Separation of Proteins with Capillary Electrophoresis in Coated Capillaries with and without Electroosmosis

Studies on Zone Broadening and Analytical Performances

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Abstract

Proteins have such structural features that they may interact with different types of surfaces by all possible forces, i.e., electrostatic, hydrogen bonding, hydrophobic. In this thesis two different types of coatings for fused silica capillaries aimed to eliminate such interactions have been studied. The first is a covalent, electroosmosis-free coating with polyacrylamide (PAA) and the second involves a non-covalent coating with the quaternary ammonium compound N, N-didodecyl –N, N-dimethylammonium bromide (DDAB) with a strong anodic electroosmosis. Optimal conditions regarding efficiency and resolution were established by variations of the composition and ionic strengths of buffers at pH below the isoelectric point of the proteins. To achieve high efficiency and resolution the choice of buffer constituents was extremely important.

The PAA coating was very stable at neutral and acidic conditions. Ammonium acetate (0.12 M) and ammonium hydroxyacetate (0.15 M) both at pH 4 provided the best separations with plate numbers up to 1 700 000 plate/m that is among the highest reported in the literature. Capillaries coated with DDAB were stable enough to, without recoating, permit consecutive separations of the proteins up to 9 hours (90 injections). High apparent efficiencies (over 1 million plates/m) were achieved with ammonium acetate (0.07 M), ammonium hydroxyacetate (0.08 M) and sodium phosphate (0.1 M) at pH 4.

Zone broadening was studied by determination of the variance contributions from all main parameters. Significant variances were contributions from longitudinal diffusion, capillary curvature, injection plug, detector time response and detector slit width while other variances, e.g., variances for Joule heat and vertical sedimentation were negligible. The remaining undetermined variance may have its origin in all types of relatively slow interactions including adsorption onto the capillary surfaces and protein-buffer component interactions. The results indicate that the latter is the main cause to zone broadening in protein separations.

Keywords: Capillary electrophoresis, capillary coatings, proteins, zone broadening, zone sharpening, efficiency, resolution, protein-surface adsorption, protein-buffer interaction, analytical performance

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Änglar, visst finns de .......

Till mina änglar Pegah och Afra
List of Papers

This thesis is based on the following papers, which will be referred to in the text by their Roman numerals:

I The influence of ignored and well-known zone distortions on the separation performance of proteins in capillary free zone electrophoresis with special reference to analysis in polyacrylamide-coated fused silica capillaries in various buffers

II The influence of ignored and well-known zone distortions on the separation performance of proteins in capillary free zone electrophoresis with special reference to analysis in polyacrylamide-coated fused silica capillaries in various buffers

III Easy applicable, non-covalent coating with N, N-didodecyl -N, N-dimethylammonium bromide (DDAB) for separation of basic proteins by capillary electrophoresis in acidic buffers in 25- and 50-µm capillaries
Studies on zone broadening and analytical performance. S. Mohabbati, S. Hjertén, D. Westerlund, J. Chromatogr. A. In manuscript

IV Improved coating properties with N, N-didodecyl - N, N-dimethylammonium bromide for separation of basic proteins by capillary electrophoresis in acidic buffers in 25-µm capillaries. S. Mohabbati, D. Westerlund, J. Chromatogr. A. In press

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# Abbreviations

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<td>BGE</td>
<td>Background electrolyte</td>
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<td>CE</td>
<td>Capillary electrophoresis</td>
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<tr>
<td>CLOD</td>
<td>Concentration limit of detection</td>
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<tr>
<td>DDAB</td>
<td>$N, N$-didodecyl $-N, N$-dimethylammonium bromide</td>
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<tr>
<td>EOF</td>
<td>Electroosmotic flow</td>
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<td>ESI</td>
<td>Electrospray ionization</td>
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<td>FAI</td>
<td>Field-amplified injection</td>
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<tr>
<td>HPLC</td>
<td>High performance liquid chromatography</td>
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<tr>
<td>i.d.</td>
<td>Inner diameter</td>
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<tr>
<td>LOD</td>
<td>Limit of detection</td>
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<tr>
<td>MS</td>
<td>Mass spectrometry</td>
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<td>N</td>
<td>Number of theoretical plates</td>
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<tr>
<td>o.d.</td>
<td>Outer diameter</td>
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<tr>
<td>PAA</td>
<td>Polyacrylamide</td>
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<tr>
<td>PI</td>
<td>Isoelectric point</td>
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<tr>
<td>PVA</td>
<td>Poly vinyl alcohol</td>
</tr>
<tr>
<td>R</td>
<td>Radius</td>
</tr>
<tr>
<td>$R_s$</td>
<td>Resolution</td>
</tr>
<tr>
<td>spPC</td>
<td>Solid phase preconcentration</td>
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<tr>
<td>TEMED</td>
<td>$N, N, N', N'$–tetramethylethylenediamine</td>
</tr>
<tr>
<td>t-ITP</td>
<td>Transient isotachophoresis</td>
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<td>UV</td>
<td>Ultraviolet</td>
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Introduction

Capillary electrophoresis (CE) has become one of the most powerful techniques in protein separations due to its strong benefits such as simple method development, high separation efficiency, low sample consumption and short analysis times [1-7]. It has been an important tool in chiral separations [8, 9], DNA [10, 11] and proteomics research [12, 13], and chip technology [14, 15]. However a major drawback of CE is poor detection sensitivity due to low loading capacity and short optical path length because of the small capillary dimensions. The concentration limits of detection are in general 10-100-fold higher than those in HPLC when UV detection is used. Many efforts have been made to improve the detection limits; on-line preconcentration methods in addition to increasing the detector pathlength with novel optical designs such as bubble cells, Z-cells, and other devices to improve the sensitivity of the detectors [16, 17] have been investigated. The most studied techniques are electrophoresis based preconcentration such as sample stacking based on the conductivity difference between sample and the running buffer [18-23], field-amplified injection (FAI) [24, 25] and transient isotachophoresis (t-ITP) [26-32].

Protein adsorption onto the negatively charged capillary wall through several factors like electrostatic, hydrophobic and hydrogen-bonding interactions leads to poor separation efficiencies and recoveries and bad reproducibilities. To overcome or suppress these effects, a variety of techniques have been developed; from simply using BGE at high pH, to coating the capillary permanently or dynamically.

Capillary electrophoresis

History

The theoretical basis of electrophoresis was introduced by F. Kohlrausch in 1897 [33]. Moving boundary theory and electrophoresis as a separation technique was introduced by Tiselius in 1937 at Uppsala University [34]. By placing protein mixtures between buffer solutions in a tube and applying an electric field, he found that sample components migrated in a direction at a rate determined by their charge and size. For this work and other achievements in separation science he was awarded a Nobel Prize in 1948.
Initial work in open tube electrophoresis was described by Hjertén in 1967. Since only wide, millimeter-bore glass capillaries were available, Hjertén rotated them along their longitudinal axis to suppress the effects of convection [35]. Virtanen performed electrophoresis in 1974 in approximately 200-µm inner diameter (i.d.) capillaries made from glass and Teflon [36].

In 1981 Jorgenson and Lukacs published an article with the first electrophoretic separation as we know it today where they used a 75-µm i.d. Pyrex glass capillary and a 30 kV voltage for separation of derivatized amino acids, peptides and amines [37].

In 1987 the first CE-mass spectrometry (MS) coupling with an electrospray interface was made by Smith et al. [39] and the first complete CE instrument on the market was introduced in 1988 [38].

**Principles**

In CE the separation is often performed in fused silica capillaries, typically 25- to 75-µm i.d., which are filled with electrolytes, most often buffers. Use of silica capillaries has the advantage of applying very high electrical fields (100-500 V/cm) and, consequently, short migration times, high efficiency and resolution with minimal Joule heat generation due to their excellent thermal conductivity. Furthermore, the large surface area/volume ratio of the capillaries dissipates the generated heat.

An advantage in CE is simplicity of the instrumentation; the ends of a narrow-bore fused silica capillary are placed in electrolyte vials, which also contain the electrodes (Fig. 1).

![Figure 1: Diagram of capillary electrophoresis system.](image-url)
Sample is loaded into the capillary by applying either an electric field or external pressure after replacing one of the vials with the sample vial. After replacing the electrolyte vial the electric field is applied and the separation is performed. Detection in CE can be carried out both on-line and off-line by several different devices. The majority of commercial systems use UV or UV-Vis absorbance as their primary mode of detection. In these systems a section of the capillary, which must be optically transparent, is used as the detection cell. The use of on-capillary detection enables detection of separated analytes with no loss of resolution. Other detection devices used in CE are for example fluorescence and MS.

Fluorescence detection is used in capillary electrophoresis for samples that naturally fluoresce or are chemically modified to contain fluorescent tags. This mode of detection offers high sensitivity and improved selectivity for these samples. Laser-induced fluorescence has been used in CE systems with detection limits as low as 8 nM [40]. The sensitivity of the technique is attributed to the high intensity of the incident light and the ability to accurately focus the light on the capillary.

In order to obtain the identity of sample components, capillary electrophoresis can be directly coupled to MS. In most systems, the capillary outlet is introduced into an ion source that utilizes electrospray ionization (ESI). The resulting ions are then analyzed by the mass spectrometer. This set-up requires volatile buffer solutions, which may affect the degree of resolution and the detection sensitivity.

Electrophoresis is the migration of charged compounds in a solution under the influence of an electric field and the separation is based on differences in solute velocities because of different charge/size ratios [41]. The size of a molecule is determined by the molecular weight, the degree of solvation and the three-dimensional structure.

The velocity of an ion is proportional to the field strength, \( E \) (a function of the applied voltage and capillary length, \( V/cm \)), and is given by:

\[
\nu = \mu_{ep} E
\]

(1)

where \( \nu \) is the ion velocity and \( \mu_{ep} \) the electrophoretic mobility, which for a given ion and medium is a constant and characteristic of that ion.

**Electroosmotic mobility**

Under aqueous conditions (at pH > 2-3) the fused silica capillary is negatively charged from ionization of the free silanol groups, which then exist in deprotonated anionic form, SiO\(^-\). When a BGE is in contact with the inner capillary wall the cation components of the buffer will be electrostatically attracted to the negatively charged surface and an electric double layer is formed (Fig. 2).
Figure 2. Interior of a fused-silica capillary in the presence of a buffer solution.

This potential difference, which is created very close to the wall, is known as zeta potential; the layer closest to the capillary wall which is called the Stern layer is immobile (Fig. 3) while the cations in the second layer, the diffuse layer, will migrate towards the cathode if a voltage is applied across the separation capillary.

Figure 3. Zeta potential.

The movement of the solvated cations will by friction forces spread to the whole bulk BGE solution which will be dragged toward the cathode with a velocity directly proportional to the electric field strength with a plug profile, the so-called electroosmotic flow EOF. The magnitude of the EOF can be expressed by

$$v_{EOF} = -\frac{E \zeta}{\eta}$$  \hspace{1cm} (2)
where $V_{EOF}$ is EOF velocity, $\varepsilon$ the dielectric constant and $\zeta$ the zeta potential. Since the zeta potential is determined by the surface charge and, thereby, is strongly pH dependent, the magnitude of the EOF varies with pH.

Increasing the ionic strength increases the thickness of the electric double layer, and, therefore, decreases the EOF due to an increase of the zeta potential. A rise in the temperature will decrease the viscosity and, hence, increase the EOF by about 2\% per degree.

EOF causes movement of cations, neutral components and anions (if their electrophoretic mobilities are smaller than electroosmotic mobility, i.e., $\mu_{ep} < \mu_{eo}$) towards the detector and, thus, simultaneous detection of all the solutes with different charges (Fig. 1).

The flow profile of the electroosmosis is flat which deviates from the laminar flow in high performance liquid chromatography (HPLC) created by a pump. Consequently, the flat profile minimizes zone broadening and increases the efficiency (Fig. 4).

Electrophoretic mobility
A substance moves in an electric field only when it is charged. The electrophoretic mobility of a protolyte is associated with the fraction of the substance that is charged ($\alpha$) at the actual pH:

$$u_{ep} = \alpha \cdot u_{em} \quad (3)$$

where $u_{em}$ is the ionic mobility of the protolyte.

The electrophoretic mobility is superimposed by the electroosmotic mobility and depends on the size and charge of the substance, the temperature and the properties of the BGE. The resulting total or apparent mobility $u_{app}$ is thus the sum of those two mobilities [42]:

$$u_{app} = u_{eo} + u_{ep} \quad (4)$$
Which all are signed quantities (anions with negative and cations with positive effective mobilities). The apparent mobility can be calculated by:

\[ u_{\text{app}} = \frac{L_d L_{\text{tot}}}{V t_m} \]  \hspace{1cm} (5)

where \( L_d \) and \( L_{\text{tot}} \) are the capillary length till detector and the total capillary length, respectively, \( V \) the applied voltage and \( t_m \) the migration time.

Some crucial parameters

**Efficiency: true and apparent**

The efficiency of capillary electrophoresis separations or the number of theoretical plates, generally expressed by \( N \) is typically much higher than the efficiency of other separation techniques like HPLC. Unlike HPLC, in CE there is no mass transfer between phases. In addition, the flow profile in EOF-driven systems is flat, rather than the rounded laminar flow profile characteristic of the pressure-driven flow in chromatography columns.

At a very high electroosmotic flow the zone will pass the detector at a high speed resulting in a narrow peak with the apparent plate number, \( N_{\text{app}+\text{eo}} \). However, for a correct comparison of the performance of different CE-systems, the efficiencies corresponding only to the electrophoretic mobilities should be employed, since the electroosmotic flow in general only displaces a zone, but does not change its width [43]. Therefore, the terms apparent and true plate numbers is used for efficiencies with and without the electroosmotic effect, respectively.

The apparent plate numbers (\( N_{\text{app}+\text{eo}} \) or \( N_{\text{app}} \)) can be transformed to the true plate numbers (\( N_{\text{ep}} \) or \( N_{\text{true}} \)) using the following equation [43]:

\[ N_{\text{ep}} = \left( \frac{\mu}{\mu_{\text{ep}} + \mu_{\text{eo}}} \right)^2 N_{\text{app}+\text{eo}} \]  \hspace{1cm} (6)

Obviously the true plate numbers are always smaller than the apparent ones when the electrophoretic and electroosmotic mobilities move in the same direction. However, when the direction of the two mobilities is opposite the apparent plate numbers are equal to the true plate numbers when the \( \mu_{\text{eo}} \) is twice the \( \mu_{\text{ep}} \).

When \( \mu_{\text{eo}} < 2 \cdot \mu_{\text{ep}} \) the apparent plate numbers are lower than the true plate numbers, the ratio reaching a minimum (= 0) when \( \mu_{\text{eo}} = \mu_{\text{ep}} \) (the
peak has an apparent mobility = 0). The apparent plate numbers > the true plate numbers in all experiments where $|u_{eo}| > 2|u_{ep}|$.

**Resolution**

The resolution describes the degree of separation of two adjacent peaks and can be calculated from the experimental data according to:

$$R_s = \frac{2 \cdot \Delta t}{w_1 + w_2} \quad (7)$$

where $\Delta t$ is the difference in migration times between the two analytes and $w_1$ and $w_2$ are the width of the peaks (in time units) often at the baseline.

Eq. 7 can be transformed to:

$$R_s = \frac{\sqrt{N}}{4} \cdot RMD \quad (8)$$

where $RMD$ is Relative mobility difference:

$$RMD = \frac{2\Delta u_{app}}{u_{ep1} + u_{ep2} + 2u_{eo}} \quad (9)$$

The EOF cannot decrease or increase the width of a zone in the capillary; only displace the zone, provided that the EOF does not cause extra band broadening. Accordingly, the separation pattern in the capillary for cations is the same in the absence and in the presence of electroosmosis, provided that the duration of these two experiments is the same [44], i.e., the time is the same for the velocities $v_{ep\pm eo}$ and $v_{ep}$. Therefore also the true resolution is the same in the absence and presence of an electroosmosis with a perfect plug flow.

For anions, if the electrophoretic mobility is more than half of the electroosmotic mobility, the resolution is higher than under conditions without electroosmosis; on the other hand if the electrophoretic mobility is lower, resolution is worse and reduction of the electroosmotic flow results in an improvement of the resolution [45].
Zone broadening

The sample is often introduced as a very short plug at the tip of the capillary but gets broader by a number of factors and the resulted zone broadening is described by the variance of peaks, \( \sigma^2 \) [43, 46, 47, 48, 49 (III)].

Many factors may affect the width of a zone in capillary zone electrophoresis, e.g., longitudinal diffusion \( \sigma^2_{\text{dif}} \), the injected plug length \( \sigma^2_{\text{inj}} \), laminar flow \( \sigma^2_{\text{lam}} \), sedimentation in the vertical section of the capillary \( \sigma^2_{\text{sed}} \), development of Joule heat \( \sigma^2_{J} \), effect of electroosmotic flow \( \sigma^2_{\text{EOF}} \), curvature of the capillary \( \sigma^2_{\text{curv}} \), adsorption of analytes to the capillary surface \( \sigma^2_{\text{ads}} \), the detector slit width \( \sigma^2_{\text{det}} \), the detector time response \( \sigma^2_{r} \). In many cases a rest variance \( \sigma^2_{\text{rest}} \) remains, i.e., variance for which there is no obvious explanation. Since independent variances are additive the total variance for zone broadening is given by the following general relationship:

\[
\sigma_{\text{tot}}^2 = \sigma_{\text{dif}}^2 + \sigma_{\text{inj}}^2 + \sigma_{\text{lam}}^2 + \sigma_{\text{sed}}^2 + \sigma_{J}^2 + \sigma_{\text{EOF}}^2 + \sigma_{\text{curv}}^2 + \sigma_{\text{ads}}^2 + \sigma_{\text{det}}^2 + \sigma_{r}^2 + \sigma_{\text{rest}}^2
\]

Diffusion

Under ideal circumstances diffusion is the only source of zone broadening and this broadening is determined by the Einstein equation:

\[
\sigma_{\text{diff}}^2 = 2Dt
\]

where \( D \) is the diffusion constant (cm\(^2\)/s) and \( t \) the migration time.

Injection

Basically there are two different methods to introduce the sample into the capillary: hydrodynamic and electrokinetic.

**Hydrodynamic injection**

The most common injection method in commercial automated systems is hydrodynamic injection driven by a pressure difference over the capillary. The pressure difference is accomplished by gas pressure on the sample vial, by vacuum suction at the capillary end or by applying a height difference between the inlet and the outlet. The average length of the concentrated sample zone introduced at 25 °C in aqueous BGE solutions is given by [49 (III)]:

\[
\Delta X_0 = \frac{PR^2 \cdot t_{\text{inj}} \cdot \kappa^2}{8\eta L_{\text{tot}}} \left[ 1 + \frac{\sqrt{\mu}}{3 \cdot r_p} \right]
\]
where $P$ is the pressure difference (dyne cm$^{-2}$), $R$ the capillary radius (cm), $t_{inj}$ the injection time (s) and $\eta$ the viscosity of the buffer (~0.01 poise) $\mu$ the ionic strength of the BGE, $\kappa^{II}$ and $\kappa^{I}$ the conductivities of the sample zone ($\Omega^2$ cm$^{-1}$) and the BGE, respectively, and $r_p$ the radius of the protein (cm).

The variance of the injection length is given by:

$$\sigma_{inj}^2 = \frac{\Delta X_0^2}{12}$$  \hspace{1cm} (13)

**Electrokinetic injection**

The sample may be introduced by applying a voltage for a short time. The amount of an analyte ion introduced is dependent on its mobility, the electroosmosis and the conductivity of the sample solution.

The width of the starting zone ($\Delta X_0$) and, thereby, the variance ($\frac{\Delta X_0^2}{12}$) can easily be determined experimentally in the conventional way from velocity and migration time when the sample is dissolved in the buffer. If this is not the case, for instance when the sample is subjected to zone sharpening, the following approach can be employed when diffusion and injection are the only parameters causing zone broadening [50 (I)]:

$$\sigma_{tot}^2 = \frac{\Delta X_0^2}{12} + \frac{2D}{uE}$$  \hspace{1cm} (14)

The width of the starting zone is obtained from the intercept after plotting the total variance against the inverted field strength.

**Hydrodynamic flow**

Hydrodynamic- or laminar flow caused by mismatch between electroosmotic velocities in different sections of the capillary is an additional source of peak broadening [49 (III)]. The hydrodynamic flow must be the same in all cross-sections of the capillary in order to completely eliminate this type of zone broadening. In other cases local axial and radial circulations of the BGE will therefore occur between areas with different EOF’s, causing zone broadening. The bigger the difference in conductivity between two buffers, the larger the pressure at the concentration boundary, and the larger the resulting variance will be.
Sedimentation zone broadening

When the sample zone has an electrical conductivity much lower than that of the running buffer, the proteins will be efficiently trapped and enriched at the boundary between low and high buffer concentration. This boundary is virtually stationary. Therefore the proteins will be highly concentrated very close to the start end of the capillary and therefore start to sediment.

Convective zone broadening, i.e. sedimentation caused by differences in the density of a solute zone and that of the buffer was calculated according to Eq. 15 [51], which is valid for the sedimentation of a zone in the vertical section of the capillary, including the influence of radial diffusion.

The variance for sedimentation zone broadening can be calculated from [49 (III), 50 (I), 52 (II)]:

\[ \sigma_{sed}^2 = \frac{R^2 t}{96D} \left[ \frac{gR^2}{4\eta} \left( \rho_s - \rho_b \right) \right]^2 \] (15)

where \( g \) = the gravitational acceleration (981 dyne.cm\(^{-2}\)), \( \rho_s \) and \( \rho_b \) = the densities of the sample and buffer solutions, respectively (\( \rho_s - \rho_b = 0.0003 \) g cm\(^{-3}\) for 0.1% solution), \( R \) = the radius of the capillary (cm), \( t \) = the sedimentation time (s), and \( \eta \) = the viscosity (0.001 poise cm\(^{-1}\)s\(^{-1}\)).

Sedimentation effects are most pronounced when the sample is introduced by electrokinetic injection. Application of a short plug of the low conductivity buffer after the sample zone would help to avoid loss of sample by sedimentation which is especially pronounced when larger concentrations and wider capillaries are used.

Joule heat

This zone broadening can be divided into zone broadening due to temperature gradients across the capillary diameter, and diffusional zone broadening due to overall increased temperature within the capillary. The temperature gradients are stronger at a given field strength in wide capillaries, and the counteracting effect of radial diffusion is smaller. However, a reduction in capillary diameter means in practice an increased risk of analyte adsorption onto the capillary wall and also a decreased sensitivity.

The variance for Joule heat can be estimated according to the equation [50 (I)]:

\[ \sigma_{J, rad.diff}^2 = \frac{1}{12} \left( \frac{B \kappa}{\lambda} \left( \frac{RE}{2T_0} \right)^2 \right) \cdot \frac{L_d^2}{t} \cdot \frac{R^2}{8D} \] (14)

where \( B = 2400 \) (at 25°C), \( \kappa \) is the electrical conductivity (\( \Omega \cdot \text{cm}^{-1}\)), \( L_d \) the length of the capillary to the detector (cm), \( \lambda \) the thermal conductivity of the
buffer \((\Omega^{-1}\mathrm{cm}^{-1})\), \(R\) the radius of the capillary (cm), \(E\) is the field strength \((\mathrm{V/cm})\), \(T_0\) the temperature of the coolant \((^\circ\mathrm{K})\), \(t\) is the migration time (s), \(D\) is the diffusion constant \((\mathrm{cm}^2/s)\). Control of temperature differentials is critical since a one-degree change in temperature results in a 2.7\% change in mobility.

**EOF**

In a capillary with electroosmosis, the lagging sheath of liquid in the diffuse layer near the column wall causes a radial diffusion flux of the lagged part of the analyte from the wall to the centre. Consequently, the axial profile of the sample moving in a capillary column will be dispersed. This will be the contribution of the uniform EOF to the total peak dispersion.

The variances can be calculated according to [53]:

\[
\sigma^2_{\text{EOF}} = \frac{\beta^2}{D} \cdot v^2_{\text{EOF}} t
\]

where \(\beta\) is the effective thickness of the diffuse layer \((\approx \frac{3}{\sqrt{\mu}} \mathrm{Å})\) (1.1 nm for ammonium acetate \((0.07 \mathrm{M})\), ammonium hydroxyacetate \((0.08 \mathrm{M})\) and 0.9 nm for phosphate buffer \((0.1 \mathrm{M})\) in the experiments in Ref. 49 (III)).

**Curvature of the capillary**

In a coiled capillary the field strength is lower at the outer circumference compared to that at the inner circumference, and the resulting difference in migration represents a peak broadening, or in other words, it accounts for an increase of the dispersion of the moving analyte especially when there are many capillary turns \([54, 55]\). The effect is larger for macromolecules with a small diffusion coefficient. For each complete loop, the zone broadening is \([56]\):

\[
\sigma^2_{\text{curv}} = \frac{L_i d^2}{16r_i^2 L_d}
\]

where \(d\) is the inner diameter of the capillary and \(r_i\) is the sum of internal radius of the capillary coils.

Consequently, the zone broadening caused by the curvature of the capillary may be significant in several commercial instruments. In microchip electrophoresis, where the zones sometimes pass several curvatures of the separation channel and the analysis times can be very short (seconds) and the diffusional zone broadening thus is negligible, the variance related to the curvature may be even more disturbing.
Detector slit width

A large detector slit width may account for some loss of efficiency and the variance is determined in a similar way to the contribution from the injection [57]:

\[ \sigma_{\text{det}}^2 = \left( \frac{\omega_{\text{det}}}{12} \right)^2 \]  \hspace{1cm} (17)

where \( \omega_{\text{det}} \) is the detector slit width.

Detector time constant

The dynamic response of the detector expressed by the time constant \( \tau \) (s) contributes to the total zone broadening and the variance is determined by [57]:

\[ \sigma_{\tau}^2 = \tau^2 E^2 \left( u_{\text{ep}} + u_{\text{eo}} \right)^2 \]  \hspace{1cm} (18)

where \( E \) is the field strength, \( u_{\text{ep}} \) the electrophoretic mobility and \( u_{\text{eo}} \) the electroosmotic mobility.

Adsorption

Reversible adsorption is a significant contributor to band broadening if the adsorption/desorption kinetics with the capillary wall is slow with respect to the migration velocity of analytes through the capillary. The adsorption onto the capillary wall increases with decreasing diameter of the capillary, due to an increasing surface/volume ratio. In case the adsorption is of electrostatic nature it will increases with decreasing ionic strength (conductivity) of the buffer. However, when hydrophobic interactions are predominant, the adsorption decreases when the ionic strength decreases. It is, thus, of great importance to have an efficient method to suppress adsorption when the diameter of the capillary is as narrow as 25 µm.

Protein-small ion interaction

Small buffer ions may interact selectively with the proteins and alter the separation performance. Electrophoresis in different buffers of the same pH gave rise to different separation profiles and plate numbers [49 (III), 50 (I), 52 (II)] which is an indication of the existence of interactions between protein and buffer constituents, but also an indication that the choice of buffer is a critical step in the pursuit of high resolution. When the on/off
kinetics of the interaction is slow a significant zone broadening will be observed.

**Zone sharpening**

The most applied zone sharpening methods in CE can be categorized in two groups: **electrophoresis based** and **chromatography based** preconcentration.

There are three main electrophoretic preconcentration mechanisms: sample stacking, field amplified injection and isotachophoresis.

In **sample stacking**, the conductivity in the sample plug is lower and, consequently, the electric field strength higher than that in the BGE. Hence, the velocity of the analyte is higher in the sample plug than in the BGE and it will be focused in the interface between these two phases. Generally, dissolving the proteins in very diluted BGE or water has a number of disadvantages, e.g., inadequate pH control in the sample zone, an increased risk of precipitation of proteins, an increase of the Joule heat in the starting zone and, therefore, expansion and loss of the sample zone and the risk of protein adsorption onto the capillary wall, as well as EOF-induced disturbances [49 (III), 52 (II)].

A related principle for zone sharpening is **field-amplified injection (FAI)**, which is also based on the differences between the velocity of the analyte in the sample plug and in the BGE. The focusing process is performed during the injection time with an applied voltage in electrokinetic injection [58].

In **transient isotachophoresis** the cationic analyte is dissolved in a leading buffer containing ions with a higher mobility than the analytes, often an ammonium solution, and introduced in the capillary using hydrodynamic injection enabling filling of about 70% of the capillary with the sample [26-32]. Subsequently the inlet of the capillary is placed in a terminating buffer vial, containing ions with a lower mobility than the analytes. After applying voltage the leading and terminating ions and the analytes migrate towards the detection side of the capillary. Because the difference in mobility, the analytes will be focused and concentrated between the two electrolyte zones. A hydrodynamic backpressure at the detection side of the capillary is often used to compress the analyte zone close to the injection end of the capillary. In the next step a normal CZE is performed using the leading electrolyte as BGE.

There are different mechanisms available for on-line preconcentration based on chromatographic retention e.g. solid adsorptive phase chromatography including solid phase preconcentration (spPC) [59, 60] and membrane preconcentration (mPC) [61] which both have the advantage of cleaning up the sample.
Solid phase preconcentration (spPC) system consists of a preconcentration column containing HPLC-packing material coated with a hydrophobic adsorptive phase, such as C₂, C₈ or C₁₈ directly connected to the separation capillary. It is possible to inject much higher sample volumes compared to CE in the loading step. After washing the sample elutes by injecting a small plug of an organic solvent followed by a separation. Among the disadvantages of this method is the large volume of organic solvent required to remove analytes from the solid phase within the CE capillary which can lead to zone broadening and tailing with reduced resolution [59, 60].

In membrane preconcentration (mPC) a suitable coated or impregnated membrane is used which is installed in a cartridge often prepared from Teflon tubing [61]. The loading, removal and separation processes are similar as spPC, however, there are several advantages such as higher loading capacity because of the high adsorptive capacity of the membrane, lower elution volume and more reproducible EOF. Both spPC and mPC are valuable methods for analysis of biological samples with injection volumes of up to 100 µl with an improvement in detection with 3-4 order of magnitude [Table 1 in Ref. 58].

Another mechanism for zone sharpening combined with sample clean up applies in the use of hollow fibers [62]. The hollow fiber technique is based on the transport of water out of the fiber through the pores. A hollow fiber, preferably with a suitable cut-off for clean up purposes, is connected to the inlet of the separation capillary. The analytes electromigrate into the hollow fiber according to their charge during the electrokinetic injection and will be concentrated. Subsequently the separation electric field is switched on and separation is carried out. This promising method has been used with advantage in trace analysis of proteins where fast and efficient on-line preconcentration has been achieved with removal of small molecules, including salts.

Capillary electrophoresis of proteins

Different properties of the fused silica capillary surfaces present a problem in protein analysis technique, especially for basic proteins due to electrostatic analyte-capillary wall interactions. Generally, adsorption of proteins onto the capillary wall through Columbic interactions, hydrogen bonding, and/or hydrophobic interactions leads to poor resolution and poor reproducibility, loss of efficiency, low protein recovery and decreased sensitivity that has to be overcome in protein analysis in CE [1-4, 62].

Modification of the capillary wall is generally aimed at preventing the undesirable interactions between the proteins and the active sites (ionisable silanol groups and siloxane bridges) on the inner wall of the fused silica
capillary. The simplest strategy consists of the use of electrolyte solutions at high pH values, considerably higher than the isoelectric point (pI) of the proteins to make both the proteins and the capillary wall negative, thus provoking strong electrostatic repulsion [1, 63, 64]. However, a simple increase in the pH of the background electrolyte (BGE) is often not sufficient to completely eliminate adsorption of proteins onto the capillary wall since hydrophobic and other interactions are not negligible. This strategy has further the disadvantage of limiting the operational range of pH [1]. In addition, prolonged exposure to highly alkaline pH may cause structural changes in proteins. High ionic strength buffers are also effective in decreasing protein-wall interactions but will lead to increased Joule heating in the capillary, which can degrade the efficiency and stability of the separation. Therefore, other methods to reduce the wall adsorption have expanded in popularity, including dynamic and static coating.

Proteins have functional groups such as hydrogen-bonding regions and hydrophobic interaction sites that may interact to different extents with the components of the BGE [49 (III), 50 (I), 52 (II), 65, 66]. Such interactions can lead to significant variations in the electrophoretic mobility of these analytes with deterioration of the efficiency as a result [49 (III), 50 (I), 52 (II), 66]. In addition, the different functionalities of proteins may interact with a variety of active sites of the coating component and, accordingly, give rise to peak broadening and asymmetry and irreproducible migration times [49 (III), 50 (I), 52 (II), 66].

Different coatings

**Covalent wall coatings**

Neutral polymers as methyl cellulose [35, 67] dextran [68], polyacrylamide (PAA) [69], polyvinylalcohol (PVA) [70] and polyethylene glycol (PEG) [71] have been used for modifying the fused silica capillary permanently. The multilayer coating often consists of a sublayer, which is used to mask the silanol groups and a top layer, the neutral polymer, for eliminating protein adsorption.

Capillaries coated with linear polyacrylamide have been widely used in applications of CE. The coating procedure includes several steps; etching, leaching, dehydration and silylation to obtain high surface coverage of the inner capillary wall by the silylating agent employed. The next step is the polymerization of acrylamide to high-molecular mass polymer in the presence of free radicals generated by a mixture of N, N, N', N' – tetramethylethlenediamine (TEMED) and ammonium persulfate. The capillary wall will then be coated with a uniform layer of linear polyacrylamide covalently attached to the silyl derivatives [68, 69].
Cationic polymers such as polyethyleneimine (PEI) [72], polybrene [73], PolyE-323 [74] have also been used for permanent coating of fused silica capillaries and gives rise to an anodic EOF. The Columbic repulsion of positively charged basic proteins with the positive charges of the coating would minimize the interactions with the capillary wall; however, the cationic coatings are not appropriate for separation of acidic proteins because of the electrostatic interactions.

Dynamic wall coatings
Dynamic coating is performed by either adding neutral polymers to the BGE to shield the capillary wall [35, 69] or suitable cations to decrease, neutralize or reverse the EOF [67, 75]. The buffer additive equilibrates with the surface of the capillary and alters its properties. The main disadvantage with these additives is a decrease in the detector signal, incompatibility with mass spectrometric detection and interactions with proteins.

Cationic surfactants for instance cetyltrimethylammonium bromide (CTAB) [76] and \(N, N\)-didodecyl \(N, N\)-dimethylammonium bromide (DDAB) [77-79] have been added to the separation buffer to form non-covalently attached wall coatings in CE with a positive zeta potential and thus a reversed EOF from the cathode towards the anode; with increasing modifier concentration the EOF is first suppressed and finally reversed. They can be firmly held in the region of the electric double layer by electrostatic and hydrophobic interactions. Some of the cations, e.g., DDAB, are so strongly adsorbed to the surface that their presence in the BGE during the separation is not needed [49 (III), 66 (IV), 77-79].
Aim of study

Analytical performance

- Rapid, effective and reproducible separations of basic proteins with comparative studies of several parameters of particular importance for the separation performance e.g., different buffers, their pH and ionic strength, field strength and injection procedure.

- Preparation of simple, effective and stable capillary coatings in order to minimize adsorption of the positively charged proteins onto the capillary wall as well as an effective washing procedure in between the runs.

- Increasing the detection sensitivity of the analytes by different zone sharpening procedures.

Zone broadening

- Comparison of separation performances and different parameters such as plate number and resolution in capillary electrophoresis with and without EOF.

- Study in detail the different mechanisms responsible for zone broadening effects both quantitatively and qualitatively.
Experimental

Apparatus
The CE experiments were performed with an Agilent capillary electrophoresis system (Agilent Technologies, Waldborn, Germany), interfaced to an HP Pentium II personal computer. Detection was accomplished on-capillary by recording UV absorbance at a short wavelength (200 nm) for high sensitivity.

Fused silica capillaries (25- and 50- µm i.d. x 365-µm outer diameter (o.d.)) were obtained from Polymicro Technologies (Phoenix, AZ, USA) and MicroQuartz (Munich, Germany). A 0.25-cm section of the polyimide coating was burned off by electrical heating for on-column detection [33]. The total lengths of the capillaries were 41.0 - 48.5 cm (8.5 cm after the detection window). The number of theoretical plates was supplied by the software, based on the statistical moment method.

Coating procedures
Covalent coating (Papers I and II)
The capillaries were coated with polyacrylamide to suppress electroosmosis and adsorption according to a modification of a procedure previously described [69]. In the pre-treatment step the capillaries were washed with 1 M NaOH, 1 M HCl and finally water in order to obtain a fresh and clean inner capillary surface. Following drying with an air stream, the capillaries were filled with a 50% solution of γ-methacryloxypropyltrimethoxysilane in acetone, left for 20 hours and then rinsed with acetone. The polymerization was carried out with 150 µl of acrylamide, 3 µl of ammonium persulfate and 3 µl of TEMED, all three components being 5% aqueous solutions (v/v for TEMED and w/v for the other two compounds). Non-covalently attached polymer was removed after 20 hours simply by rinsing with water by an HPLC pump. The quality of the coating was tested with acetone (10% aqueous solution injected for 6 s at 50 mbar) as a neutral electroosmotic marker, detected at 280 nm; the coating was considered efficient at electroosmotic mobility less than 10^{-6} cm^{2}/V.s.
Non-covalent coating (Papers III and IV)

Fresh capillaries were used for each buffer system to avoid memory effects. Previous to the coating the new capillaries were rinsed by high-pressure (935 mbar) with 1 M NaOH, 0.1 M NaOH, and water for 20 minutes, respectively, to produce a surface capable to bind the surfactant homogeneously.

**Coating method 1 (Paper III):**

The non-covalent coating was created by a high-pressure rinse (5 min) with DDAB dissolved in water (at a concentration of 0.1 mM) followed by a high-pressure rinse (1 min) with the BGE in order to flush out the excess surfactant. [49 (III)].

**Coating method 2 (Paper IV):**

This modified coating procedure was a combination of rinse (5 min at 935 mbar) with DDAB and an equilibration step (5 minutes) with the surfactant remaining in the capillary, repeated three times, and finally a high-pressure rinse with the BGE for 3 minutes and application of voltage for equilibration in 10 minutes [66].

The neutral marker mesityloxide (5µl in 2 ml of water) was injected into the capillary (50 mbar for 6 s) in order to calculate the EOF and, thereby, get some information about the quality of the coating in both cases. The electroosmotic mobility was calculated according to:

\[
\mu_{\text{EOF}} = \frac{L_d L_t}{t_m V}
\]

where \(L_d\) is the length of the capillary to the detector, \(L_t\) the total length of the capillary, \(t_m\) the migration time of mesityloxide and \(V\) the applied voltage. Detection of mesityloxide was accomplished on-capillary by UV-monitoring at 254 nm.

**Injection**

In capillaries coated with PAA the proteins were introduced electrokinetically in 10- to 1000-fold diluted BGE. The most repeatable separations were achieved with low voltage and long injection time (1 kV for 16 s).

In DDAB-coated capillaries the proteins were injected hydrodynamically dissolved in 10-fold diluted BGE with 50 mbar and varying injection times (6-120 s).
Results and discussions

Paper I & Paper II

The influence of ignored and well-known zone distortions on the separation performance of proteins in capillary free zone electrophoresis with special reference to analysis in polyacrylamide-coated fused silica capillaries in various buffers

Paper I. Theoretical studies

Paper II. Experimental studies at acidic pH with on-line enrichment

Analytical performance
The separation of acidic and basic proteins was studied in a polyacrylamide-coated, EOF free capillary at pH below their isoelectric points (pI) with UV detection at 200 nm. Buffers with various pH and ionic strengths were used which gave rise to different separation performance with regards to efficiency and resolution.

Ammonium acetate (0.12 M) and ammonium hydroxyacetate (0.15 M) both at pH 4 provided the best separations with plate numbers up to 1 700 000 plate/m (Fig. 5) corresponding to a zone width (2σ) of only 1 mm when injecting the proteins in 10-fold diluted BGE. The experimental value is rather close to the theoretical maximum number (Nmax), which was 2 660 000 (assuming that diffusion is the only source of zone broadening). The separation performance with buffers at pH 3 (e.g., phosphate buffer) was not satisfactory and loss of proteins was observed because of possible denaturation and conformation changes of the proteins. Application of more dilute BGE in the sample zone (up to 1000-fold) decreased the efficiency, partly due to conductivity and/or pH difference between the sample and the buffer zone giving rise to hyper-sharp peaks (when the conductivity difference is so large that one boundary of the sample zone is sharp (90º relative to the baseline) during the run whereas the other boundary becomes successively broader, i.e., the diffusional broadening at one boundary is not counteracted by the zone sharpening caused by the conductivity difference).
The enrichment effects were not as the theory predicted [Eq. 1 in Ref. 52 (II)], and there was no or only a small increase in peak heights or even a small decrease when the dilution factor exceeded 200 (Fig. 11 d-e in Ref. 52 (II)) probably due to vertical sedimentation of the highly concentrated sample zone and possible thermal expansion as a result of the low conductivity, which in both cases results in a loss of sample. Moreover, upon high dilution of the sample zone the inevitable changes in pH may decrease the mobility of the proteins and, thus, cause the enrichment factor to become still lower than expected.

Fig. 5. Separation of four proteins in PAA coated capillaries. BGE: 0.12 M ammonium acetate at pH 4. The proteins: (1) cytochrome c, (2) lysozyme, (3) ribonuclease A and (4) α-chymotrypsinogen A were introduced in ten times diluted BGE. Electrophoretic sample application: 1 kV for 16 s.

The concentration limits of detection were in the range 4-40 pmol/ml for the ammonium acetate and ammonium hydroxyacetate when the proteins were dissolved in 100- and 200-fold diluted BGE, respectively.

Zone broadening

Different mechanisms responsible for zone broadening were studied in detail. The variance for longitudinal diffusion was larger than all other calculated variances. Other significant variances were contributions from capillary curvature effect (1.6-5.6%) and injection plug (0.5-13%) while the variance for Joule heat was negligible (0.02-0.5%). The variances due to detector time response (0.1 s for these experiments), which were not taken into consideration in papers I and II [Refs. 50 (I), 52 (II)] were by calculations made after the publication also found to be significant (1.9-2.3%). However, in most cases, the main contribution to the total zone broadening was the rest variance, i.e., the difference between the experimentally determined total variance and the sum of all other calculated variances. Since the efficiencies increased with increasing field strength
(Fig. 4 in Ref. 52 (II)), the rest variance was probably not caused by adsorption of the proteins onto the capillary wall, but may have its origin in relatively slow interactions between proteins and the buffer constituents as well as hyper-sharp peaks at high dilution of the sample. The rest variance increased strongly with the mobility of the proteins, which is an indication of protein-buffer interactions.

Paper III

Easy applicable, non-covalent coating with \( N, N\)-didodecyl \(-N, N\)-dimethylammonium bromide (DDAB) for separation of basic proteins by capillary electrophoresis in acidic buffers in 25- and 50-\( \mu \)m capillaries

Studies on zone broadening and analytical performance

Analytical performance

Capillaries (25- and 50-\( \mu \)m i.d.) were coated with DDAB [Fig. 6] and were used for separation of four basic standard proteins [Table 1 in Ref. 49 (III)] in buffers with varying ionic strengths at pH 4.

\[
\begin{align*}
\text{Figure 6. } N, N\text{-didodecyl-}N, N\text{-dimethylammonium bromide (DDAB).}
\end{align*}
\]

This coating has the advantage that the surfactant is not present (or at an extremely low concentration) in the running buffer, which probably makes it compatible with MS detection. In contrast to single-chained surfactants, which form spherical micelles, double-chained DDAB aggregate in an aqueous solution and form vesicles at concentration above the critical vesicle concentration (CVC: 0.035 mM in water), which then adsorb to the capillary surface and form a flat bilayer structure (Fig. 7 [77]).
The excess of positive surface charges gave a strong electroosmotic flow towards the anode (anodic or reversed EOF) of opposite direction compared to bare fused-silica and permitted rapid separations [Fig. 9 in Ref. 49 (III)]. The proteins were dissolved in 10-fold diluted BGE and injected hydrodynamically; the number of plates decreased 40% and 65% when the proteins were introduced in 100-fold diluted BGE and in water, respectively. A probable reason is rotation of the sample zone caused by local differences in EOF with an attendant broadening of the starting zone [80]. Additionally, adsorption of the proteins onto the capillary surface due to lower ionic strength might also contribute to the decrease in the plate numbers.
Choice of buffer component, ionic strength and pH were critical for high plate numbers; for instance in 0.1 M phosphate buffer at pH 4 true efficiencies up to 600 000 per m ($N_{\text{max}}$: 5 million per m) were obtained in 25 µm capillaries (apparent plate numbers up to 4 million/m corresponding to $N_{\text{max}}$ up to 7 million/m) (Tables 5 and 6 and Fig. 6a in Ref. 49 (III)). Other buffers, which gave satisfactory results after optimization, were 0.07 M ammonium acetate and 0.08 M ammonium hydroxyacetate both at pH 4 (Fig. 8).

Protein/phosphate interaction

Exceptionally high efficiencies were obtained with phosphate buffer in the 25-µm capillary (Tables 5 and 6 in Ref. 49 (III)) and the true mobilities of the proteins were lower in this buffer. The efficiencies decreased significantly with decreasing ionic strength (Fig. 5 in Ref. 49 (III)). There is, with evidence from several reports in the literature [81-85], indication of association between proteins and phosphate ions. The hypothesis is that the protein-phosphate complex, which has a lower net charge, adsorbs to the capillary surface to a lower degree than the protein due to less electrostatic attractions to the capillary wall. Such a mechanism will obviously give less zone broadening.

Paper IV

**Improved coating properties with $N, N$-didodecyl - $N, N$-dimethylammonium bromide for separation of basic proteins by capillary electrophoresis in acidic buffers in 25-µm capillaries**

A modified non-covalent coating with DDAB was used for separation of standard basic proteins in CE in 25 µm capillaries. Improved coating stability and repeatability was achieved compared to previous studies [49 (III)] and adsorption of the proteins onto the capillary surface was more inhibited as a result of more efficient covering of the silanol groups. The procedure involved repeated treatments with DDAB incorporating equilibration steps. It is probable that this action gives adequate time for the DDAB molecules to diffuse into the capillary surface to give a more complete coverage. As a result, the coated capillary allowed separation of proteins up to 9 hours (90 injections) without recoating in between the runs with a good precision in migration time and area under the curves (AUC) (Fig. 9).

In order to improve the limited concentration sensitivity different procedures were studied. The simplest strategy was to optimize the injection
parameters and thus increasing loading capacity in order to have the highest sensitivity with preserved high efficiency and resolution (Fig. 3 in Ref. 66 (IV)). The separation method was rather robust and allowed a 5-fold increase of the injection time (from 6 to 30 seconds with the same hydrodynamic pressure, 50 mbar) with an improved peak area repeatability compared to previous studies [49 (III)].

![Fig. 9. Separation of four basic proteins in DDAB coated capillaries with no recoating between the runs. A) first separation B) after 1 hour C) after 5 hours D) after 9 hours. Sample: 1) α-chymotrypsinogen, 2) ribonuclease A, 3) lysozyme and 4) cytochrome c, dissolved in ten times diluted BGE and injected by pressure (50 mbar for 6 seconds).]

Exceptionally high apparent and true efficiencies were obtained with phosphate buffer in the 25-µm capillaries in previous studies [49 (III)] probably due to a complex formation between the positively charged proteins and the negatively charged phosphate ions.

The mobilities of the proteins decreased with increasing ionic strength of the phosphate buffer (Fig. 7 in Ref. 66 (IV)), which further supports the occurrence of selective interaction of the buffer counter-ions with the analytes. The mobilities reached a plateau at ionic strengths ≥ 0.1 M indicating the saturation of the binding sites at these concentrations.

The effect of increased temperature on the coating stability and a possible effect on protein-phosphate ion interaction were investigated (Fig. 9 in Ref. 66 (IV)). Capillaries were heated to 35°C and the separation performance...
was compared to non-heated ones (room temperature 25°C). No significant changes in performance regarding efficiency and peak area were observed which indicates a coating with high stability.
Conclusions

The separation of basic proteins was studied in capillary free zone electrophoresis with and without EOF in buffers of different compositions, concentrations and pH to establish optimum conditions. To achieve high efficiency and resolution the choice of buffer constituents was extremely important.

The **EOF-free polyacrylamide coating** was very stable at neutral and acidic conditions; washing of the capillary with 2 M HCL increased the repeatability of migration times and peak areas.

The buffers of choice were 0.12 M ammonium acetate and 0.15 M ammonium hydroxyacetate at pH 4 with plate numbers up to 1,700,000 plates/m, corresponding to a zone width \((2\sigma)\) of only 1 mm. The separation performance at pH 3 studied with several different buffers was not satisfactory and loss of proteins was observed probably due to denaturation and precipitation.

A simple zone sharpening procedure by dissolving the analytes in 10- to 1000-fold diluted BGE was studied. Efficient zone sharpening was achieved with dilution factors as high as 100 -200, improving the sensitivity and giving limits of detection at 200 nm in the order of 4-8 pmoles/ml of proteins for ammonium acetate and 12-41 pmoles/ml for ammonium hydroxyacetate. However, a 10-fold dilution of the running buffer gave the highest plate numbers most likely because hyper-sharp peaks were obtained at high dilutions of the sample for which conductivity and/or pH effects cause significant zone broadening. The results were not reproducible at the highest dilution factors, 500–1000 times, and there was no or only a small increase in peak heights or even a small decrease when the dilution factor exceeded 200 (Fig. 11 d-e in Ref. 52 (II)). This is probably due to vertical sedimentation of the concentrated sample and lower mobilities of the proteins because of the higher pH in the sample zone and possibly thermal expansion effects owing to the lower conductivity.

All other kinds of zone broadenings besides those considered in table 1 in Ref. 52 (II) have together a variance (rest variance), which was in the most cases the largest contribution to the total variance. The rest variance probably refer to protein-buffer ion interactions, sedimentation in the horizontal section of the capillary (Fig. 2b in Ref. 52 (II)) and zone distortions caused by so large conductivity and pH differences between a
protein zone and surrounding buffer that one boundary is hyper-sharp. Protein-capillary wall interactions seem to be negligible.

The DDAB coating (Paper III) in 25- and 50-µm capillaries was stable enough to, without recoating, permit consecutive separations of the proteins up to 100 minutes with good precisions in migration times (0.9-2.9% and 0.2-1.4% for 25- and 50-µm capillaries, respectively) and peak areas (3.0-8.1% and 1.0-5.0% for 25- and 50-µm capillaries, respectively).

Introducing the proteins in 10-fold diluted BGE gave rise to good repeatability and adequate apparent resolutions and excellent apparent efficiencies at pH 4.0 in some volatile buffers compatible with mass spectrometry detection, such as 0.07 M ammonium acetate, 0.08 M ammonium hydroxyacetate and in a non-volatile buffer, 0.1 M sodium phosphate with limits of detection in the order of 2, 14 and 4 µM and 100, 200 and 600 nM in 25- and 50-µm capillaries, respectively.

The dominating contributions were from the rest variances, diffusion (3.5-14.5%), capillary coiling (0.1-1.3%), detector time response (1-3%), injection (0.1-1.2%) and detector slit width (1.2-2.0% for 50 µm capillaries) while all other variances being negligible (Table 8 in Ref. 49 (III)). Experimental evidence indicated that the main contribution was due to slow interactions between proteins and buffer components and adsorption of the proteins onto the capillary wall caused by inadequate coating. An incomplete surface coverage of DDAB and, thus, less packed bilayer, had apparently resulted in a capillary surface that still exposed many free silanol groups.

Apparent and true efficiencies corresponding to efficiencies in capillaries with and without EOF were calculated. The presence of strong electroosmosis in most cases gives a high apparent efficiency (related to the speed of migration) not accompanied by corresponding improvements in resolution.

Apparent efficiencies in excess of 4,000,000 plates per meter were obtained with sodium phosphate in 25-µm capillaries, while the other buffers gave about 1,000,000 apparent plates per meter. A hypothesis is that this difference may be due to the formation of a protein-dihydrogenphosphate complex, which is adsorbed to a smaller degree onto the capillary surface than the free proteins.

After an improved coating procedure (Paper IV) the DDAB coating was stable enough to, without recoating, permit consecutive separations of the proteins up to 9 hours (90 injections) with good precisions in peak areas (RSD = 1.1%) and migration times and with high apparent efficiencies (over 1 million theoretical plates per m). The optimized coating method gave more stable capillaries that could withstand a temperature rise at least of 10°C. The adsorption of the proteins onto the capillary surface was minimized and slow protein-buffer component interactions seemed to be the main contributions to zone broadening.
The complex formation of the proteins with phosphate buffer was further explored. In a set of experiments the protein mobilities decreased with increasing ionic strength of the buffer, which is a further verification of the presumed complex formation. The mobilities became constant at ionic strengths \( \geq 0.1 \) M indicating a saturation of the binding sites at these concentrations.

The advantage with this type of coating with DDAB is its simplicity in preparation. The reproducible EOF measurements indicate a high stability of this wall coating, which in the absence of an interfering additive to the BGE probably will permit mass spectrometric detection of proteins.

Future studies

Future research is needed to further investigate important issues regarding zone broadening and analytical performance:

- Development of theoretical and experimental tools to secure the mechanism behind the large rest variances obtained with both PAA- and DDAB-coated capillaries.
- Improving the detection limits by the development of reliable enrichment procedures at sample introduction.
- Coupling both types of systems to mass spectrometry for the development of methods for purity and stability studies of proteins.
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References

A doctoral dissertation from the Faculty of Pharmacy, Uppsala University, is usually a summary of a number of papers. A few copies of the complete dissertation are kept at major Swedish research libraries, while the summary alone is distributed internationally through the series Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Pharmacy. (Prior to January, 2005, the series was published under the title “Comprehensive Summaries of Uppsala Dissertations from the Faculty of Pharmacy”.)