanol groups on the capillary wall may interact with the positively charged moieties on the proteins. Electrostatic interactions are believed to play a key role in the adsorption of proteins on the capillary wall. The analyte may be attracted to the capillary surface by Coulombic forces and once there hydrogen bonding and hydrophobic interactions can join in immobilizing the protein at the interface between the capillary wall and the electrolyte solution. Therefore, masking the negatively charged silanol groups on the capillary wall by an amino compound incorporated in the electrolyte solution has been considered by several researchers as a method of choice to avoid protein adsorption. The numerous types of amino compounds that have been employed as additives of the electrolyte solutions to minimize protein–capillary wall interactions and to improve peak efficiency and resolution, are summarized in two main groups: one comprising diaminalkanes and monovalent amines, the other including non-polymeric and polymeric polyamines.

3.3.1. Diaminoalkanes and monovalent amines

The utility of diaminoalkanes as additives of the electrolyte solution to aid in suppressing protein–capillary wall interactions was demonstrated first by

Lauer and McManigill [9]. These researchers reported that the addition to the electrolyte solution of as low as 5 m*M* 1,4-diaminobutane (putrescine) is effective at eliminating peak tailing and improving resolution of basic proteins subjected to CZE in bare fused-silica capillaries at neutral and moderately alkaline pH values. They also observed a significant non-linear decreasing of the electroosmotic flow with increasing the concentration of 1,4-diaminobutane in the electrolyte solution from 0–5 m*M*. This effect has been confirmed at different pH values ranging from pH 7–8.5, but has not been observed with other monovalent cations, such as ammonium and trimethyl ammonium (concentration and type of salt are not specified). These data are consistent with a variation of the zeta potential related to the modification of the electric double layer at the interface between the capillary wall and the electrolyte solution caused by the adsorption of 1,4-diaminobutane.

Similar results have been reported by Landers et al. [103] who incorporated low concentrations of 1,4-diaminobutane (0.25–1.0 m*M*) into 100 m*M* borate buffer (pH 8.5) with the purpose of enhancing the resolution of ovalbumin glycoforms by reducing the electroosmotic flow through the adsorption of the diaminoalkane at the interface between the capillary wall and the electrolyte solution. Many other studies have demonstrated the utility of using 1,4-diaminobutane as an additive of the electrolyte solutions employed for the separation of the various glycoforms of different glycoproteins. For example, the carbohydrate-dependent microheterogeneity of recombinant coagulation factor VIIa (r-FVIIa) has been characterized by subjecting to CZE the native and the neuraminidase treated recombinant protein [104]. The separation of the glycoforms of r-FVIIa into six or more peaks has been achieved using 25 m*M* putrescine in 100 m*M* phosphate buffer (pH 8.0). In another example [105], the recombinant human erythropoietin (r-HuEPO) has been resolved in its individual glycoforms using a combination of 1,4-diaminobutane (to diminish protein–capillary wall interactions) with urea (to break-up protein aggregations). In addition, Watson and Yao [106] have demonstrated that the incorporation of 1,4-diaminobutane into the electrolyte solution allows the separation of recombinant granulocyte-colony-stimulating factor glycoforms according to the increasing number of the sialic acids in the glycoprotein molecule. More recently, the resolution of various tranferrin glycoforms differing in the sialic acid content (sialoforms) has been obtained using an electrolyte solution containing 1 m*M* 1,4-diaminobutane in 100 m*M* borate buffer at pH 8.3 [107].

Other diaminoalkanes have been proposed for suppressing capillary wall interactions and for controlling the electroosmotic flow. Rohlicek and Deyl [108] were the first to use 1,5-diaminopentane (cadaverine), demonstrating the beneficial effect of this additive on the separation of standard and naturally occurring protein mixtures with high pH electrolyte solutions. This additive was also successfully employed for the determination of the microheterogeneity of glycoproteins, including recombinant coagulation factor VIIa [104] and ovalbumin [109]. The resolution of the different glycoforms of several glycoproteins induced by the incorporation of a diaminoalkane into the electrolyte solutions is believed to be not only caused by a reduction of the electroosmotic flow, but also by ion-pair formation between the additive and the negatively charged moieties on the glycoproteins [104,110].

The utility of 1,3-diaminopropane for analyzing basic proteins at pH values well below their isoelectric points has been evaluated by Bullock [112] and Bullock and Yuan [111]. They demonstrated that the addition to phosphate or formate buffers of 1,3-diaminopropane at concentrations ranging from 30–50 m*M*, along with moderate levels of sodium or potassium salts, provides a series of electrolyte solutions suitable for the efficient separation of basic proteins in the pH range 3.5–9.0. At neutral pH, most proteins among those investigated by Bullock and Yuan [111] irreversibly adsorb, whereas the others migrate with severe peak tailing, when 1,3-diaminopropane is at concentrations below 5.0 m*M* in the electrolyte solution containing 40 m*M* sodium sulfate. Peak shape improves and migration times become longer by increasing the concentration of the additive, while maintaining constant the level of sodium sulfate. Above 50 m*M* 1,3-diaminopropane, further improvements in peak shape are modest for any protein, whereas migration times continue to increase as a result of decreasing in the electroosmotic flow. Similar results have been reported by

Song et al. [113] who investigated the dependence of peak efficiency and migration behavior of several basic proteins on the concentration of ethylene diamine in the range 20–80 m*M* at different pH values (6.5, 8.0 and 9.5) and in the presence of a constant level of sodium sulfate in the electrolyte solution (35 m*M*). The presence of different alkali metal salts (sodium and potassium sulfate, chloride and phosphate) at various concentrations in the electrolyte solutions containing 50 m*M* 1,3-diaminopropane, has shown to produce variations in selectivity and protein migration times that have been attributed to specific effects induced by the salt, such as changes in protein conformation or in the ionization of the individual amino acid residues [111]. Examples of practical applications of this additive have been reported by Morbeck et al. [114] for the analysis of the microheterogeneity of the glycoprotein–human chorionic gonadotropin (see Fig. 7) and by Lindner et al. [97] for the separation of histone H1 subtypes.

The investigation of the dependence of the electroosmotic mobility on the concentration of ethylenediamine, 1,3-diaminopropane and 1,4-diaminobutane in the electrolyte solution has confirmed the trend observed by Lauer and McManigill [9] with 1,4-diaminobutane that the electroosmotic flow decreases non-linearly with increasing the concentration of the diamine in the buffer [115]. This indicates that the diaminoalkanes adsorb at the interface between the capillary wall and the electrolyte solution with the result of lowering the zeta potential, which is also affected by the reduction in the thickness of the diffuse double layer as a consequence of the increased ionic strength. In addition, besides controlling the electroosmotic flow, the adsorption of the amino modifier in the immobilized region of the electric double layer is effective at masking the silanolic adsorption sites for proteins.

Diaminoalkanes in which the diamine groups have been replaced with quaternary ammonium moieties have also been employed as electrolyte additives. Theoretically, these additives are expected to be advantageous over diaminoalkanes are a result of their non pH-dependent ionization and the absence of pH alteration upon their addition to the electrolyte solution. The α,ω bis-quaternary ammonium alkanes hexamethonium chloride and bromide and decamethonium bromide, having alkyl chain of six and ten carbon atoms respectively, have successfully been employed by the group of Landers for the analysis of glycoprotein microheterogeneity (Oda and Landers [107], Oda et al. [116]). Glycoform resolution has been found to be higher with decamethonium bromide than with hexamethonium bromide and with 1,4-diaminobutane in the order, indicating a correlation between additive effectiveness at resolving the different glycoprotein isoforms and alkyl chain length. In addition, glycoform resolution is obtained at much lower additive concentration with bis-quaternary ammonium alkyl com-

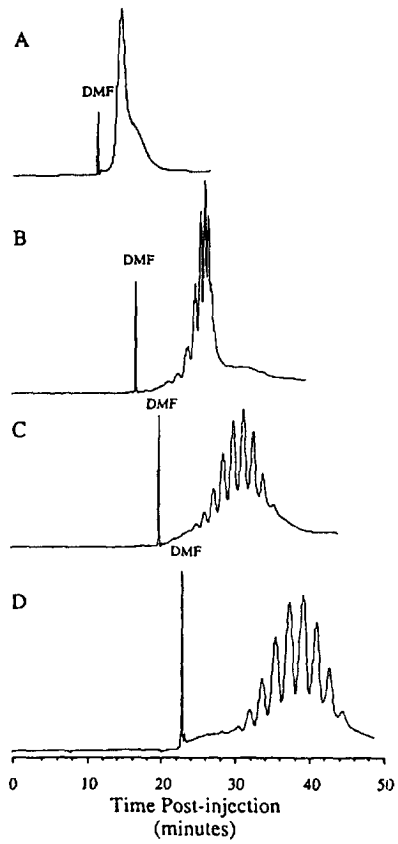

Fig. 7. Effect of various concentrations of 1,3-diaminopropane on the electrophoretic migration and separation of human chorionic gonadotropin (hCG). Capillary, bare fused-silica 100 cm total length (78 cm to the detector)×50 μm I.D.; applied voltage, 20 kV; temperature, 28°C; electrolyte solution, 25 m*M* borate buffer (pH 8.8) containing 0 (A), 1.0 (B), 2.5 (C) and 5.0 m*M* (D) 1,3 diaminopropane. Adapted from Ref. [114].

pounds (100–300 μ*M*) than with their alkyl diamine counterparts (e.g. 1–5 m*M*).

More recently, Corradini and Cannarsa [59] demonstrated the effectiveness of N,N,N′,N′-tetramethyl-1,3-butanediamine (TMBD) at preventing protein–capillary wall interactions and at controlling the electroosmotic flow in bare fused-silica capillaries at different pH values. This study evaluated the effect of the additive concentration on the electroosmotic flow, separation efficiency and migration behavior of model basic proteins at three different pH values in the range 4.0–6.5. The similar Langmuirian-type dependence of the zeta potential on the additive concentration observed at three different pH values (see Fig. 3 and Section 2.3) is indicative of the specific adsorption of TMBD in the immobilized region of the electric double layer, which follows a common trend at all pH values, consistent with a predicted Langmuirian-type adsorption model [54]. Depending on pH of the electrolyte solution, which affects the ionization of both the silanol groups on the capillary wall and the amino functions of the additive, the incorporation of a given concentration of TMBD in the electrolyte solution results either in the drastic reduction of the electroosmotic flow or in the inversion of its direction from cathodic to anodic, while masking the protein adsorption sites. This results in the possibility of controlling the electroosmotic flow to modulate the electrophoretic resolution for a given separation by appropriate selection of the electrolyte pH and the additive concentration, without losing the effectiveness of the additive at preventing protein capillary wall interactions, as is shown in Figs. 8 and 9, respectively.

Monovalent amines have found relatively limited applications as additives of the electrolyte solutions employed in protein CZE as a result of the generally reported poor effectiveness at reducing protein–capillary wall interactions at pH values between the extremes of the pH scale. The use of triethylamine (100 m*M*) and propylamine (50 m*M*) has been reported by Bullock and Yuan [111] who have evidenced that neither of these monovalent amines were as effective as putrescine in efficiently separating basic proteins at neutral pH in bare fused-silica capillaries. Similar results have recently been reported by Cifuentes et al. [57] in a paper discussing the comparative effectiveness of different electrolyte

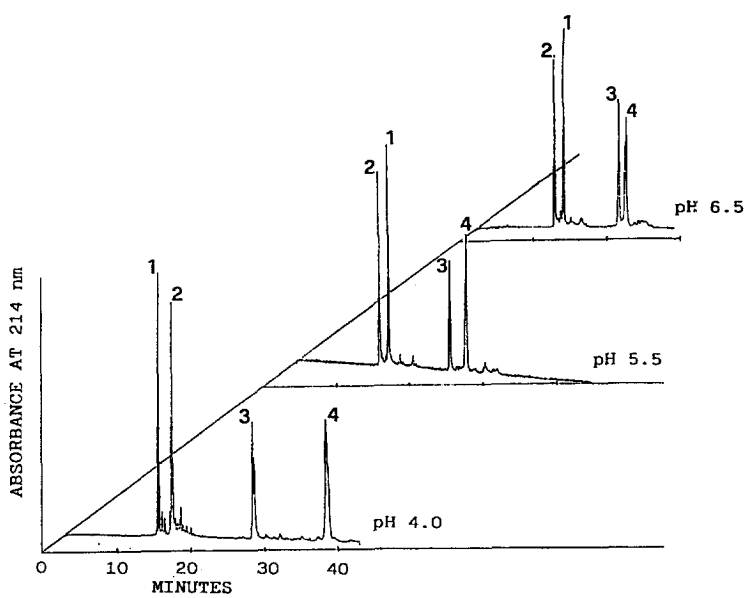

Fig. 8. Separation of basic proteins with electrolyte solutions containing 40 m*M* N,N,N′,N′-tetramethyl-1,3-butanediamine (TMBD) titrated to different pH values with phosphoric acid. Capillary, bare fused-silica 37 cm total length (30 cm to the detector)×75 μm I.D.; applied voltage, 10 kV; temperature, 28°C; detection wavelength, 214 nm at the cathodic end. Proteins, 1=cytochrome *c*, 2=lysozyme, 3=ribonuclease A, 4=α-chymotrypsinogen A. Adapted from Ref. [59] with permission.

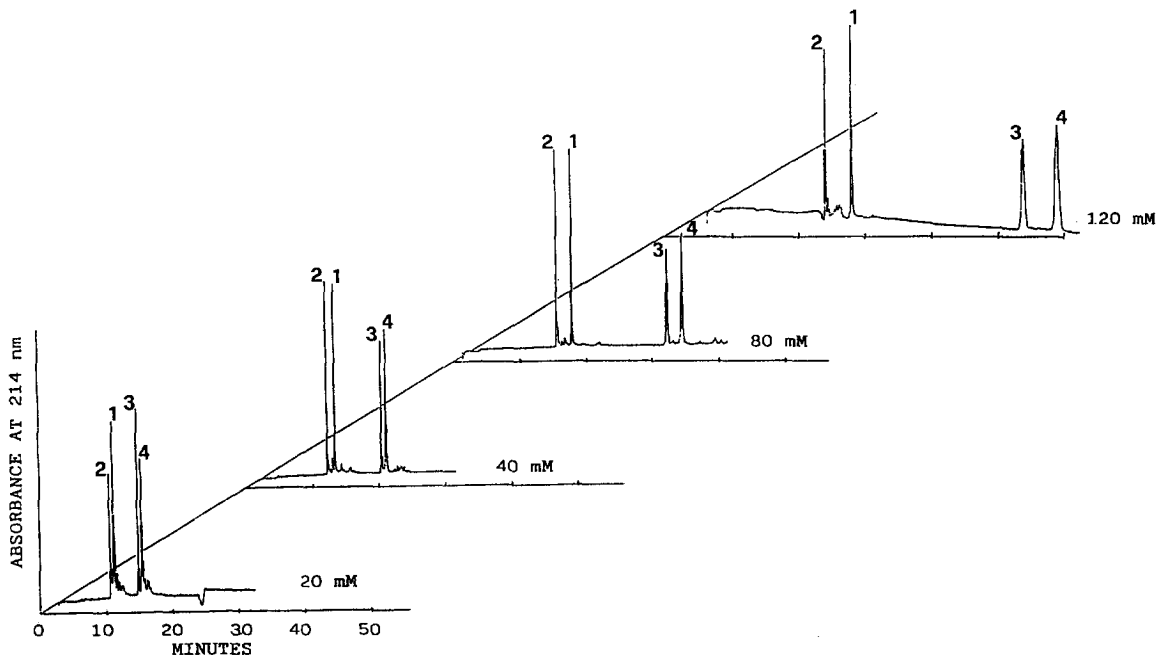

Fig. 9. Separation of basic proteins with electrolyte solutions containing different concentrations of N,N,N′,N′-tetramethyl-1,3-butanediamine (TMBD) titrated to pH 6.5 with phosphoric acid. All other conditions as in Fig. 8. Adapted from Ref. [59] with permission.

additives in reducing protein–capillary wall interactions at pH 7.0.

On the other hand, triethylamine (250 m*M*) and morpholine (250 m*M*) have been found to be effective at masking the silanol groups in uncoated capillary when added to electrolyte solutions at pH 5.5 [117]. Morpholine has also been employed to separate the recombinant human insulin and the recombinant human growth hormone from their degradation products in uncoated capillary with tricine buffer (pH 8.0) containing 20 m*M* sodium chloride [118,119]. In another paper, Rickard et al. [120] reported the use of morpholine to minimize wall interactions either at pH 2.35 or at pH 8.0 in a study aimed at correlate CZE electrophoretic mobilities with physicochemical properties of recombinant human insulin, human growth hormone, human proinsulin, human insulin-like growth factor, bovine somatostatin and their enzymatic digests.

The usefulness of glucosamine and galactosamine as additives for the capillary electrophoresis of basic proteins in untreated capillaries at low pH has been demonstrated in this laboratory and their effectiveness has been positively compared with that of triethylamine and triethanolamine [54]. Another example of the employment of monovalent amines for improving the separation of basic proteins at low pH has been reported by Lindner et al. [97] who demonstrated the effectiveness of triethylamine in increasing peak efficiency of HI histone subtypes.

The effect of four closely related monovalent alkylamines, namely triethylamine, N,N-diethylethanolamine, N-ethyldiethanolamine and triethanolamine, on the electroosmotic flow and on the electrophoretic behavior of model basic proteins in bare fused-silica capillary has been investigated in this laboratory [55]. It has been shown that at pH 2.5 and constant ionic strength the dependence of the anodic electroosmotic mobility on the concentration of the four additives in the range 4–120 m*M* is described by four non-intersecting curves, all Langmuirian in shape. At any additive concentration the value of the electroosmotic mobility is in the order of increasing basicity and decreasing molecular size of the additive. This may indicate that the observed differences in the electroosmotic flow may reflect

differences in the adsorbability of the four closely related monovalent alkylamines, which it seems to be directly proportional to the additive basicity and inversely proportional to their molecular size.

Triethylamine and other monovalent amines have also been found to improve peak efficiency of basic proteins at pH 11 [24]. Other examples of using monovalent amines at alkaline pH values have been reported by Paterson et al. [121], who proposed a combination of ethanolamine and Tween 20 to separate the β-lactoglobulin A, B, and C variants of bovine whey, and by Legaz and Pedrosa [109], who tested the effect of hydroxylamine and ethylamine on the separation of ovalbumin glycoforms.

3.3.2. Polyamines

A number of polyamines have been examined as additives of the electrolyte solutions for their ability in reducing protein–capillary wall interactions and controlling the electroosmotic flow. Multiple functional amino modifiers are expected to be strongly adsorbed at the interface between the capillary wall and the electrolyte solution as a results of the multiple interaction sites possessed by these additives. The adsorption, which may be also irreversible for polymeric polyamines, results in masking the silanol groups and the other adsorption active sites on the capillary wall and in altering the electroosmotic flow, which is lowered and in some cases, reversed from cathodic to anodic.

Besides acting as masking agents for the silanol groups, aliphatic polyamines are also suitable for use as components of the buffer system. For example, phosphate and/or trifluoroacetate salts of selected aliphatic di-, tri- and tetra-amines, having pK_a values in the pH range 3.25–9.84, have been used by Kalman et al. [122] to separate eleven mutants and the wild type of the enzyme *Staphylococcal nuclease* at different pH values in acrylamide coated capillary. A list of aliphatic polyamines, originally introduced in reversed-phase chromatography, which have a potential use as additives in protein CZE is reported in [123]. Examples of the usefulness of tri- and tetra-amines as electrolyte additives with bare fused-silica capillaries have been reported by Corradini and Cannarsa [56], who successfully employed triethylenetetramine for the separation of model basic proteins in bare fused-silica capillaries and by Legaz and Pedrosa [109] who demonstrated the beneficial effect of adding low concentrations of spermine or spermidine to borate buffer for the resolution of ovalbumin glycoforms.

The usefulness of tetraazomacrocycles as electrolyte additives in enhancing the efficiency for the separation of basic proteins in cross-linked polyacrylamide coated capillaries, which may exhibit residual silanolic activity as a result of partial polymeric coverage, has been demonstrated by Cifuentes et al. [124]. Tetraazomacrocycles are cyclic polyamines bearing four amino functions symmetrically distributed around the cavity of a rigid cyclic structure, whose size depends on the number of carbon atoms of the alkyl chain forming the ring. It is reported that the incorporation of 1,4,7,10-tetraazocyclodecane (cyclen) into the electrolyte solution drastically reduces protein–capillary wall interactions, as evidenced by the enhancement of peak efficiency in the presence of this additive.

According to the expectation that the efficacy of a silanol masking agent can be enhanced by increasing the number of amino functions, several researchers have reported the use of polymeric polyamines as additives of the electrolyte solutions employed in protein CZE. An example of this strategy is represented by the use of chitosan, a cationic natural polymer composed of D-glucosamine and N-acetyl-D-glucosamine, which is obtained by partial deacetilation of chitin [125]. As low as 0.002% (w/v) chitosan in the electrolyte solution is sufficient to reverse the direction of the electroosmotic flow from cathodic to anodic and to ensure reasonable separation efficiency of basic proteins as a result of the adsorption of the polycationic additive onto the capillary wall. The anodic electroosmotic flow increases with increasing the concentration of chitosan in the electrolyte solution. However, above 0.05% (w/v) chitosan the electroosmotic flow is relatively constant, indicating a saturation of chitosan coverage at the interface between the capillary wall and the electrolyte solution. A similar trend is reported for migration time repeatability and peak efficiency which increase non-linearly with increasing the concentration of chitosan in the electrolyte solution and reach a plateau at about 0.05% (w/v) chitosan. Since the cationic character of chitosan arises from the primary amino group of the monomer glucos-

amine which has a pK_a value of around 6.3, the positive net charge exhibited at acidic pH values decreases with increasing the electrolyte pH, causing a reduction in the adsorbability of the additive and, thus, in the effectiveness at masking the protein adsorption sites on the capillary wall. In addition, the solubility of chitosan decreases at pH values above 6.0. On the other hand, both anodic electroosmotic flow and protein electrophoretic mobility decrease with lowering the electrolyte pH below 3.5, resulting in a reduction in resolution and peak efficiency. This effect has been attributed to the increased intrinsic viscosity of the diluted chitosan solution observed at the lower pH values. Such increase in the intrinsic viscosity would result from the expansion and stiffening of the chitosan molecules due to the repulsive forces between neighboring charged groups which increase with lowering the electrolyte pH as a result of the increased number of charges of the chitosan backbone. The result of these limitations is that the useful application of chitosan as an electrolyte additive is restricted to the pH range 3.5–5.5.

In other examples [57,126], high molecular mass polyethyleneimine (PEI) has been used as an electrolyte additive for the efficient separation of proteins either in bare fused-silica [57] or in PEI physically coated capillaries [126]. This polyamine has been used as a coating agent by several authors who have proposed either simple physical coating procedures [127] or cross-linking of the adsorbed polyamine [128].

When incorporated into the electrolyte solution at concentration as low as 0.1% (w/v) and used with bare fused-silica capillaries, PEI reverses the direction of the electroosmotic flow from cathodic to anodic, as a result of the strong adsorption of the additive onto the capillary wall. Therefore, basic proteins migrate toward the electroosmotic flow. At pH values at which the electroosmotic mobility results to be greater then the electrophoretic mobility, the polarity of the applied voltage must be reversed from positive to negative in order to displace the proteins to the detection window. The result is that the migration order is reversed compared to that obtained with positive applied voltage.

PEI may interact with negatively charged proteins, resulting in selectivity variations and undesirable interactions with the PEI molecules adsorbed onto the capillary wall. These interactions are believed to be responsible for the poor efficiency observed with acidic proteins at pH values above their isoelectric points. Due to the strong adsorption of PEI molecules on the silica surface, a PEI layer can be coated on the capillary wall by just flushing the capillary with a solution containing high molecular mass PEI. The physically adsorbed layer appears to be very stable and can be used in the pH range 3–11 with electrolyte solutions without PEI [127]. The combination of PEI physically coated capillary and electrolyte solutions containing PEI at different concentrations and pH values has resulted in the possibility of controlling the separation selectivity and resolution of proteins [126]. The observed variations in selectivity and resolution can be attributed to the different changes in the electrophoretic mobility of proteins resulting from the addition of PEI to the electrolyte solution which can be explained by the possible electrostatic interactions occurring between the negatively charged moieties on the proteins and the amino functions of PEI. In addition, hydrophobic interactions and hydrogen bonding are believed to play a role as well.

A further example of using a polyamine as an electrolyte additive has been reported by Cohen and Grushka [129] who proposed polydimethyldiallylammonium chloride, a linear polyquaternary amine polymer. This polymer is strongly adsorbed onto the inner surface of fused-silica capillaries to give a stable dynamic coating that reverses the direction of the electroosmotic flow from cathodic to anodic and enables the highly efficient separation of basic proteins at neutral pH (see Fig. 10). The presence of 0.1 m*M* polydimethyldiallylammonium chloride in the running electrolyte solution is needed to maintain the stability of the dynamic coating which ensures high reproducibility of the anodic electroosmotic flow and, thus, of the protein migration times.

Other polyamines, such as polybrene (or hexadimetrine bromide), a linear hydrophobic polyquaternary amine polymer of the ionene type [130], have been used as coating agents [51,131,132]. These polymers are believed to be irreversibly adsorbed onto the capillary wall by a combination of multi-site electrostatic and hydrophobic interactions and once flushed in the capillary may give very stable coatings which maintain their characteristics also in absence

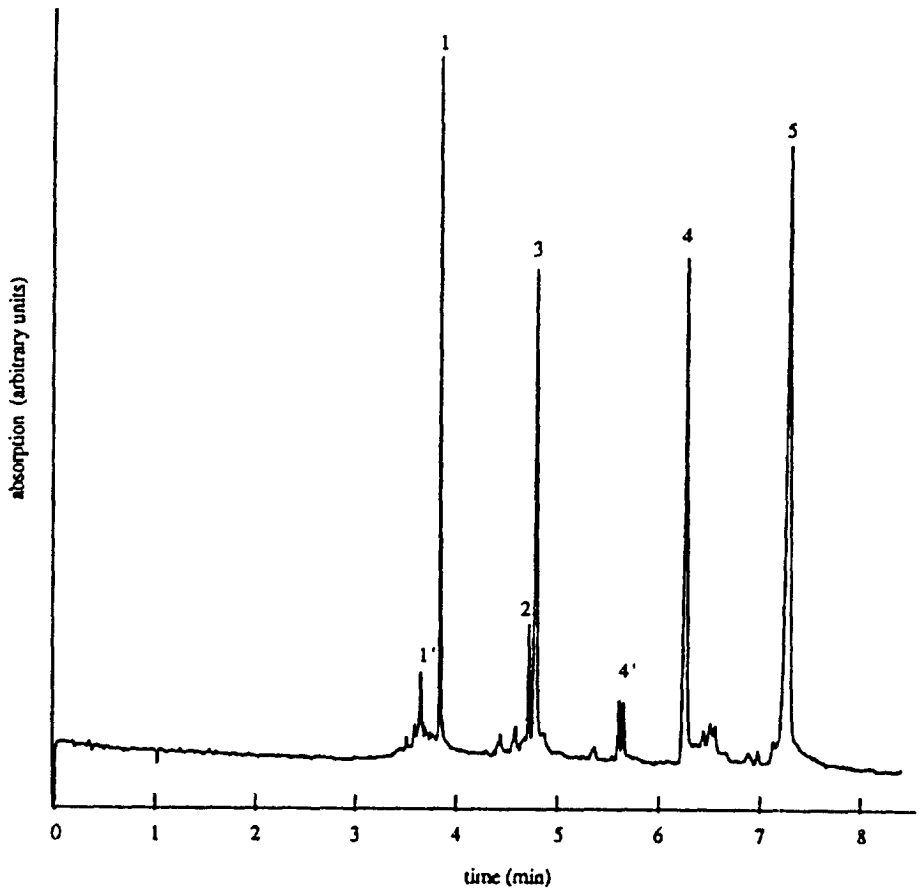

Fig. 10. Separation of basic proteins in a untreated fused-silica capillary using a running electrolyte solution containing polydimethyldiallylammonium chloride (PDADMAC). Capillary, fused-silica 68-70 cm total length (29–31 cm to the detector)×50 μm I.D.; applied voltage, 25 kV; temperature, 28°C; detection wavelength, 220 nm at the anodic end; electrolyte solution, 30 m*M* hydroxylamine–HCl (pH 7.0) containing 5 mg/ml PDADMAC. Proteins, 1=myoglobin, 2=ribonuclease A, 3=α-chymotrypsinogen A, 4=cytochrome *c*, 5=lysozyme. Adapted from Ref. [129] with permission.

of the coating agent in the running electrolyte solution.

3.4. Surfactants

Surfactants are amphiphatic molecules containing both hydrophilic and hydrophobic regions. At low concentration in the electrolyte solution the surfactant molecules are in a molecular-disperse stage, where they may be associated as dimers, trimers or oligomers, depending on. the chemical structure of the surfactant and on pH, ionic strength, composition and temperature of the electrolyte solution. At a well defined concentration, recognized as the critical micelle concentration (CMC), the surfactant molecules aggregate to form clusters, known as micelles, which are in dynamic equilibrium with the surfactant monomers.

Surfactants may bind to proteins either to the same extent, resulting in protein–surfactant complexes of approximately constant charge-to-mass ratio (e.g. protein–SDS complexes), or to a different degree which can enlarge differences in the electrophoretic mobilities between proteins. In addition, the surfactant molecules may adsorb at the interface between the inner surface of the capillaries (either coated or

uncoated) and the electrolyte solution, with the result of altering the electroosmotic flow and masking the protein adsorption sites on the capillary wall.

Surfactants have been investigated extensively in capillary electrophoresis for the separation of both charged and neutral molecules using a technique based on the partitioning of the analytes between the hydrophobic micelles formed by the surfactant and the electrolyte solution, which is termed micellar electrokinetic capillary chromatography (MECC or MEKC) [133]. This technique has proven to be useful for the analysis of a variety of low-molecular mass species, whereas it has exhibited limitations in separating proteins and other biopolymers, as well as small highly hydrophobic molecules. The poor selectivity of MECC for the separation of proteins may be attributed to several factors, including the strong interactions between the hydrophobic moieties on the protein molecules and the micelles, the inability of large proteins to penetrate into the micelles, and the binding of the monomenic surfactant to the proteins. The result is that even though the surfactant concentration in the electrolyte solution exceeds the CMC, the protein–surfactant complexes are not likely to be subjected to partition in the micelles as are smaller molecules. As well as maintaining hydrophobic proteins in the molecular dispersion in aqueous solutions, surfactants have been incorporated into the electrolyte solutions employed in protein CZE as additives for minimizing protein–capillary wall interactions, for improving selectivity and resolution, and for controlling the electroosmotic flow.

3.4.1. Ionic and zwitterionic surfactants

Cationic surfactants adsorb at the interface between the inner surface of bare fused-silica capillaries and the electrolyte solutions, determining drastic variations in the zeta potential which becomes positive when the charge density of the adsorbed surfactant exceeds that resulting from the ionization of the superficial silanol groups. The result of charge reversal is that proteins at pH values below their isoelectric points are repelled from the capillary wall. In addition, the direction of the electroosmotic flow is reversed from cathodic to anodic (see Section 2.4) with the result that the electrophoretic migration of the positively charged proteins occurs in the opposite direction of the bulk liquid. Therefore, if the magnitude of the electroosmotic mobility is greater than that of the electrophoretic mobility of the protein sample, the polarity of the applied voltage must be reversed from positive to negative in order to displace the proteins to the detection window.

This approach was first reported by Emmer et al. [134] who demonstrated that the addition of micromol amounts (50–100 μg/ml) of a fluorinated cationic surfactant (marked by 3M as Fluorad FC 134) to the electrolyte solution reverses the direction of the electroosmotic flow and allows the efficient separation of model basic proteins at neutral pH (see Fig. 11) [134] and closely related forms of recombinant human insulin-like growth factor I [52] in bare fused-silica capillaries. Similar results have been reported by Muijselaar et al. [135] who also investigated the effect of pH on the anodic electroosmotic flow at two different concentrations of the fluorinated cationic surfactant in the electrolyte solution.

A fluorinated zwitterionic surfactant has also been investigated as an additive, either added to the electrolyte solution alone or in combination with the cationic fluorosurfactant FC 134, for the separation by CZE of model proteins [136], isoenzymes and subunits of pig liver esterase [137]. The isoelectric point of this amphoteric additive is about 8. Therefore, at pH below this value the zwitterionic fluoro-

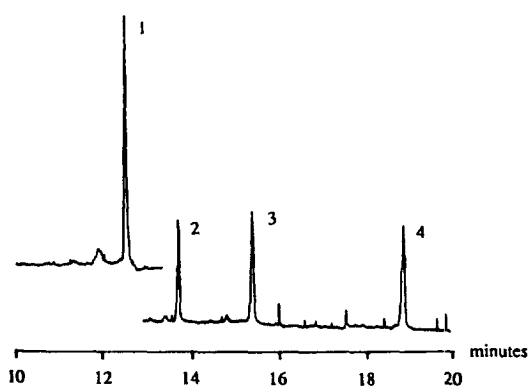

Fig. 11. Electropherogram of four basic proteins (myoglobin run separately) obtained with the cationic fluorosurfactant buffer additive FC134. Capillary, fused-silica 100 cm total length (90 cm to the detector)×50 μm; applied voltage, 30 kV; detection wavelength, 230 nm at the anodic end; electrolyte solution, 10 m*M* phosphate buffer (pH 7.0) containing 50 μg/ml, FC134. Proteins, 1=myoglobin, 2=ribonuclease A, 3=cytochrome *c*, 4=lysozyme. Adapted from Ref. [134].

surfactant is positively charged and behaves similarly to the cationic fluorosurfactant FC 134, reversing the direction of the electroosmotic flow from cathodic to anodic and masking the active protein adsorption sites on the capillary wall. The significant differences in the protein selectivity observed with the two additives incorporated in the electrolyte solution either alone or in various combinations, have been attributed to the different interactions between the distinct fluorosurfactants and the proteins, which influence to diverse extents the charge-to-mass ratio of the analytes and, hence, their electrophoretic mobilities.

A further improvement in this strategy is represented by the use of mixtures of cationic and anionic fluorosurfactants added in micromolar amounts to the electrolyte solution which allows the simultaneous separation of basic and acidic proteins at neutral pH, as displayed in Fig. 12 [138]. The composition of the surfactant mixture has proven to affect both the electrophoretic mobility of proteins and the magnitude and direction of the electroosmotic flow which turns out to be anodic when the cationic surfactant fraction in the mixture is preeminent and anodic in the opposite case. Accordingly, the electroosmotic flow proves to be suppressed when equal proportion of the two fluorosurfactants are incorporated into the electrolyte solution.

The binding of ionic or zwitterionic surfactants to protein molecules alter both the hydrodynamic (Stokes) radius and the effective charge of proteins. This causes a variation in the electrophoretic mobility which is directly proportional to the effective charge and inversely proportional to the Stokes radius [96]. The binding of the surfactant molecules to proteins may vary with the surfactant species and its concentration and is influenced by the experimental conditions, such as pH, ionic strength and temperature of the electrolyte solution. For example, SDS binds to proteins to the same degree, resulting in complexes of approximately constant charge-to-mass ratio, whereas cetyl trimethylammonium bromide (CTAB) binds to each protein to a different extent which may enlarge the differences in the charge-to-mass ratio between these analytes, with the consequence of improving selectivity. It follows that ionic and zwitterionic surfactants may act as hydrophobic selectors which can be added to the electrolyte solution to modulate the electrophoretic selectivity in the CZE system.

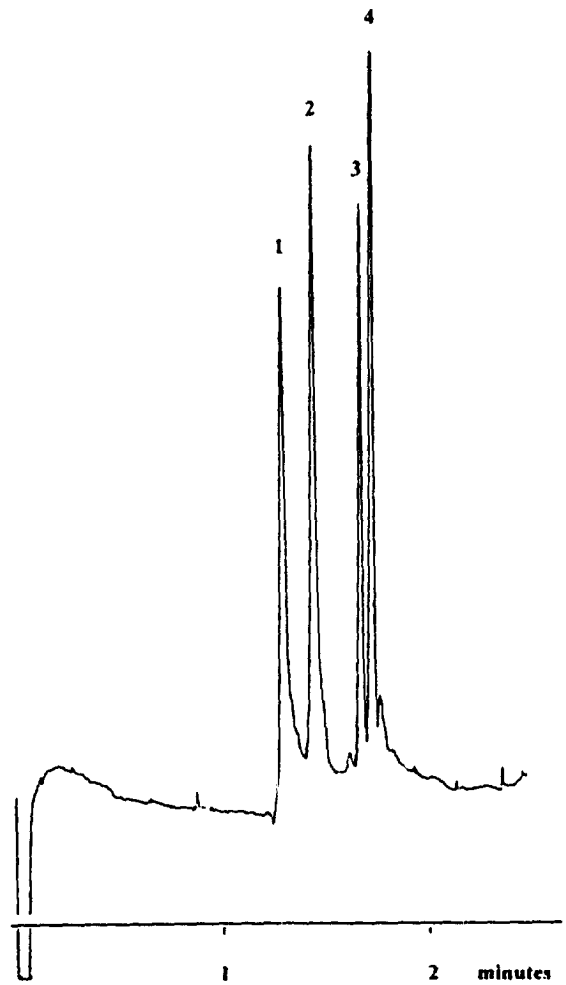

Fig. 12. Rapid separation of acidic and basic proteins. Capillary, fused-silica 50 cm total length (39 cm to the detector)×50 μm I.D.; applied voltage, 30 kV; detection wavelength, 210 nm at the cathodic end; electrolyte solution, 10 m*M* phosphate buffer (pH 7.0) containing 20% (w/v) cationic (FC134) and 80% (w/v) anionic (FC128) fluorosurfactants. Proteins, 1 = myoglobin, 2 = transferrin, 3 = β-lactoglobulin I, 4 = β-lactoglobulin II. Total additive concentration, 54 μg/ml. Adapted from Ref. [138].

The influence of various surfactants on the electrophoretic mobilities of apolipoproteins, whose distribution in human plasma seems to be a better marker for coronary heart diseases than cholesterol level, has been reported by Tadey and Purdy [139]. These authors demonstrated that the addition to the

electrolyte solution of either anionic surfactants sodium deoxycholate (DOC) or sodium dodecyl sulfate allows complete separation of the apolipoproteins from plasma low-density lipoproteins (LDLs) and high-density lipoproteins (HDLs) which, however, exhibit different selectivity and resolutions with the two distinct surfactants as a result of their different affinity for the apolipoproteins. Similarly, the differences in selectivity and resolution observed for the separation of over seven different components of the apolipoproteins from plasma very-low-density lipoproteins (VLDLs), obtained by using either SDS or CTAB in the electrolyte solution, have also been interpreted as the effect of the different binding of this distinct surfactants to the apolipoproteins.

In another example of this strategy [140], the recombinant insulin-like growth factor I variants, differing mainly in the hydrophobic character, have been resolved in a coated capillary by using mixed aqueous–organic electrolyte solutions containing a suitable amount (5.0 m*M*) of the zwitterionic surfactant N-dodecyl-N,N-dimethyl-3-amino-1-propanesulfonate (DAPS). The role of the zwitterionic surfactant is believed to be that of probing the exposed surface of the protein variants for hydrophobic differences, whereas the organic solvents plays the role of modulating the hydrophobic interactions between the different protein variants and the surfactant.

In analytical methods employing UV detection at low wavelengths, the employment of cetyl trimethylammonium chloride (CTAC) may be advantageous in comparison to the use of CTAB as a result of the less background absorbance at the lower wavelengths generated by the chloride ions. An example on the use of CTAC for the separation of basic and acidic proteins in both bare fused-silica and octadecylsilane modified capillary with UV detection at 214 nm has been reported by Strege and Lagu [141].

3.4.2. Non-ionic surfactants

Towns and Regnier [142] have demonstrated that non-ionic surfactants can be hydrophobically adsorbed onto an alkylsilane derivatized capillary inner surface to generate a hydrophilic layer that prevents protein–capillary wall interactions and controls the electroosmotic flow. The incorporation of a non-ionic surfactant into the electrolyte solution may be also needed to maintain hydrophobic proteins in molecular dispersion in aqueous solution or/and to modulate the selectivity of the CZE system.

Different oxyethylene based surfactants of the Brij and Tween series have been employed to develop non-ionic surfactant coated capillaries [142–145]. These surfactants are particularly suitable for use as electrolyte additives for protein separation due to their commercial availability, high purity, moderate cost, low toxicity, high cloud temperature and low UV wavelength transparency. The non-ionic surfactant coated capillaries are prepared by derivatization of the inner silica surface with an alkylsilane followed by the adsorption of a layer of the surfactant from an aqueous solution above the critical micelle concentration. It is proposed that the hydrophobic chain of the surfactant molecule strongly interacts with the alkylsilane derivatized surface whereas the hydrophilic head group of the surfactant creates a hydrophilic layer that masks the underlying alkylsilane and the residual free silanol groups. The incorporation of the non-ionic surfactant into the electrolyte solution below the CMC is aimed at preventing the gradual loss of surfactant from the alkylsilane surface and may play a role in tuning the selectivity by interacting with the protein molecules.

CZE systems employing non-ionic surfactants and alkylsilane coated capillaries exhibit cathodic electroosmotic flow which is lower compared to that in bare fused-silica capillaries and increases only slightly throughout the pH range 4–11 [142]. This allows adjustment of the pH of the electrolyte solution to optimize selectivity without significantly affecting the rate of the electroosmotic flow. Polyoxyethylene ether (Brij 35) coated alkylsilane derivatized capillaries have been used with electrolyte solutions containing low levels of this non-ionic surfactant for the separation of both model proteins and complex protein samples. These include the seeds albumins from the *Leguminosae vicia* species [145] and the recombinant proteins granulocyte macrophage colony stimulating factor [144] and human tumor necrosis factor [146].

Electrolyte solutions containing non-ionic surfactants have also been employed for the separation of proteins by CZE in both bare fused-silica and polymer coated capillaries. For example, Paterson et al. [121] have shown that the addition of the non-ionic surfactant Tween 20 to the electrolyte solution employed to analyze the β-lactoglobulin variants A,

B and C in bovine whey enhance the resolution of these proteins in bare fused-silica capillaries. Another non-ionic surfactant of the polyoxyethylene sorbitan monoalkylate series, the Tween 80, (which differs from Tween 20 for the longer alkyl chain) has been reported to be essential to obtain full protein recovery from polyvinyl alcohol coated capillaries and highly repeatable migration times in the separation of the different glycoforms of the recombinant protein tissue plasminogen activator (rtPA) [147]. In a previous paper from Yim [148], the rtPA glycoforms were partially separated in polymer coated capillaries by using another non-ionic surfactant, the Triton X-100 which was chemically reduced to minimize UV absorbance.

In another example [149], the electrophoretic behavior of the three closely related intrinsic thylacoid membrane proteins of the photosystem II light harvesting complex (LHC II) in higher plants were reported to depend on the type and concentration of the surfactant incorporated into the electrolyte solution to maintain these proteins in molecular dispersion in the aqueous medium. The complete and repeatable separation of the LHC II proteins has been accomplished in a bare fused-silica capillary with the electrolyte solution containing *n*-octyl-β-D-glucopyranoside at concentration of 7.0 m*M* (see Fig. 13). It is shown that the electrophoretic behavior of these proteins are significantly affected by the surfactant concentration in the electrolyte solution. Similar observations have been reported by Towns and Regnier for model proteins [142]. Distinct electrophoretic behavior can be observed below and above the CMC of the surfactant incorporated in the electrolyte solution. Below the CMC, the variations in the migration behavior obtained by adding different amounts of the surfactant into the electrolyte solution may be related to the distinct degrees of complexation of the proteins by the surfactant at the different concentrations, which are expected to affect to different extents the charge-to-mass ratio of the proteins and, thus, their electrophoretic mobilities. Above the CMC, the micelles in the electrolyte solution may interact with either the protein–surfactant complexes or with the free proteins or with both, causing the reduction in peak efficiency observed with both model proteins [142] and intrinsic membrane proteins [149].

3.5. Other ion-pairing agents

Several additives among those reviewed in the previous sections have been reported to give rise to ion-pair formation with proteins which may result in enhanced efficiency, altered selectivity and improved resolution. Most of these additives are amino-modifiers (similar to those employed in HPLC), zwitterions, and surfactants (either ionic or zwitterionic) incorporated into the electrolyte solutions below their CMC. Other conventional HPLC ion-pair agents,

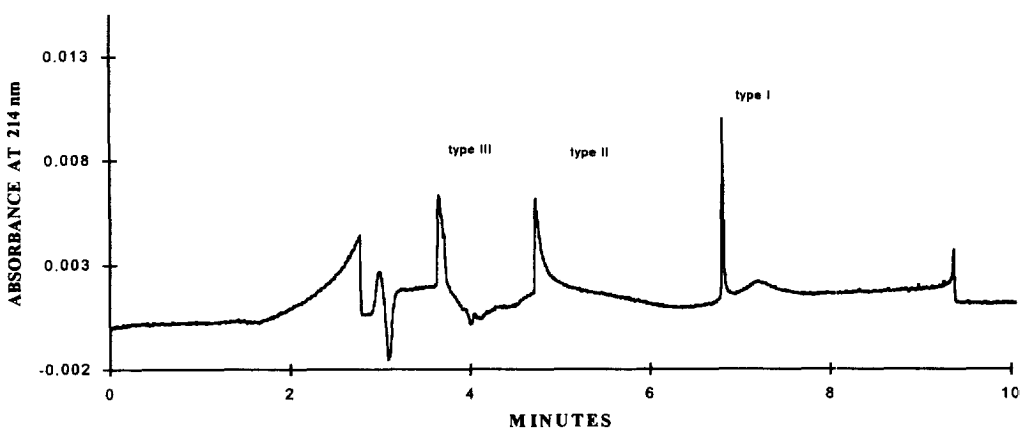

Fig. 13. Separation of LHC II intrinsic thylacoid membrane proteins by CZE with running electrolyte solution containing *n*-octyl-β-D glucopyranoside. Capillary, bare fused-silica 37 cm total length (30 cm to the detector)×50 μm I.D.; electrolyte solution 25 m*M* Tris, 192 m*M* glycine (pH 8.8) containing 7 m*M* *n*-octyl-β-D glucopyranoside; applied voltage, 15 kV; detection wavelength, 280 nm at the cathodic end; temperature, 28°C. Adapted from Ref. [149] with permission.

such as the short-chain alkyl sulfonates have found more application in peptide [150] rather than in protein CZE [151]. The preferential use of cationic ion-pairing additives can be explained in terms of their capability at competing with proteins for the negatively charged silanol groups on the capillary inner surface which reduces protein–capillary wall interactions. On the other hand, several anions, such as phosphate and borate, which can be present in the buffer solutions employed as the background electrolyte may also act as ion-pairing agents influencing the electrophoretic mobility of proteins and, hence, selectivity and resolution.

Recently, the use of the sodium salt of myoinositol hexakis-(dihydrogen phosphate), communally known as phytic acid, has been reported to improve separation efficiency and resolution of proteins [152,153] and peptides [152,154,155]. Phytic acid is a naturally occurring compound containing six phosphate groups with pK_a values ranging from 1.9–9.5. Besides having a polyanionic character over a wide pH range, phytic acid is not toxic and its sodium salt is highly soluble in aqueous media and is UV transparent down to 200 nm.

The enhanced efficiency and resolution obtained by the incorporation of the sodium salt of phytic acid into the electrolyte solution has been attributed to ion-pair formation between the strongly negative polyanionic phytic acid and the positive charged amino acid residues, particularly lysine and arginine [152,153]. The resulting effect of these ion-pair formation is the reduction of the net positive charge of basic proteins with the consequent reduction of their Coulombic interaction with the negatively charged capillary wall. Because the ion-pairing effect depends on the number of the basic amino acid residues exposed to the electrolyte solution, basic proteins of similar p*I* and different content of lysine and arginine residues can be selectively separated on the basis of the different extent of their complexation by phytic acid.

Selectivity enhancement for protein CZE can also be obtained by using the more traditional ion-pairing agents alkylsulfonates and tetraalkylammonium salts. A recent example of this strategy has been reported by Weldon et al. [151] who have described the effects of the anionic ion-pairing agents butanesulfonic acid, heptanesulfonic acid and decanesulfonic acid on the resolution of ferri- and ferrohemoglobin and on the separation of three model proteins at acidic pH values.

3.6. Denaturing agents

The tendency of hydrophobic proteins, such as certain intrinsic membrane proteins, to undergo aggregation or self-aggregation may result either in solubility problems or insufficient selectivity and resolution. These problems can be avoided or at least controlled by incorporating surfactants or/and other solubilizing or denaturing reagents into the electrolyte solution.

The use of surfactants, either ionic or non-ionic has been described in Sections 3.4.1 and 3.4.2, respectively. Other denaturing agents include urea at elevated concentrations (higher than 1.0 *M*) and formic and trifluoroacetic acid. Urea is widely employed as an additive in protein separation by slab gel electrophoresis and HPLC. The effectiveness of urea in these separation systems has mainly been attributed to its capability at promoting deaggregation and disruption of hydrophobic and non-covalent interactions.

The advantage of incorporating urea into the electrolyte solution employed for the separation of calcium-binding proteins from liver plasma membranes and for a mixture of intrinsic membrane glycoproteins has been demonstrated by Josic et al. [156]. Urea has also been successfully employed to resolve caseins in milk [68,157]. In this case the action of urea is that of preventing the formation of casein micelles during electrophoresis. The majority of caseins are present in milk in the form of micelles which are disrupted during sample preparation by the combined action of the reducing reagent DL-dithiothreitol (DDT) and urea. Their reformation during electrophoresis is prevented by adding 6 *M* urea to the electrolyte solution.

The capability of urea at breaking hydrogen bonds may be helpful at improving the resolution of the different glycoforms arising from N- and O-linked glycosilation of glycoproteins. An example of the successful employment of urea for this purpose has been reported by Watson and Yao [105] who have described the separation of recombinant human erythropoietin into its individual glycoforms using 7 *M* urea in combination with 1,4-diaminobutane as the electrolyte additive.

Appendix A

Additive	BGE	Capillary	Proteins	Reference
Non-ionic polymers				
Methyl cellulose (0.05% w/v)	0.1 *M* acetate (pH 4.8)	Uncoated, 50 cm×50 μm I.D.	Albumin	[158]
Methyl cellulose (0.5%, w/v)	0.1 *M* borax, 0.5 m*M* SDS (pH 10)	Uncoated, 27 cm×75 μm I.D.	IgG, alkaline phosphatase (calf intestine)	[159]
Methyl cellulose (0.04%, w/v)	40 m*M* borate (pH 10)	Coated, CElect P-150 (Supelco), 55 cm×50 μm I.D.	Cerebrospinal fluid proteins	[69]
Methylhydroxyethyl cellulose (0.05%, w/v)	10 m*M* phosphate, 6 *M* urea (pH 2.5) or 10 m*M* citrate, 6 *M* urea (pH 3.0)	Coated, hydrophilic (SGE) and CElect P-I (Supelco), 57 cm×50 μm I.D.	Milk proteins (bovine)	[68]
Hydroxypropylmethyl cellulose (0.03%, w/v)	100 m*M* phosphate (pH 2.0)	Uncoated, 58.8 cm×75 μm I.D.	Histones, acetylated histones	[66]
Hydroxypropylmethyl cellulose (0.03%, w/v)	0.1 *M* phosphate (pH 2.0)	Uncoated, 58.8 cm×75 μm I.D.	Phosphorylated histone H1 variants	[67]
Hydroxypropylmethyl cellulose (0.02%, w/v)	0.1–0.5 *M* phosphate (pH 2.0); 0.1–0.5 *M* methanephosphonate–TEA (pH 2.0); 50 m*M* phosphate–90 m*M* perchlorate–TEA (pH 2.0)	Uncoated, 50–60 cm×75 μm I.D.	Histone H1 subtypes and their phosphorylated modifications	[97]
Hydroxypropylmethyl cellulose (0.05%, w/w)	50 m*M* phosphate (pH 3.0)	Uncoated, 70 cm×75 μm I.D.	Standard basic proteins	[71]
Hydroxyethyl cellulose (0.05%, w/w)	50 m*M* phosphate (pH 3.0)	Uncoated, 70 cm×75 μm I.D.	Standard basic proteins	[71]
Dextran (M_r 2 000 000) (10%, w/v)	0.06 *M* 2-amino-2-methyl-1-3-propanediol–cacodylic acid, 0.1% SDS (pH 8.8)	Coated 18 cm×75 μm I.D.	Standard proteins	[86]
Dextran (M_r 2 000 000) (10%, w/v)	0.1 *M* Tris–CHES 0.1% SDS (pH 8.6)	Coated 27 cm×100 μm I.D.	Standard proteins	[160]
Dextran (M_r 2 000 000) (0.05%, w/w)	50 m*M* phosphate (pH 3.0)	Uncoated, 70 cm×75 μm I.D.	Standard basic proteins	[71]
Dextran (M_r 70 000; 500 000 2 000 000) (10%, w/v)	0.1 *M* Tris/CHES 0.1% SDS (pH 8.8)	Coated, 38 cm×50 μm I.D. 38 cm×100 μm I.D.	Standard proteins	[88]
Dextran (M_r 2 000 000) (10%, w/v)	60 m*M* 2-amino-2-methyl-1,2-propanediol–cacodylic acid (pH 8.8)	Coated, 34 cm×50 μm I.D.	Standard proteins	[95]
Dextran (M_r 2 000 000) (0–12%)	0.4 *M* TRIS/borate, 0.1% SDS, 10% glycerol (pH 8.3)	Uncoated, 24 cm×50 μm I.D.	Myoglobin molecular mass markers, standard proteins	[161]

Continued

Additive	BGE	Capillary	Proteins	Reference
Steaoryl dextran (1%)	10 m*M* Tris–acetate (pH 8.6)	Coated, 12.5 cm×50 μm I.D.	Human serum albumin, transferrin	[15]
Pullulan (M_r 50 000–100 000) (1–10% w/v)	0.1 *M* Tris–CHES, 0.1% SDS (pH 8.8)	Coated, 50 cm×75 μm I.D.	Standard proteins	[92]
Pullulan (M_r 50 000–100 000) (5% w/v)	0.1 *M* Tris/CHES 0.1% SDS (pH 8.7)	Coated, 50 cm×75 μm I.D.	Standard proteins	[93]
Pullulan (M_r 50 000–100 000) (3–10% w/v)	0.1 *M* Tris–CHES 0.1% SDS (pH 8.7)	Coated, 5.0 cm×75 μm I.D.	Standard proteins	[94]
Poly(vinyl alcohol) (0.05%, w/w PVA 15 000)	20 m*M* phosphate, 30 m*M* NaCl (pH 3.0)	Uncoated, 70 cm×75 μm I.D.	Standard basic proteins	[70]
Poly(vinyl alcohol) (0.05%, w/v PVA 15 000)	10 m*M* phosphate, 6 *M* urea (pH 2.5)	Coated, hydrophilic (SGE) 57 cm×50 μm I.D.	Milk proteins (bovine)	[68]
Poly(vinyl alcohol) (0.05%, w/w PVA 50 000)	50 m*M* phosphate (pH 3.0); 70 m*M* phosphate (pH 3.0 or pH 3.5) 50 m*M* or 100 m*M* phosphate (pH 5.5)	Uncoated, 70 cm×75 μm I.D. PVA thermally Coated, 70 cm×75 μm I.D.	Standard basic proteins	[71]
Poly(vinyl alcohol) (0.05–1%, w/v, PVA 72 000)	50 m*M* MES (pH 7.0)	Uncoated, 57 cm×75 μm I.D.	Standard basic proteins	[57]
Poly(vinyl alcohol) (1–6%, w/v, PVA)	60 m*M* 2-amino-2-methyl-1,3-propanediol–cacodylic acid (pH 8.8)	Coated, 34 cm×50 μm I.D.	Standard proteins	[95]
Poly(ethylene glycol) (5%, PEG 35 000)	0.1 *M* phosphate (pH 2.5)	Coated, 20 cm×50 μm I.D.	Bovine serum albumin (monomer, dimer, trimer)	[76]
Poly(ethylene glycol) (3% w/v, PEG 100 000)	0.1 *M* Tris–CHES, 0.1% SDS (pH 8.8)	Coated, 40 cm×100 μm I.D.	Standard proteins	[86]
Poly(ethylene glycol) (5%, PEG 40 000)	25 m*M* phosphate (pH 2.0)	Uncoated, 37 cm×50 μm I.D.	Native and reduced trypsinogen	[77]
Poly(ethylene oxide) (3% w/v, PEO)	0.1 *M* Tris–CHES, 0.1% SDS (pH 8.8)	Uncoated, 47 cm×100 μm I.D.	Standard proteins	[87]
Poly(ethylene oxide) (1–5% w/v, PEO 10 000–1000 000)	0.1 *M* Tris–CHES, 0.1% SDS (pH 8.5)	Uncoated, 27 cm×100 μm I.D.	Standard proteins	[89]
Poly(ethylene oxide) (0.2% w/v, PEO 8000 000)	30 m*M* phosphate (pH 6.0)	Uncoated, 50 cm×75 μm I.D.	Standard proteins	[72]
PEO+PEG (eCAP SDS 14-200 kit; Beckman)	Replaceable polymer-buffer (Beckman)	Coated, 27 cm×100 μm I.D.	Standard proteins	[90]
PEO+PEG (eCAP SDS 14-200 kit; Beckman)	Replaceable polymer-buffer (Beckman)	Coated, 27 cm×100 μm I.D.	Standard proteins	[91]
Propylene glycol	0.25 *M* phosphate (pH 6.8)	Uncoated, 48 cm×25 μm I.D.	Recombinant human growth hormone	[162]

Continued

Additive	BGE	Capillary	Proteins	Reference
Ionic and zwitterionic salts				
KCl (10–20 m*M*)	20 m*M* CAPS (pH 11), 20 m*M* Tricine (pH 8.22)	Uncoated, 100 cm×52 μm I.D.	Standard proteins	[9]
CsCl, KCl, NaCl, LiCl, K_2SO_4 (0.1–1.0 *M*)	0.1 *M* CHES, 1 m*M* EDTA (pH 9.0)	Uncoated, 50 cm×75 μm I.D.	Standard proteins	[11]
KCl (40 m*M*)	0.01 *M* tricine (pH 8.24)	Uncoated, 100 cm×50 μm I.D.	Standard proteins	[96]
KCl (100 m*M*)	0.2 *M* phosphate (pH 7.0)	Coated, 100 cm×20 μm I.D.	Standard proteins	[5]
KCl (10–100 m*M*)	50 m*M* MES (pH 7.0)	Uncoated, 57 cm×75 μm I.D.	Standard basic proteins	[57]
NaCl (0.12 *M*)	0.1 *M* phosphate (pH 2.5)	Coated, 50 cm×50 μm I.D.	Histones (from Chinese hamster cells)	[163]
LiCl (25 m*M*)	50 m*M* borate (pH 8.3)	Uncoated, 70 cm×75 μm I.D.	Humanized anti-TAC monoclonal antibody	[98]
K_2SO_4 (0.25 *M*)	40 m*M* phosphate (pH 7.0)	Uncoated, 75 cm×25 μm I.D.	Lysozyme, α-chymotrypsinogen A	[12]
Na_2SO_4, K_2SO_4, NaCl, KCl, K_2HPO_4 (40–80 m*M*)	50 m*M* phosphate, 50 m*M* 1,3-diaminopropane	Uncoated, 75 cm×50 μm I.D.	Standard basic proteins	[111]
K_2HPO_4 (10–80 m*M*)	10–80 m*M* phosphate (pH 3.8)	Coated, 63 cm×50 μm I.D.	Standard basic proteins	[31]
K_2SO_4 (0.25 *M*)	20 m*M* phosphate (pH 5.5)	Uncoated, 50 cm×25 μm I.D.	Standard proteins	[117]
Na^+ (0.5 *M*) (as phosphate)	500 m*M* phosphate (pH 5–10)	Uncoated, 23 cm×25 μm I.D	Standard proteins	[164]
Na^+ (0.5 *M*) (as phosphate)	500 m*M* phosphate, 4 *M* urea (pH 7.0)	Uncoated, 23 cm×25 μm I.D	Milk proteins	[164]
$(NH_4)_3PO_4$ (125–500 m*M*)	125–500 m*M* phosphate (pH 7.0)	Coated 100 cm×20 μm I.D.	Standard proteins	[5]
Li^-, Na^+, K^+, Cs^+ (as methanephosphonate)	100 m*M* methanephosphonate, 0.02% hydroxypropylmethyl cellulose (pH 2.0)	Uncoated, 50 cm×75 μm I.D.	Histone H1 subtypes	[97]
Trimethylglycine (betaine) (2.0 *M*)	40 m*M* phosphate, 0.1 *M* K_2SO_4 (pH 7.5)	Uncoated, 75 cm×25 μm I.D.	Lysozyme, α-chymotrypsinogen A	[12]
Methylglycine (sarcosine) (2.0 *M*)	40 m*M* phosphate (pH 6.7)	Uncoated, 75 cm×25 μm I.D.	Lysozyme, α-chymotrypsinogen A	[12]
Trimethylammonium propyl sulfonate (0.5 *M*) (Accupure Z-1 methyl)	50 m*M* borate (pH 8.3)	Uncoated, 70 cm×75 μm I.D.	Humanized anti-TAC Monoclonal antibody	[98]
Trimethylammonium propyl sulfonate (0.5 *M*) (Accupure Z-1 methyl)	0.1 *M* phosphate (pH 9.0)	Uncoated, 57 cm×50 μm I.D.	Ricin	[99]
Trimethylammonium propyl sulfonate (2.0 *M*)	0.1 *M* phosphate (pH 6.9)	Uncoated, 70 cm×75 μm I.D.	β-Lactoglobulin A, β-Lactoglobulin B	[165]
Trimethylammonium butyl sulfonate (2.0 *M*)	0.1 *M* phosphate (pH 6.9)	Uncoated, 70 cm×75 μm I.D.	Cytochrome *c*	[165]

Continued

Additive	BGE	Capillary	Proteins	Reference
Z4A, Z6C, Z60 (multicomponent mixtures of zwitterionic species)	0.1 *M* acetate (pH 4.8)	Coated, 50 cm×50 μm I.D.	Hemoglobin	[158]
ZB-4 (Beckman's proprietary zwitterion)	100 m*M* phosphate (pH 5.6)	Uncoated, 23 cm×25 μm I.D	Standard proteins	[164]
Mono- and divalent amines				
Triethylamine (100 m*M*)	50 m*M* phosphate, 40 m*M* Na_2SO_4 (pH 7.0)	Uncoated, 75 cm×50 μm I.D.	Standard basic proteins	[111]
Triethylamine (250 m*M*)	20 m*M* phosphate (pH 5.5)	Uncoated, 27 cm×25 μm I.D. Coated, 50 cm×25 μm I.D.	Standard basic proteins	[117]
Triethylamine (10 m*M*)	20 m*M* phosphate (pH 11)	Uncoated, 72–74 cm×50 or 100 μm I.D.	Lysozyme, myoglobin, trypsinogen	[24]
Triethylamine (10–80 m*M*)	50 m*M* phosphate (pH 2.5)	Uncoated, 37 cm×75 μm I.D.	Standard basic proteins	[54]
Triethylamine	100 m*M* methanephosphonate–TEA, 0.02% hydroxypropylmethyl cellulose (pH 2.0)	Uncoated, 50–60 cm×75 μm I.D.	Histone H1 subtypes and their phosphorylated modifications	[97]
Triethanolamine (10–80 m*M*)	50 m*M* phosphate (pH 2.5)	Uncoated, 37 cm×75 μm I.D.	Standard basic proteins	[54]
N,N-Diethylethanolamine (10–120 m*M*)	50 m*M* phosphate (pH 2.5)	Uncoated, 37 cm×75 μm I.D.	Standard basic proteins	[55]
N-Ethyldiethanolamine (10–120 m*M*)	50 m*M* phosphate (pH 2.5)	Uncoated, 37 cm×75 μm I.D.	Standard basic proteins	[55]
Ethanolamine (0.1% v/v)	50 m*M* borate, 0.1% Tween 20 (pH 7.0)	Uncoated, 75 cm×50 μm I.D.	(β-Lactoglobulin A, B, C variants of bovine whey	[121]
Hydroxylamine (0.1–2.1 m*M*)	25 m*M* borate (pH 9.0)	Uncoated, 69 cm×75 μm I.D.	Ovalbumin glycoforms	[109]
Ethylamine (34.5–200 m*M*)	25 m*M* borate (pH 9.0)	Uncoated, 69 cm×75 μm I.D.	Ovalbumin glycoforms	[109]
n-Propylamine (100 m*M*)	50 m*M* phosphate, 40 m*M* Na_2SO_4 (pH 7.0)	Uncoated, 75 cm×50 μm I.D.	Standard basic proteins	[111]
Morpholine (5.8 m*M*)	10 m*M* tricine, 20 m*M* NaCl (pH 8.0)	Uncoated, 95.5 cm×50 μm I.D.	Recombinant human insulin and its derivatives	[119]
Morpholine (5.8 m*M*)	10 m*M* tricine, 20 m*M* NaCl (pH 8.0)	Uncoated, 105 cm×50 μm I.D.	Recombinant human growth hormone and its derivatives	[119]
Morpholine (5.8 m*M*)	10 m*M* tricine, 20 m*M* NaCl (pH 8.0)	Uncoated, 105 cm×50 μm I.D.	Recombinant human growth hormone and its derivatives, recombinant human insulin and its derivatives	[118]
Morpholine (45 m*M*)	10 m*M* tricine, 20 m*M* NaCl (pH 8.0)	Uncoated, 50 μm I.D.	Recombinant human growth hormone, human insulin, human proinsulin, human insulin-like factor, bovine somatostatin	[120]

Continued

Additive	BGE	Capillary	Proteins	Reference
Morpholine (0.1–0.5 *M*)	20 m*M* phosphate (pH 5.5)	Uncoated, 27 cm×25 μm I.D. Coated, 50 cm×25 μm I.D.	Standard basic proteins	[117]
Morpholine	100 m*M* methanephosphonate–morpholine, 0.02% hydroxypropylmethyl cellulose (pH 2.0)	Uncoated, 50–60 cm×75 μm I.D.	Histone H1 subtypes and their phosphorylated modifications	[97]
Morpholine (0.1–0.5 *M*)	20 m*M* phosphate (pH 5.5)	Coated, 30 cm×25 μm I.D.	Standard basic proteins	[124]
Morpholine (0.1–0.2 *M*)	50 m*M* MES (pH 7.0)	Uncoated, 57 cm×75 μm I.D.	Standard basic proteins	[57]
Tetramethylammonium chloride (25 m*M*)	50 m*M* borate (pH 8.3)	Uncoated, 70 cm×75 μm I.D.	Humanized anti-TAC monoclonal antibody	[98]
1,4-Diaminobutane (putrescine) (5.0 m*M*)	10 m*M* tricine, 20 m*M* NaCl (pH 8.2)	Uncoated, 100 cm×52 μm I.D.	Standard proteins	[9]
1,4-Diaminobutane (1.0 m*M*)	100 m*M* borate (pH 9.0)	Uncoated, 87 cm×50 μm I.D.	Ovalbumin glycoforms	[103]
1,4-Diaminobutane (2.5 m*M*)	10 m*M* tricine, 10 m*M* NaCl, 7.0 *M* urea (pH 6.2)	Uncoated, 50 cm×75 μm I.D.	Recombinant erythropoietin (r-HuEPO) glycoforms	[105]
1,4-Diaminobutane (2.5 m*M*)	50 m*M* phosphate 50 m*M* borate (pH 8.0)	Uncoated, 50 cm×75 μm I.D.	Glycoforms of recombinant human granulocyte-colony-stimulating factor	[106]
1,4-Diaminobutane (30 m*M*)	phosphate (pH 8.5)	Uncoated, 70 cm×50 μm I.D.	Standard basic proteins	[115]
1,4-Diaminobutane (30 m*M*)	20 m*M* K_2SO_4 (pH 7.0)	Uncoated, 57 cm×50 μm I.D.	Standard basic proteins ricin	[99]
1,4-Diaminobutane (5–25 m*M*)	100 m*M* phosphate (pH 8.0)	Uncoated, 88 or 45cm×75 μm I.D.	Recombinant human factor VIIa	[104]
1,4-Diaminobutane (1.83–5.48 m*M*)	25 m*M* borate (pH 9.0)	Uncoated, 69 cm×75 μm I.D.	Ovalbumin glycoforms	[109]
1,4-Diaminobutane (1.0 m*M*)	100 m*M* borate (pH 8.3)	Uncoated, 87 cm×50 μm I.D.	Transferrin glycoforms	[107]
1,5-Diaminopentane (cadaverine) (5.0 m*M*)	2.5 m*M* borate (pH 9.8)	Uncoated, 60 cm×100 μm I.D.	Standard proteins, crude muscle protein extract, elastins	[108]
1,5-Diaminopentane (25 m*M*)	100 m*M* phosphate (pH 8.0)	Uncoated, 88 or 45 cm×75 μm I.D.	Recombinant human factor VIIa	[104]
1,5-Diaminopentane (5.48 m*M*)	25 m*M* borate (pH 9.0)	Uncoated, 69 cm×75 μm I.D.	Ovalbumin glycoforms	[109]
1,3-Diaminopropane (10–50 m*M*)	Phosphate 35 m*M*, Na_2SO_4 (pH 8.0) or phosphate, 30 m*M* Na_2SO_4 (pH 9.0)	Uncoated, 75 cm×50 μm I.D.	Standard proteins	[111]
1,3-Diaminopropane (50 m*M*)	Phosphate, 40 m*M* Na_2SO_4 (pH 7.0)	Uncoated, 57 cm×50 μm I.D.	Recombinant human interleukin-4	[112]

Continued

Additive	BGE	Capillary	Proteins	Reference
1,3-Diaminopropane (30 m*M*)	phosphate (pH 8.5) 70 cm×50 μm I.D.	Uncoated,	Standard basic proteins	[115]
1,3-Diaminopropane (5.0 m*M*)	25 m*M* borate (pH 8.0) 100 cm×50 μm I.D.	Uncoated,	Human chorionic gonadotropin glycoforms	[114]
1,3-Diaminopropane	100 m*M* methanephosphonate/1, 3-diaminopropane, 0.02% hydroxypropyl-methyl cellulose (pH 2.0)	Uncoated, 50–60 cm×75 μm I.D.	Histone H1 subtypes and their phosphorylated modifications	[97]
Ethylenediamine (20–80 m*M*)	Phosphate, 35 m*M* Na_2SO_4 (pH 6.5, 8.0, and 9.5)	Uncoated, 75 cm×50 μm I.D.	Standard basic proteins	[113]
Ethylenediamine (60 m*M*)	Phosphate (pH 8.5)	Uncoated, 70 cm×50 μm I.D.	Standard basic proteins	[115]
N,N,N′,N′-Tetramethyl-1,3-butanediamine (TMBD) (20–120 m*M*)	20–120 m*M* TMBD–phosphate (pH 4.0, 5.5 and 6.5)	Uncoated, 37 cm×75 μm I.D.	Standard basic proteins	[59]
Hexamethonium bromide (30 μ*M*–1.0 m*M*)	100 m*M* borate (pH 8.4)	Uncoated, 87 cm×50 μm I.D.	Ovalbumin glycoforms	[116]
Decamethonium bromide (300 μ*M*)	100 m*M* borate (pH 8.4)	Uncoated, 87 cm×50 μm I.D.	Ovalbumin glycoforms	[116]
Hexamethonium bromide (1 m*M*)	25 m*M* borate (pH 8.4)	Uncoated, 87 cm×50 μm I.D.	Human chorionic gonadotropin	[116]
Hexamethonium chloride (30 μ*M*–1.0 m*M*)	100 m*M* borate (pH 8.4)	Uncoated, 87 cm×50 μm I.D.	Ovalbumin glycoforms	[116]
Hexamethonium bromide (750 μ*M*)	100 m*M* borate (pH 8.3)	Uncoated, 87 cm×50 μm I.D.	Serum transferrin glycoforms	[107]
Decamethonium bromide (1 or 2 m*M*)	100 m*M* borate (pH 8.3)	Uncoated, 87 cm×50 μm I.D. or 67 cm×50 μm I.D.	Serum transferrin glycoforms	[107]
Polyamines				
Diethylenetriamine (DIEN) (25.0 m*M*)	25 m*M* DIEN–phosphate (pH 4.1)	Coated, 47 cm×75 μm I.D.	Staphylococcal nuclease mutants and wild type	[122]
Triethylenetetramine (TETA) (12.5 m*M*)	12.5 m*M* TETA–trifluoroacetic acid (pH 6.8–9.5)	Coated, 47 cm×75 μm I.D.	Staphylococcal nuclease mutants and wild type	[122]
Triethylenetetramine (TRIEN) (25 m*M*)	25 m*M* TRIEN–phosphate (pH 4.0)	Uncoated, 37 cm×75 μm I.D.	Standard basic proteins	[56]
N,N′-Bis(3-aminopropyl)-1,4-butanediamine (spermine) (0.14 m*M*)	25 m*M* borate (pH 9.0)	Uncoated, 69 cm×75 μm I.D.	Ovalbumin glycoforms	[109]
N-(3-Aminopropyl)-1,4-butanediamine (0.087–0.87 m*M*)	25 or 100 m*M* borate (pH 9.0)	Uncoated, 69 cm×75 μm I.D.	Ovalbumin glycoforms	[109]
1,4,7,10,-	20 m*M* phosphate	Coated,	Standard basic proteins	[124]

Continued

Additive	BGE	Capillary	Proteins	Reference
Tetraazocyclodecane (cyclen)	(pH 5.5)	30 cm×25 μm I.D.		
Chitosan (0.002–0.1% w/v)	50 m*M* acetate (pH 3.5–5.5)	Uncoated, 53 cm×50 μm I.D.	Standard basic proteins	[125]
Polyethyleneimine (PEI) (600 000–1000 000) (0.1%)	50 m*M* acetate (pH 5.5)	PEI-coated, 50 cm×75 μm I.D.	Standard basic proteins	[126]
Polyethyleneimine (PEI) (600 000–1000 000) (0.1%)	50 m*M* MES (pH 7.0)	Uncoated, 57 cm×75 μm I.D.	Standard proteins	[57]
Polydimethyldiallyl-ammonium chloride (120 000) (0.1 m*M*)	30 m*M* hydroxylamine-HCl (pH 7.0)	Uncoated, 70 cm×50 μm I.D.	Standard proteins	[129]
Ionic and zwitterionic surfactants				
Fluorosurfactant fluorad FC 134 (50–100 μg/ml)	10–50 m*M* phosphate (pH 7.0)	Uncoated, 100 cm×50 μm I.D.	Standard proteins	[134]
Fluorosurfactant Fluorad FC 134 (100 μg/ml)	5–50 m*M* phosphate (pH 6.0–7.0)	Uncoated, 100 cm×50 μm I.D.	Human insulin-like growth factor I (IGF-I)	[52]
Fluorosurfactant FC 135 (50 μg/ml)	10 m*M* phosphate (pH 4.5 and pH 7.0)	Uncoated, 99.65 or 64.54 cm×40 μm I.D.	Standard proteins	[135]
Fluorosurfactant fluorad FC 134 (100 μg/ml)	50 m*M* acetate–phosphate (pH 3.5 and pH 4.0)	Uncoated, 100 cm×50 μm I.D.	Pig liver esterase	[137]
Fluorosurfactant fluorad FC I34 (10–50 μg/ml)	10–20 m*M* phosphate–25–400 μg/ml zwitterionic fluorosurfactant (pH 3–5)	Uncoated, 60 cm×50 μm I.D.	Standard proteins	[136]
Fluorosurfactant fluorad FC 134 and FC 128 in mixture (50–100 μg/ml)	10 m*M* phosphate (pH 7.0)	Uncoated, 100 cm×50 μm I.D.	Standard basic and acidic proteins	[138]
Cetyl trimethyl ammonium bromide (3.0–3.5 m*M*)	30 m*M* borate (pH 9.0)	Uncoated, 75 cm×50 μm I.D. Coated, 80 cm×50 μm I.D.	Plasma apolipoproteins	[139]
Cetyl trimethyl ammonium chloride (0.01–1.0%, w/v)	50 m*M* acetate (pH 5.1)	Coated, 120 cm×50 μm I.D.	Standard proteins and a proprietary recombinant protein	[141]
Cetyl trimethyl ammonium bromide (10 m*M*)	50 m*M* MES (pH 7.0)	Uncoated, 57 cm×75 μm I.D.	Standard basic proteins	[57]
Sodium deoxycholate (10 m*M*)	30 m*M* borate (pH 9.0)	Uncoated, 75 cm×50 μm I.D. Coated, 80 cm×50 μm I.D.	Plasma apolipoproteins	[139]
Zwitterionic fluorosurfactant DuPont (200–400 μg/ml)	20 m*M* acetate (pH 3.5)	Uncoated, 100 cm×50 μm I.D.	Pig liver esterase	[137]
N-Dodecyl-N,N-dimethyl-3-amino-1-propanesulfonate (DAPS) (5.0 m*M*)	20 m*M* β-alanine–citric acid (pH 3.8), containing 15–50% (v/v) acetonitrile	Coated, 50 cm×50 μm I.D.	Recombinant insulin-like growth factor I variants	[140]

Continued

Additive	BGE	Capillary	Proteins	Reference
Non-ionic surfactants				
Brij 35 (0.001% w/w)	10 m*M* phosphate (pH 7.0)	Coated, 50 cm×75 μm I.D.	Standard basic proteins	[142]
Brij 35 (0.05% w/v)	50 m*M* phosphate (pH 6.8)	Coated, 37 cm×50 μm I.D.	Recombinant granulocyte macrophage colony stimulating factor	[144]
Brij 35 (0.05% w/v)	10 m*M* phosphate (pH 8.0)	Coated, 57 cm×75 μm I.D.	Seed albumins	[145]
Brij 35 (0.001% w/v)	50 m*M* acetate (pH 4.5)	Coated, 53 cm×50 μm I.D.	Recombinant human necrosis factor	[146]
Triton X-100 (0.01%)	0.2 *M* ϵ-amino-*n*-caproic acid–ammonium phosphate (pH 4.6)	Coated, 20 cm×25 μm I.D.	Recombinant human tissue plasminogen activator glycoforms	[148]
Tween 20 (0.1%, v/v)	50 m*M* MES, 0.1% ethanolamine (pH 8.0)	Uncoated, 72 cm×50 μm I.D.	β-Lactoglobulin A, B, and C variants	[121]
Tween 80 (0.01%, v/v)	0.2 *M* ϵ-amino-*n*-caproic acid–50 m*M* acetic acid (pH 5.1)	Coated, 47 cm×50 μm I.D.	Recombinant tissue plasminogen activator	[147]
n-Octyl-β-D-glucopyranoside (3–10 m*M*)	25 m*M* Tris–192 m*M* glycine (pH 8.8)	Uncoated, 37 cm×50 μm I.D.	Intrinsic thylacoid membrane proteins of the photosystem II light harvesting complex (LHC II)	[149]
Ion-pairing agents				
Sodium salt of phytic acid [myo-inositol hexakis-(dihydrogen phosphate)] (15 m*M*)	150 m*M* borate (pH 9.5)	Uncoated, 27 cm×50 μm I.D.	Standard proteins	[152]
Sodium salt of phytic acid (1–75 m*M*)	25–100 m*M* phosphate (pH 6.0 or 7.5) 500 m*M* borate (pH 8.4)	Uncoated, 37 cm×50 μm I.D.	Standard basic proteins, metallo β-lactamase, aprotinin	[153]
Butanesulfonic acid (100 m*M*)	20 or 50 m*M* phosphate (pH 2.5)	Uncoated, 75 cm×50 μm I.D.	Standard proteins, ferri- and ferrohemoglobin	[151]

References

[1] K.K. Unger, Porous Silica: Its Properties and Use as Support in Column Liquid Chromatography, Elsevier, Amsterdam, 1979.
[2] S. Hjerten, Chromatogr. Rev. 9 (1967) 122–127.
[3] J.W. Jargenson, K. DeArman Lukacs, Science 222 (1983) 266–272.
[4] S. Hjerten, J. Chromatogr. 347 (1985) 191–198.
[5] S.A. Swedberg, Anal. Biochem. 185 (1990) 51–56.
[6] W. Nashabeh, Z. El Rassi, J. Chromatogr. 559 (1991) 367–383.
[7] Z. Zhao, A. Malik, M.L. Lee, Anal. Chem. 65 (1993) 2747–2752.
[8] R.M. McCormick, Anal. Chem. 60 (1988) 2322–2338.
[9] H.H. Lauer, D. McManigill, Anal. Chem. 58 (1986) 166–170.
[10] K.J. Lee, G.S. Heo, J. Chromatogr. 559 (1991) 317–324.
[11] J.S. Green, J.W. Jorgenson, J. Chromatogr. 478 (1989) 63–70.
[12] M.M. Bushey, J.W. Jorgenson, J. Chromatogr. 480 (1989) 301–310.
[13] C.S. Lee, W.C. Blanchard, C.-T. Wu, Anal. Chem. 62 (1990) 1550–1552.
[14] C.T. Wu, T. Lopes, B. Patel, C.S. Lee, Anal. Chem. 64 (1992) 886–891.
[15] S. Hjerten, L. Valtcheva, K. Elenbring, D. Eaker, J. Liq. Chromatogr. 12 (1989) 2471–2499.
[16] S. Honda, A. Taga, K. Suzuki, S. Suzuki, K. Kakehi, J. Chromatogr. 597 (1992) 377–382.
[17] A.W. Adamson, Physical Chemistry of Surfaces, Wiley, New York, 5th ed., 1990.
[18] K.D. Lukacs, J.W. Jorgenson, J. High Resolut. Chromatogr. Chromatogr. Commun. 8 (1985) 407–411.

[19] K.D. Altria, C.F. Simpson, Chromatographia 24 (1987) 527–532.
[20] W.J.L. Lambert, D. Middleton, Anal. Chem. 62 (1990) 1585–1587.
[21] M.A. Hayes, I. Kheterpal, A.G. Ewing, Anal. Chem. 65 (1993) 27–31.
[22] T.L. Huang, Chromatgraphia 35 (1993) 395–398.
[23] P. Coufal, K. Stulik, H.A. Claessens, C.A. Cramers, J. High Resolut. Chromotogr. 17 (1994) 325–334.
[24] N. Cohen, E. Grushka, J. Chromatogr. A 678 (1994) 167–175.
[25] Y. Mechref, Z. El Rassi, Electrophoresis 16 (1995) 617–624.
[26] D.J. Shaw, Introduction to Colloid and Surface Chemistry, Butterworts, London, 3rd ed., 1980.
[27] T. Tsuda, K. Nomura, G. Nakagawa, J. Chromatogr. 248 (1982) 241–247.
[28] P.C. Hiemenz, Principles of Colloid and Surface Chemistry, Marcel Dekker, New York, 2nd ed., 1986.
[29] S. Fujiwara, S. Honda, Anal. Chem. 58 (1986) 1811–1814.
[30] J.H. Knox, I.H. Grant, Chromatographia 24 (1987) 135–143.
[31] G.J.M. Bruin, J.P. Chang, R.H. Kuhlman, K. Zegers, J.C. Kraak, H. Poppe, J. Chromatogr. 471 (1989) 429–436.
[32] B.B. VanOrman, G.G. Liversidge, G.L. McIntire, T.M. Olefirowicz, A.G. Ewing, J. Microcolumn Sep. 2 (1990) 176–180.
[33] H.J. Issaq, I.Z. Atamna, G.M. Janini, Chromatographia 32 (1991) 155–161.
[34] K. Salomon, D.S. Burgi, J.C. Helmer, J. Chromatogr. 559 (1991) 69–80.
[35] K. Salomon, D.S. Burgi, J.C. Helmer, J. Chromatogr. 549 (1991) 375–385.
[36] I.Z. Atamna, H.J. Issaq, G.M. Muschik, G.M. Janini, J. Chromatogr. 588 (1991) 315–320.
[37] R.H.P. Reid, J. Chromatogr. A 669 (1994) 151–183.
[38] I.Z. Atamna, C.J. Metral, G.M. Muschik, H.J. Issaq, J. Liq. Chromatogr. 13 (1990) 2517–2527.
[39] P.J. Oefner, G.K. Bonn, C.G. Huber, S. Nathakarnkitkool, J. Chromatogr. 625 (1992) 331–340.
[40] S. Nathakarnkitkool, P.J. Oefner, G. Bartsch, M.A. Chin, G.K. Bonn, Electrophoresis 13 (1992) 18–31.
[41] T. Tsuda, J. High Resolut. Chromatogr. Chromatogr. Commun. 10 (1987) 622–624.
[42] J.C. Reijenga, G.V.A. Aben, T.P.E.M. Verheggen, F.M. Everaerts, J. Chromatogr. 260 (1983) 241–254.
[43] T. Kaneta, S. Tanaka, M. Taga, J. Chromatogr. A 653 (1993) 313–319.
[44] G.M. Janini, K.C. Chan, J.A. Barnes, G.M. Muschik, H.J. Issaq, J. Chromatogr. A 653 (1993) 321–327.
[45] C.A. Lucy, R.S. Underhill, Anal. Chem. 68 (1996) 300–305.
[46] D.W. Fuerstenau, J. Phys. Chem. 60 (1956) 981–985.
[47] P. Somasundaran, T.W. Healy, D.W. Fuerstenau, J. Phys. Chem. 68 (1964) 3562–3566.
[48] J.W. Jargenson, D.K. Lukacs, Anal. Chem. 53 (1981) 1298–1302.
[49] J.C. Giddings, Sep. Sci. 4 (1968) 181–189.
[50] X. Huang, J.A. Luckey, M.J. Gordon, R.N. Zare, Anal. Chem. 61 (1989) 766–770.
[51] J.E. Wiktorowicz, J.C. Colburn, Electrophoresis 11 (1990) 769–773.
[52] A. Emmer, M. Jansson, J. Roeraade, J. High Resolut. Chromatogr. 14 (1991) 738–740.
[53] W. Nashabeh, Z. El Rassi, J. Chromatogr. 596 (1992) 251–264.
[54] D. Corradini, A. Rhomberg, C. Corradini, J. Chromatogr. A 661 (1994) 305–313.
[55] D. Corradini, G. Cannarsa, E. Fabbri, C. Corradini, J. Chromatogr. A 709 (1995) 127–134.
[56] D. Corradini, G. Cannarsa, LC–GC Int. 9 (1996) 34–40.
[57] A. Cifuentes, M.A. Rodriguez, F.J. Garcia-Montelongo, J. Chromatogr. A 742 (1996) 257–266.
[58] M.T. Galceran, L. Puignou. M. Diez, J. Chromatogr. A 732 (1996) 167–174.
[59] D. Corradini, G. Cannarsa, Electrophoresis 16 (1995) 630–635.
[60] A.D. Tran, S. Park, P.J. Lisi, O.T. Huynh, R.R. Ryall, P.A. Lane, J. Chromatogr. 542 (1991) 459–471.
[61] C. Schwer, E. Kenndler, Anal. Chem. 63 (1991) 1801–1807.
[62] G.M. Janini, K.C. Chan, J.A. Barnes, G.M. Muschik, H.J. Issaq, Chromatographia 35 (1993) 497–502.
[63] M.S. Bello, P. de Besi, R. Rezzonico, P.G. Righetti, E. Casiraghi, Electrophoresis 15 (1994) 623–626.
[64] M.R. Schure, R.E. Murphy, Electrophoresis 16 (1995) 2074–2085.
[65] S. Hjerten, Ark. Kemi 13 (1958) 151–152.
[66] H. Lindner, W. Helliger, A. Dirschlmayer, M. Jaquemar, B. Puschendorf, Biochem. J. 283 (1992) 467–471.
[67] H. Lindier, W. Helliger, A. Dirschlmayer, H. Talasg, M. Wurm, B. Sarg, M. Jaquemar, B. Puschendorf, J. Chromatogr. 608 (1992) 211–216.
[68] N. de Jong, S. Visser, C. Olieman, J. Chromatogr A. 652 (1993) 207–213.
[69] G. Cowdrey, M. Firth, G. Firth, Electrophoresis 16 (1995) 1922–1926.
[70] M. Gilges, H. Husmann, M.H. Kleemiss, S.R. Motsch, G. Schomburg, J. High Resolut. Chromatogr. 15 (1992) 452–457.
[71] M. Gilges, M.H. Kleemiss, G. Schomburg, Anal. Chem. 66 (1994) 2038–2046.
[72] N. Iki, E.S. Yeung, J. Chromatogr. A 731 (1996) 273–282.
[73] P.G. De Gennes, Scaling Concepts in Polymer Chemistry, Cornell University Press, Ithaca, NY, 1979.
[74] P.D. Grossman, D.S. Soane, J. Chromatogr. 559 (1991) 257–266.
[75] Y. Bae, D. Soane, J. Chromatogr. A 652 (1993) 17–22.
[76] M. Zhu, D.L. Hansen, S. Burd, F. Gannon, J. Chromatogr. 480 (1989) 311–319.
[77] M.A. Strege, A.L. Lagu, J. Chromatogr. A 652 (1993) 179–188.
[78] K. Weber, M. Osborn, J. Biol. Chem. 244 (1969) 4406–4412.
[79] J. Reynolds, C. Tanford, Proc. Nat. Acad Sci. USA 66 (1970) 1002–1007.
[80] S. Hjerten, J. Chromatogr. 270 (1983) 1–6.
[81] A.S. Cohen, B.L. Karger, J. Chromatogr. 397 (1987) 409–417.

[82] V. Dolnik, J. Microcolumn. Sep 6 (1994) 315–330.
[83] E. Kenndler, H. Poppe, J. Cap. Electrophoresis 1 (1994) 144–157.
[84] A. Guttman, Electrophoresis 17 (1996) 1333–1341.
[85] A. Widhalm, C. Schwer, D. Blass, E. Kenndler, J. Chromatogr. 549 (1991) 446–451.
[86] K. Ganzler, K.S. Greve, A.S. Cohen, B.L. Karger, A. Guttman, N.C. Cooke, Anal. Chem. 64 (1992) 2665–2671.
[87] A. Guttman, J. Horvath, N. Cooke, Anal. Chem. 65 (1993) 199–203.
[88] M.R. Karim, J.-C. Janson, T. Takagi, Electrophoresis 15 (1994) 1531–1534.
[89] K. Benedek, S. Thiede, J. Chromatogr. A 676 (1994) 209–217.
[90] P.C.H. Shieh, D. Hoang, A. Guttman, N. Cooke, J. Chromatogr. A 676 (1994) 219–226.
[91] A. Guttman, P. Shieh, J. Lindahl, N. Cooke, J. Chromatogr. A 676 (1994) 227–231.
[92] M. Nakatani, A. Shibukawa, T. Nakagawa, J Chromatogr. A 672 (1994) 213–218.
[93] M. Nakatani, A. Shibukawa, T. Nakagawa, Electrophoresis 17 (1996) 1210–1213.
[94] M. Nakatani, A. Shibukawa, T. Nakagawa, Electrophoresis 17 (1996) 1584–1586.
[95] E. Simò-Alfonso, M. Conti, C. Gelfi, P.G. Righetti, J. Chromatogr. A 689 (1995) 85–96.
[96] Y. Waltbroehl, J.W. Jorgenson, J. Microcolumn Sep. 1 (1989) 41–45.
[97] H. Lindner, W. Helliger, B. Sarg, C. Meraner, Electrophoresis 16 (1995) 604–610.
[98] N.A. Guzman, J. Moschera, K. Iqbal, W. Malick, J. Chromatogr. 608 (1992) 197–204.
[99] H.B. Hines, E.E. Brueggemann, J. Chromatogr. A 670 (1994) 199–208.
[100] R.B. Cole, J. Varghese, R.M. McCormick, D. Kadlecek, J. Chromatogr. A 680 (1994) 363–373.
[101] A. Nahum, C. Horváth, J. Chromatogr. 203 (1981) 53–63.
[102] K.E. Biij, C. Horváth, W.R. Melander, A. Nahum, J. Chromatogr. 203 (1987) 65–84.
[103] J.P. Landers, R.P. Oda, B.J. Madden, T.C. Spelsberg, Anal. Biochem. 205 (1992) 115–124.
[104] N.K. Klausen, T. Kornfelt, J. Chromatogr. A 718 (1995) 195–202.
[105] E. Watson, F. Yao, Anal. Biochem. 210 (1993) 389–393.
[106] E. Watson, F. Yao, J. Chromatogr. 630 (1993) 442–446.
[107] R.P. Oda, J.P. Landers, Electrophoresis 17 (1996) 431–437.
[108] V. Rohlicek, Z. Deyl, J. Chromatogr. 494 (1989) 87–99.
[109] M.E. Legaz, M.M. Pedrosa, J. Chromatogr. A 719 (1996) 159–170.
[110] M. Taverna, A. Baillet, D. Biou, M. Schluder, R. Werner, D. Ferrier, Electrophoresis 13 (1992) 359–366.
[111] J.A. Bullock, L.-C. Yuan, J. Microcolumn Sep. 3 (1991) 241–248.
[112] J. Bullock, J. Chromatogr. 633 (1993) 235–244.
[113] L. Song, Q. Ou, W. Yu, J. Chromatogr. A 657 (1993) 175–183.
[114] D.E. Morbeck, B.J. Madden, D.J. McCormick, J. Chromatogr. A 680 (1994) 217–224.
[115] L. Song, Q. Ou, W. Yu, J. Liq. Chromatogr. 17 (1994) 1953–1969.
[116] R.P. Oda, B.J. Madden, T.C. Spelsberg, J.P. Landers, J. Chromatogr. A 680 (1994) 85–92.
[117] A. Cifuentes, M. de Frutos, J.M. Santos, J.C. Diez-Masa, J. Chromatogr. 555 (1993) 63–72.
[118] R.G. Nielsen, G.S. Sittampalam, E.C. Rickard, Anal. Biochem. 177 (1989) 20–26.
[119] P.D. Grossman, J.C. Colburn, H.H. Lauer, R.G. Nielsen, R.M. Riggin, G.S. Sittampalam, E.E. Rickard, Anal. Chem. 61 (1989) 1186–1194.
[120] E.C. Rickard, M.M. Strohl, R.G. Nielsen, Anal. Biochem. 197 (1991) 197–207.
[121] G.R. Paterson, J.P. Hill, D.E. Otter, J. Chromatogr. A 700 (1995) 105–110.
[122] F. Kalman, S. Ma, R.O. Fox, C. Horváth, J. Chromatogr. A 705 (1994) 135–154.
[123] W.R. Melander, J. Stoveken, C. Horváth, J. Chromatogr. 185 (1979) 111–127.
[124] A. Cifuentes, J.M. Santos, M. de Frutos, J.C. Diez-Masa, J. Chromatogr. A 652 (1993) 161–170.
[125] Y.J. Yao, S.F.Y. Li, J. Chromatogr. A 663 (1994) 97–104.
[126] A. Cifuentes, H. Poppe, J.C. Kraak, F.B. Erim, J. Chromatogr. B 681 (1996) 21–27.
[127] F.B. Erim, A. Cifuentes, H. Poppe, J.C. Kraak, J. Chromatogr. A 708 (1995) 356–361.
[128] J.K. Towns, F.E. Regnier, J. Chromatogr. 516 (1990) 69–78.
[129] N. Cohen, E. Grushka, J. Cap. Electrophoresis 1 (1994) 112–115.
[130] W.L. Hinze, B. Moreno, F.H. Quina, Y. Suzuki, H. Wang, Anal. Chem. 66 (1994) 3449–3457.
[131] J. Wiktorowicz, U.S. Patent, 5015350, 1991.
[132] J.F. Kelly, S.J. Locke, L. Ramaley, P. Thibault, J. Chromatogr. A 720 (1996) 409–427.
[133] S. Terabe, K. Otsuka, K. Ichikawa, A. Tsuchiya, T. Ando, Anal. Chem. 56 (1984) 111–113.
[134] A. Emmer, M. Jansson, J. Roeraade, J. Chromatogr. 547 (1991) 544–550.
[135] W.G.H.M. Muijselaar, C.H.M.M. de Bruijn, F.M. Everaerts, J. Chromatogr. 605 (1992) 115–123.
[136] A. Emmer, J. Roeraade, J. Liq. Chromatogr. 17 (1994) 3831–3846.
[137] A. Emmer, M. Jansson, J. Roeraade, J. Chromatogr. A 672 (1994) 231–236.
[138] E.L. Hult, A. Emmer, J. Roeraade, J. Chromatogr. A 757 (1997) 255–262.
[139] T. Tadey, W.C. Purdy, J. Chromatogr. A 652 (1993) 131–138.
[140] W. Nashalbeh, K.F. Greve, D. Kirby, F. Foret, B. Karger, D.H. Reifingder, S.E. Builder, Anal. Chem. 66 (1994) 2148–2154.
[141] M.A. Strege, A.L. Lagu, Anal. Biochem. 210 (1993) 402–410.
[142] J.K. Towns, F.E. Regnier, Anal. Chem. 63 (1991) 1126–1132.
[143] X.-W. Yao, D. Wu, F.E. Regnier, J. Chromatogr. 636 (1993) 21–29.

[144] G.G. Yowell, S.D. Fazio, R.V. Vivilecchia, J. Chromatogr. A 652 (1993) 215–224.
[145] B.P. Salmanowicz, Chromatographia 41 (1995) 99–106.
[146] Y.J. Yao, K.C. Loh, M.C.M. Chung, S.F.Y. Li, Electrophoresis 16 (1995) 647–653.
[147] J.M. Thorne, W.K. Goetzinger, A.B. Chen, K.G. Moorhouse, B.L. Karger, J. Chomatogr. A 744 (1996) 155–165.
[148] K.W. Yim, J. Chromatogr. 559 (1991) 401–440.
[149] L. Zolla, M. Bianchetti, A.M. Timperio, G. Scarascia Mugnozza, D. Corradini, Electrophoresis 17 (1996) 1597–1601.
[150] R.S. Rush, P.L. Derby, T.W. Strickland, M.F. Rohde, Anal. Chem. 65 (1993) 1834–1842.
[151] M.K. Weldon, C.M. Arrington, P.L. Runnels, J.F. Wheeler, J. Chromatogr. A 758 (1997) 293–302.
[152] G.N. Okafo, H.C. Birrell, M. Greenaway, M. Haran, P. Camilleri, Anal. Biochem. 219 (1994) 201–206.
[153] G.N. Okafo, A. Vinther, T. Kornfelt, P. Camilleri, Electrophoresis 16 (1995) 1917–1921.
[154] T. Kornfelt, A. Vinther, G.N. Okafo, P. Camilleri, J. Chromatogr. A 726 (1996) 223–228.
[155] K.L. Kostel, A.L. Freed, S.M. Lunte, J. Chromatogr. A 744 (1994) 241–248.
[156] D. Josic, K. Zeilinger, W. Reutter, A. Bottcher, G. Schmitz, J. Chromatogr. 516 (1990) 89–98.
[157] I. Recio, C. Olieman, Electrophoresis 17 (1996) 1228–1233.
[158] M. Zhu, R. Rodriguez, D. Hansen, T. Weher, J. Chromatogr. 516 (1990) 123–131.
[159] S.J. Harrington, R. Varro, T.M. Li, J. Chromatogr. 559 (1991) 385–390.
[160] R. Lausch, T. Scheper, G.-W. Reif, T. Schlosser, J. Fleischer, R. Freitag, J. Chromatogr. A 654 (1993) 190–195.
[161] Y. Zhang, H.K. Lee, S.F.Y. Li, J. Chromatogr. A 744 (1996) 249–257.
[162] T.M. McNerney, S.K. Watson, J.-H. Sim, R.L. Bridenbaugh, J. Chromatogr. A 744 (1996) 223–229.
[163] L.R. Gurley, J.E. London, J.G. Valdez, J. Chromatogr. 559 (1991) 431–443.
[164] F.A. Chen, L. Kelly, R. Palmieri, R. Biehler, H. Schwartz, J. Liq. Chromatogr. 15 (1992) 1143–1161.
[165] X.-h. Fang, T. Zhu, V.-h. Sun, J. High Resolut. Chromatogr. 17 (1994) 749–752.