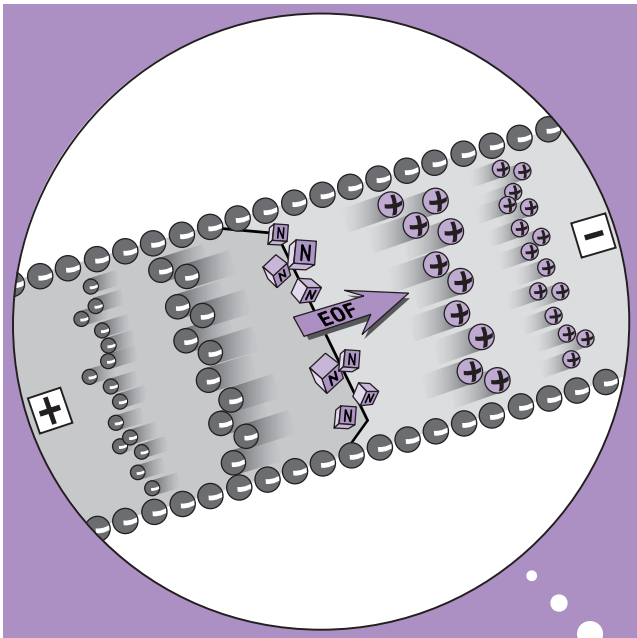


An introduction

# High performance capillary electrophoresis

Agilent Technologies
Innovating the HP Way

A primer

# High performance capillary electrophoresis

An introduction

David Heiger

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# Foreword

Capillary electrophoresis (CE) was born of the marriage of the powerful separation mechanisms of electrophoresis with the instrumentation and automation concepts of chromatography. The early phases of its evolution were mostly concerned with determining its characteristics and learning about some of the inherent capabilities of the technique. It has now entered its second decade of development. Today CE is a technique with much promise, but it is still in a somewhat immature state, especially compared to the older and more established methods of gas chromatography, liquid chromatography, and conventional gel electrophoresis. Those searching for "canned" or "off-the-shelf" solutions to problems may find the literature somewhat sparse. The number of publications concerning application of CE to practical problems is growing at an explosive rate, but is still small in volume when compared to the older and more established separation methods.

If you are coming to CE from a background of chromatography, working in CE does require mastering some new concepts and terminology, such as electrophoretic mobility and electroosmosis. If your background is in conventional gel elctrophoresis, you will be faced with a more "instrumental" approach to electrophoresis; one which includes autosamplers, on-line/real-time detection, and direct computer interfacing for experimental control and data acquisition. It may be reassuring to know that one comment repeatedly made by first time CE users is how simple CE actually is, and how quickly one can get useful results.

Today there are many modes of CE to help solve problems. These include free zone electrophoresis, gels for sieving and molecular weight based separations, isoelectric focusing, and isotachophoresis. Thanks to the clever ideas of Professor Terabe, one can even do separations using charged micelle "pseudophases" (micellar electrokinetic chromatography, or MEKC), where a molecule's hydrophobicity can play a role in the separation, and even neutral molecules can be resolved in an electric field. The field of

CE is characterized by imaginative research into new separation mechanisms and new detection principles. In fact, the lowest detection limits attained in the whole field of separations are for CE with laser induced fluorescence detection, where researchers are on the verge of single molecule detection. The prospects of extraordinary separation power, speed of analysis, and extreme sensitivity are driving intensive research on CE by many of the best analytical research laboratories around the world.

The future of CE is difficult to anticipate. As the number of practitioners continues to grow, our knowledge base will increase, as will our level of comfort in knowing its strengths as well as its limitations. We will have many "recipes" at our fingertips for successful approaches to problems. None-the-less, the field will be exciting for many years to come. CE is likely to be a principal tool in DNA sequencing, and due to its great speed, may well play a crucial role in sequencing the human genome. The successes achieved so far in interfacing CE with mass spectrometers promise remarkable capabilities for unraveling the structures of complex macromolecules with unsurpassed speed, accuracy, and sensitivity. CE-MS may offer us the possibility of sequencing proteins in a matter of days. As new detection methods and reagents are refined, CE will revolutionize our knowledge of substances such as carbohydrate polymers, where methodology has long hindered our understanding of the role of an important class of molecules. And we should not forget the extremely small sample requirement of CE, which makes it attractive as a tool for exploring the chemistry of small places, a prime example of which is the single cell. There is one thing that I am certain about; CE will provide us with many exciting surprises in the years ahead.

James W. Jorgenson
Chapel Hill, NC, USA
November 25, 1992

Table of content

# Scope

The purpose of this book is to introduce the technique of high performance capillary electrophoresis and provide to enable users information to apply it to specific applications. This is accomplished through a review of the fundamental concepts, with emphasis on basic theory, modes of operation, and instrumental considerations. Major application areas with relevant examples and a basic literature survey are also included.

# Chapter 1

# Introduction

## 1.1 High performance capillary electrophoresis (CE) – an overview

Separation by electrophoresis is obtained by differential migration of solutes in an electric field. In CE, electrophoresis is performed in narrow-bore capillaries, typically 25- to 75- µm inner diameter (id), which are usually filled only with buffer. Use of the capillary has numerous advantages, particularly with respect to the detrimental effects of Joule heating. The high electrical resistance of the capillary enables the application of very high electrical fields (100 to 500 V/cm) with only minimal heat generation. Moreover, the large surface area-to-volume ratio of the capillary efficiently dissipates the heat that is generated. The use of the high electrical fields results in short analysis times and high efficiency and resolution. Peak efficiency, often in excess of $10^5$ theoretical plates, is due in part to the plug profile of the electroosmotic flow, an electrophoretic phenomenon that generates the bulk flow of solution within the capillary. This flow also enables the simultaneous analysis of all solutes, regardless of charge. In addition the numerous separation modes which offer different separation mechanisms and selectivities, minimal sample volume requirements (1 to 10 nl), on-capillary detection, and the potential for quantitative analysis and automation, CE is rapidly becoming a premier separation technique.

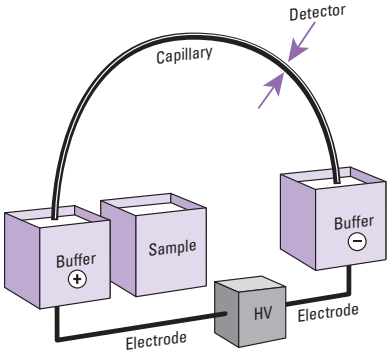



**Figure 1**
**Schematic of CE instrumentation**

One key feature of CE is the overall simplicity of the instrumentation. A schematic diagram of a generic capillary electrophoresis system is shown in figure 1. Briefly, the ends of a narrow-bore, fused silica capillary are placed in buffer reservoirs. The content of the reservoirs is identical to that within the capillary. The reservoirs also contain the electrodes used to make electrical contact between the high voltage power supply and capillary. Sample is loaded onto the capillary by replacing one of the reservoirs (usually at the anode) with a sample reservoir and applying either an electric field or an external pressure. After replacing the buffer reservoir, the electric field is applied and the separation performed. Optical detection can be made at the opposite end, directly through the capillary wall. The theoretical and practical details regarding injection,

separation, detection, quantitative analysis, equipment and automation, and so on, are discussed in this book.

## 1.2 Current state of development

CE is a rapidly growing separation technique. One of the greatest advantages is its diverse application range. Originally considered primarily for the analysis of biological macromolecules, it has proved useful for separations of compounds such as amino acids, chiral drugs, vitamins, pesticides, inorganic ions, organic acids, dyes, surfactants, peptides and proteins, carbohydrates, oligonucleotides and DNA restriction fragments, and even whole cells and virus particles.

The mechanisms responsible for separation in CE are different from those in chromatography, and thus can offer orthogonal, complementary analyses. In addition, CE may offer simpler method development, minimal sample volume requirements, and lack of organic waste.

While numerous advances are being made in CE, the technique is still in a development and growth stage. The number of publications per year on CE has risen from about 90 in 1983, to about 140 in 1987, to more than 300 in 1991. Incumbent with new technology is a lag time between published results generated by researchers developing the technique and the formation of a workable knowledge-base for the user. Scientists should be aware that CE is not totally mature, relative to HPLC for example. Both development of the theory and its application to separation problems are still somewhat incomplete. This implies, for example, that methods may need to be developed and optimized for each application by the user.

Significant advances, however, have already been achieved in the past few years which have begun to standardize the use of CE. These include improvements in migration time and peak area reproducibility as well as quantitative analy-

sis, methods for on-capillary sample preconcentration to improve sensitivity, and development of capillary coatings to both control electroosmotic flow and limit solute-wall interactions. Undoubtedly, as more researchers use CE and disseminate results, it will become a standard technique in separation science.

## 1.3 Characteristics of CE

- Electrophoresis is performed in narrow-bore (25- to 75-μm id), fused silica capillaries
- High voltages (10 to 30 kV) and high electric fields (100 to 500 V/cm) are applied across the capillary
- High resistance of the capillary limits current generation and internal heating
- High efficiency ($N>10^5$ to $10^6$) and short analysis time
- Detection performed on-capillary (no external detection cell)
- Small sample volume required (1 to 50 nl injected)
- Numerous modes to vary selectivity and wide application range
- Operates in aqueous media
- Simple methods development
- Automated instrumentation

Chapter 2

# Principles of capillary electrophoresis

## 2.1 Historical background and development

Electrophoresis has been defined as the differential movement of charged species (ions) by attraction or repulsion in an electric field. Electrophoresis as a separation technique was introduced by Tiselius in 1937. Placing protein mixtures between buffer solutions in a tube and applying an electric field, he found that sample components migrated in a direction and at a rate determined by their charge and mobility. For his work in separation science Tiselius was awarded a Nobel Prize.

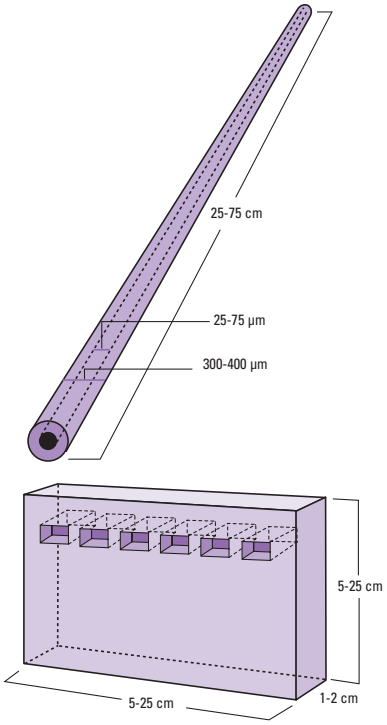



**Figure 2**
**Comparison of gel used for slab electrophoresis and capillary for HPCE**

Separation efficiency in free solution, as performed by Tiselius, was limited by thermal diffusion and convection. For this reason, electrophoresis traditionally has been performed in anti-convective media, such as polyacrylamide or agarose gels. Gels in the slab or tube format have been used primarily for the size-dependent separation of biological macromolecules, such as nucleic acids and proteins. Although it is one of the most widely used separation techniques, slab gel electrophoresis generally suffers from long analysis times, low efficiencies, and difficulties in detection and automation.

An alternative to the slab-format is to perform the electrophoretic separation in narrow-bore tubes or capillaries (figure 2). Since narrow capillaries are themselves anti-convective, gel media are not essential to perform that function. This allows the performance of free-solution (or open tube) electrophoresis, as well as the use of traditional gel media in the capillary.

Initial work in open tube electrophoresis was described by Hjérten in 1967. At that time, since only millimeter-bore capillaries were available, Hjérten rotated them along their longitudinal axis to minimize the effects of convection. Later Virtanen and then Mikkers performed electrophoresis in approximately 200-µm internal diameter (id) capillaries made from glass and Teflon, respectively. In the early 1980s Jorgenson and Lukacs advanced the technique by using

75-µm id fused silica capillaries. Jorgenson also clarified the theory, described the relationships between operational parameters and separation quality, and demonstrated the potential of high performance capillary electrophoresis (CE) as an analytical technique. Since that time, numerous reviews and a few books have been written describing various aspects of CE (see Bibliography).

## 2.2 High performance capillary electrophoresis (CE)

CE can be considered an instrumental approach to electrophoresis. In many ways the improvements in performance resulting from using capillaries instead of slab gels are analogous to those attained by performing chromatography in the column rather than the flat-bed format. Further, the mechanisms of separation are greatly extended in the CE-format, thus extending the application range of electrophoresis. To this end, electrophoresis is no longer limited to separation of macromolecules and can also be used to separate cations, anions, and neutrals in a single analysis.

## 2.3 Theory

### 2.3.1 Electrophoresis

Separation by electrophoresis is based on differences in solute velocity in an electric field. The velocity of an ion can be given by

$$\mathbf{v} = \mu_e \mathbf{E} \qquad (1)$$

where
- $v$ = ion velocity
- $\mu_e$ = electrophoretic mobility
- $E$ = applied electric field

The electric field is simply a function of the applied voltage and capillary length (in volts/cm). The mobility, for a given ion and medium, is a constant which is characteristic of

that ion. The mobility is determined by the electric force that the molecule experiences, balanced by its frictional drag through the medium. That is

$$\mu_e \propto = \frac{\text{Electric force } (F_E)}{\text{Frictional force } (F_F)} \quad (2)$$

The electric force can be given by

$$F_E = q\,E \quad (3)$$

and the frictional force (for a spherical ion) by

$$F_F = -6\,\pi\,\eta\,r\,v \quad (4)$$

where q = ion charge
$\eta$ = solution viscosity
r = ion radius
v = ion velocity.

During electrophoresis a steady state, defined by the balance of these forces, is attained. At this point the forces are equal but opposite and

$$q\,E = 6\,\pi\,\eta\,r\,v \quad (5)$$

Solving for velocity and substituting equation (5) into equation (1) yields an equation that describes the mobility in terms of physical parameters

$$\mu_e = \frac{q}{6\,\pi\,\eta\,r} \quad (6)$$

From this equation it is evident that small, highly charged species have high mobilities whereas large, minimally charged species have low mobilities.

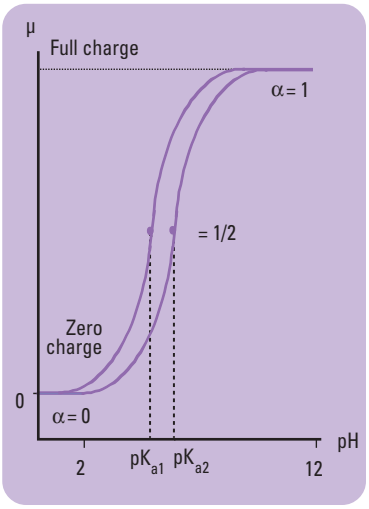



**Figure 3**
**Mobility of two weak acids as a function of pH**

The electrophoretic mobility usually found in standard tables is a physical constant, determined at the point of full solute charge and extrapolated to infinite dilution. This usually differs from that determined experimentally. The latter is called the effective mobility and is often highly dependent on pH (that is, solute $pK_a$) and composition of the running buffer.

The differences between the absolute and effective mobilities are demonstrated in figure 3. Here, two hypothetical solutes are shown to possess the same electrophoretic mobility at full charge. From a mobility table, these solutes would appear to be inseparable since there would be no differential migration. However, these species have different $pK_a$ values and different mobilities depending on their pH-controlled charge.

## 2.3.2 Electro-osmotic flow (EOF)

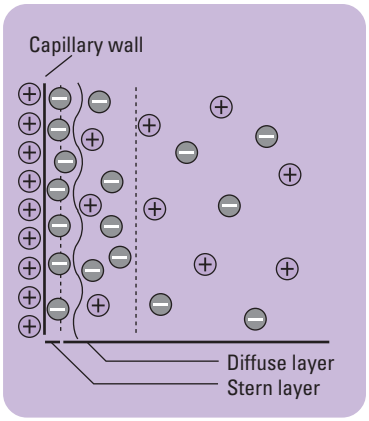



**Figure 4**
**Representation of the double-layer at the capillary wall[1]**

A fundamental constituent of CE operation is electro-osmotic, or electroendosmotic flow (EOF). EOF is the bulk flow of liquid in the capillary and is a consequence of the surface charge on the interior capillary wall. The EOF results from the effect of the applied electric field on the solution double-layer at the wall (figure 4). The EOF controls the amount of time solutes remain in the capillary by superposition of flow on to solute mobility. This can have the effect of altering the required capillary length, but does not affect selectivity.

Under aqueous conditions most solid surfaces possess an excess of negative charges. This can result from ionization of the surface (that is, acid-base equilibria) and/or from adsorption of ionic species at the surface. For fused silica both processes probably occur, although the EOF is most strongly controlled by the numerous silanol groups (SiOH) that can exist in anionic form ($SiO^-$) (figure 5a). Although

the exact pI of fused silica is difficult to determine, EOF becomes significant above pH4. Nonionic materials such as Teflon also exhibit EOF, presumably resulting from adsorption of anions.

Counterions (cations, in most cases), which build up near the surface to maintain charge balance, form the double-layer and create a potential difference very close to the wall (figure 5b). This is known as the zeta potential. When the voltage is applied across the capillary the cations forming the diffuse double-layer are attracted toward the cathode. Because they are solvated their movement drags the bulk solution in the capillary toward the cathode. This process is shown in schematic form in figure 5c.

The magnitude of the EOF can be expressed in terms of velocity or mobility by

$$\mathbf{v_{EOF} = (\varepsilon\, \zeta / \eta)\, E} \qquad (7)$$

or

$$\mu_{EOF} = (\varepsilon\, \zeta / \eta) \qquad (8)$$

where:
- $v_{EOF}$ = velocity
- $\mu_{EOF}$ = EOF "mobility"
- $\zeta$ = zeta potential
- $\varepsilon$ = dielectric constant.

(note the independence of mobility on applied electric field)

The zeta potential is essentially determined by the surface charge on the capillary wall. Since this charge is strongly pH dependent, the magnitude of the EOF varies with pH. At high pH, where the silanol groups are predominantly deprotonated, the EOF is significantly greater than at low pH where they become protonated. Depending on the

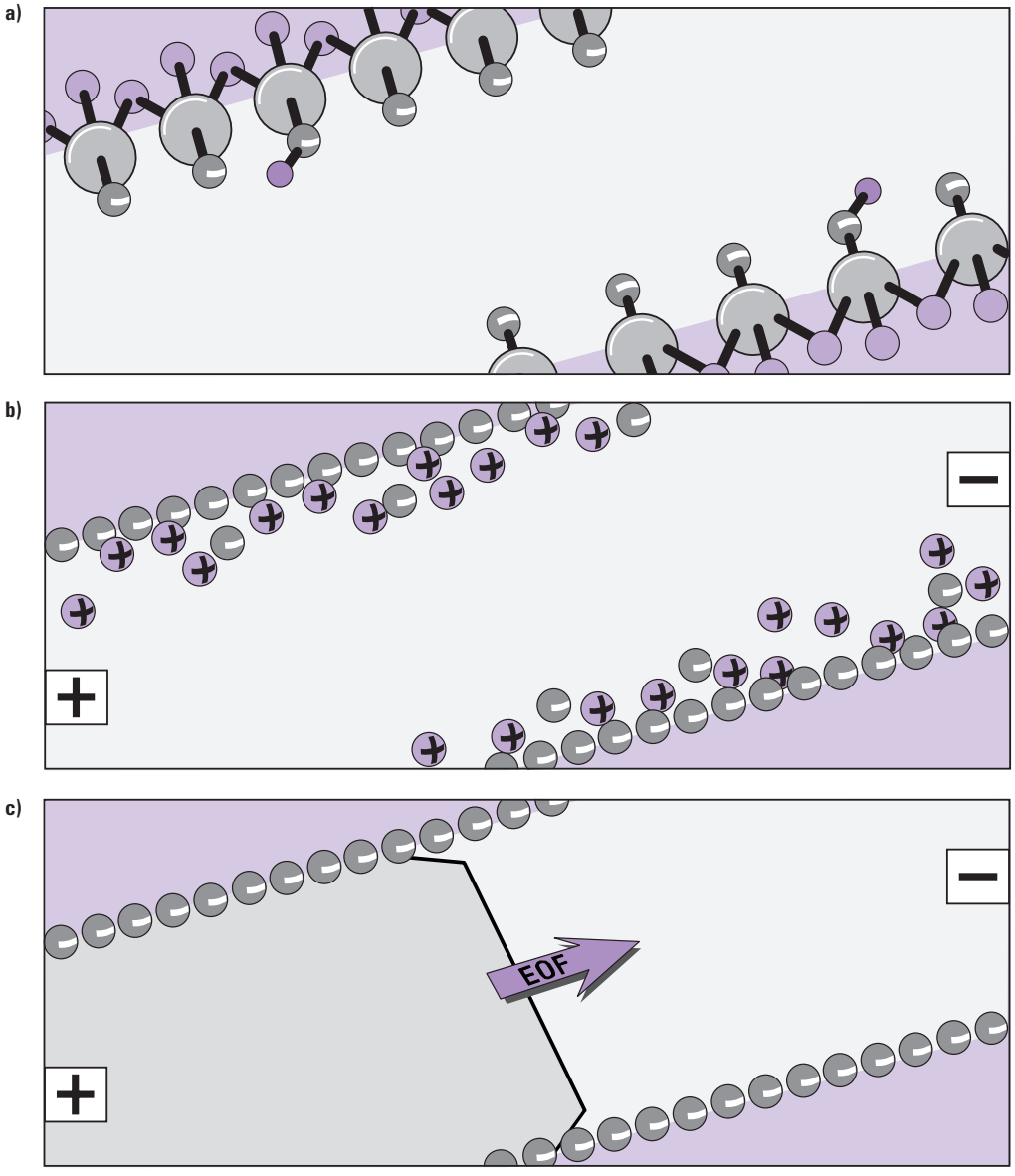



**Figure 5**
**Development of the electro-osmotic flow:**
**a) negatively charged fused silica surface ($Si\text{-}O^-$)**
**b) hydrated cations accumulating near surface**
**c) bulk flow towards the cathode upon application of electric field**

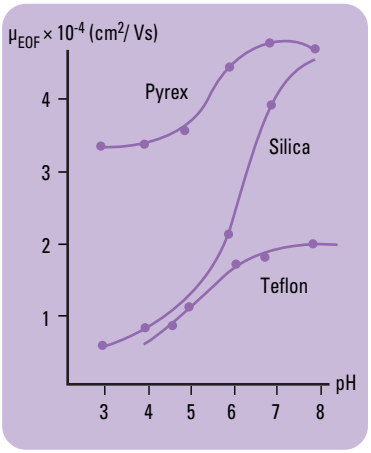



**Figure 6**
**Effect of pH on electro-osmotic flow mobility in various capillary materials[2]**

specific conditions, the EOF can vary by more than an order of magnitude between pH 2 and 12. Figure 6 illustrates this effect for fused silica and other materials.

The zeta potential is also dependent on the ionic strength of the buffer, as described by double-layer theory. Increased ionic strength results in double-layer compression, decreased zeta potential, and reduced EOF (see figure 9).

A unique feature of EOF in the capillary is the flat profile of the flow, as depicted in figure 5c. Since the driving force of the flow is uniformly distributed along the capillary (that is, at the walls) there is no pressure drop within the capillary, and the flow is nearly uniform throughout. The flat flow profile is beneficial since it does not directly contribute to the dispersion of solute zones. This is in contrast to that generated by an external pump which yields a laminar or parabolic flow due to the shear force at the wall (figure 7). Figure 7a shows that the flow rate drops off rapidly at the

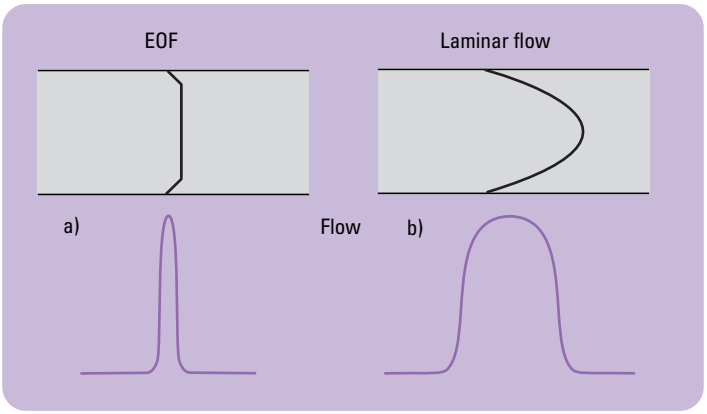



**Figure 7a, b**
**Flow profile and corresponding solute zone**

wall. This quiescent solution layer is caused by friction against flow at the surface. Since this layer extends a short way into the solution, it is relatively unimportant to the overall separation process (that is, other dispersive processes dominate). Further, the flow rate and profile are generally independent of capillary diameter. The profile will

be disrupted, however, if the capillary internal diameter is too wide (≥200 to 300 µm). In this case, surface tension becomes insufficient to uniformly drag the center portion of the liquid at the velocity generated at the walls. Details of the effect of the flow profile on peak shape are given in sections *2.3.4 Dispersion* and *2.3.4.1 Factors Affecting Efficiency*.

Another benefit of the EOF is that it causes movement of nearly all species, regardless of charge, in the same direction. Under normal conditions (that is, negatively charged capillary surface), the flow is from the anode to the cathode. Anions will be flushed towards the cathode since the magnitude of the flow can be more than an order of magnitude greater than their electrophoretic mobilities. Thus cations, neutrals, and anions can be electrophoresed in a single run since they all "migrate" in the same direction. This process is depicted in figure 8. Here, cations migrate fastest since the electrophoretic attraction towards the cathode and the EOF are in the same direction, neutrals are all carried at the velocity of the EOF but are not separated from each other, and anions migrate slowest since they are attracted to the anode but are still carried by the EOF toward the cathode.

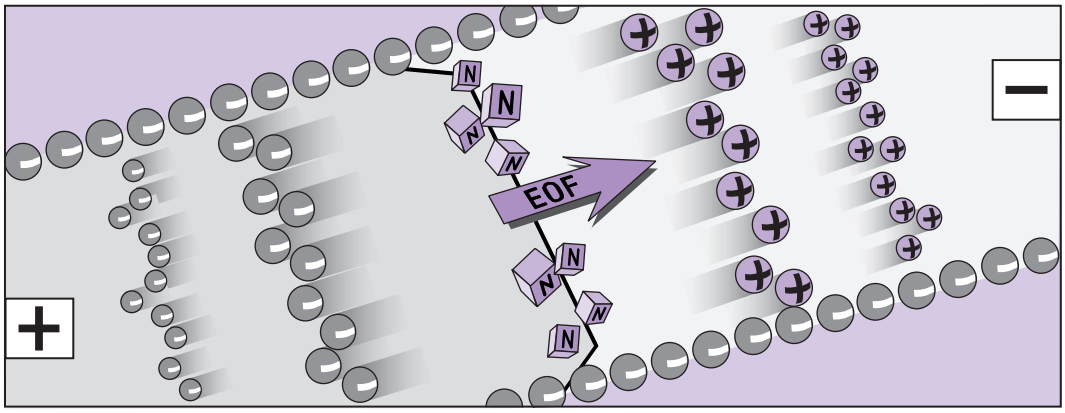



**Figure 8**
**Differential solute migration superimposed on electro-osmotic flow in capillary zone electrophoresis**

For the analysis of small ions (for example, sodium, potassium, chloride) the magnitude of the EOF is usually not greater than the solute mobilities. In addition, modification of capillary wall charge can decrease EOF while leaving solute mobility unaffected. In these circumstances, anions and cations can migrate in opposite directions.

### 2.3.2.1 EOF control

While the EOF is usually beneficial, it often needs to be controlled. At high pH, for example, the EOF may be too rapid, resulting in elution of solute before separation has occurred. Conversely, at low or moderate pH, the negatively charged wall can cause adsorption of cationic solutes through coulombic interactions. This latter phenomenon has been especially problematic for basic protein separations. In addition, electrophoretic separation modes such as isoelectric focusing, isotachophoresis, and capillary gel electrophoresis often require reduction of EOF.

Fundamentally, control of EOF requires alteration of the capillary surface charge or buffer viscosity. There are several methods to accomplish this, as detailed in table 1 and in the following paragraphs. Note that conditions that affect the surface charge of the wall often affect the solute (such as buffer pH). Successful separations are usually obtained when the conditions optimize both EOF and solute mobility properties.

The rate of EOF can most easily be decreased by lowering the electric field, as described by equation (7). This action, however, has numerous disadvantages with regard to analysis time, efficiency, and resolution. From a practical point of view, the most dramatic changes in EOF can be made simply by altering the pH of the buffer, as described above (figure 6). Adjusting the pH, however, can also affect the solute charge and mobility. Low pH buffers will proto-

| Variable | Result | Comment |
|---|---|---|
| **Electric field** | Proportional change in EOF | • Efficiency and resolution may decrease when lowered<br>• Joule heating may result when increased |
| **Buffer pH** | EOF decreased at low pH and increased at high pH | • Most convenient and useful method to change EOF<br>• May change charge or structure of solute |
| **Ionic strength or buffer concentration** | Decreases zeta potential and EOF when increased | • High ionic strength generates high current and possible Joule heating<br>• Low ionic strength problematic for sample adsorption<br>• May distort peak shape if conductivity different from sample conductivtiy<br>• Limits sample stacking if reduced |
| **Temperature** | Changes viscosity 2 – 3% per°C | • Often useful since temperature is controlled instrumentally |
| **Organic modifier** | Changes zeta potential and viscosity (usually decreases EOF) | • Complex changes, effect most easily determined experimentally<br>• May alter selectivity |
| **Surfactant** | Adsorbs to capillary wall via hydrophobic and/or ionic interactions | • Anionic surfactants can increase EOF<br>• Cationic surfactant can |
| **Neutral hydrophilic polymer** | Adsorbs to capillary wall via hydrophobic interactions | • Decreases EOF by shielding surface charge and increasing viscosity |
| **Covalent coating** | Chemical bonding to capillary wall | • Many modifications possible (hydrophilicity or charge)<br>• Stability often problematic |

**Table 1**
**Methods to control electroosmotic flow**

nate both the capillary surface and the solute, while high pH buffers deprotonate both. Knowledge of solute pI is often useful in selecting the appropriate pH range of the running buffer.

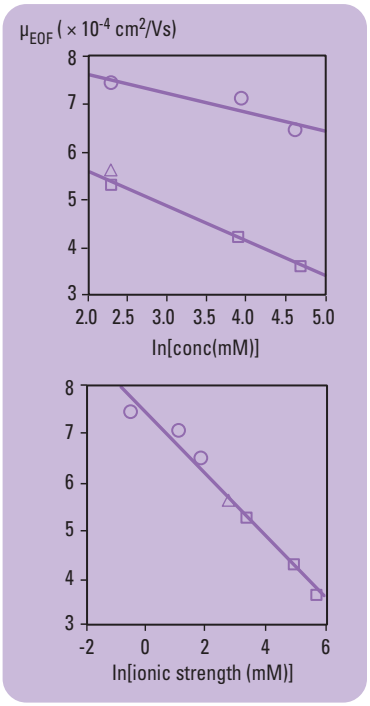



**Figure 9**
**Electro-osmotic flow mobility as a function of buffer concentration and ionic strength[3]**
circles = borate buffer
squares = phosphate buffer
triangles = carbonate buffer, all pH 8

both the capillary surface and the solute, while high pH buffers deprotonate both. Knowledge of solute pI is often useful in selecting the appropriate pH range of the running buffer.

EOF can also be affected by adjusting the concentration and ionic strength of the buffer. The magnitude of this effect is illustrated in figure 9. High buffer concentrations are also useful in limiting coulombic interactions of solute with the walls by decreasing the effective charge at the wall. Heating within the capillary, however, constrains the use of high concentration buffers (the effects of heating are described in *2.3.3. Analytical parameters*). Typical buffer concentrations range from 10 to 50 mM, although 100 to 500 mM and higher have also been used.

Lastly, EOF can be controlled by modification of the capillary wall by means of dynamic coatings (that is, buffer additives) or covalent coatings. These coatings can increase, decrease, or reverse the surface charge and thus the EOF. Details regarding the nature of coatings are given in *section 3.1*.

## 2.3.3 Analytical parameters

The analytical parameters for capillary electrophoresis can be described in similar terms to those for column chromatography. Capillary zone electrophoresis (CZE) is the simplest mode of CE and all subsequent discussions in this section refer to it. Below, fundamental and practical descriptions of time, mobility, solute zone dispersion, efficiency, and resolution are presented.

### 2.3.3.1 Mobility and migration time

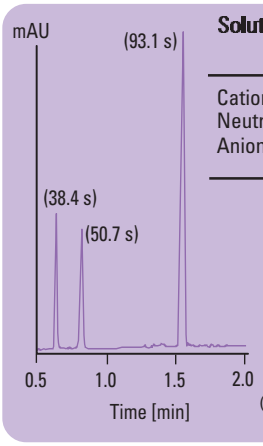
The time required for a solute to migrate to the point of detection is called the “migration time”, and is given by the quotient of migration distance and velocity. The migration

time and other experimental parameters can be used to calculate the apparent solute mobility using

$$\mu_a = \frac{l}{tE} = \frac{lL}{tV} \qquad (9)$$

where: $\mu_a = \mu_e + \mu_{EOF}$.
V = applied voltage
l = effective capillary length (to the detector)
L = total capillary length
t = migration time
E = electric field

In the presence of EOF, the measured mobility is called the apparent mobility, $\mu_a$. The effective mobility, $\mu_e$, can be extracted from apparent mobility by independently measuring the EOF using a neutral marker that moves at a velocity equal to the EOF. Examples of neutral markers include DMSO, mesityl oxide, and acetone. A sample calculation using a vitamin separation is illustrated in figure 10.




| Solute | Migration time(s) | $\mu_a$ (cm²/Vs) | $\mu_e$ (cm²/Vs) |
|---|---|---|---|
| Cation | 38.4 | $3.05 \times 10^{-3}$ | $7.40 \times 10^{-4}$ |
| Neutral | 50.7 | $2.31 \times 10^{-3}$ | $2.31 \times 10^{-3}$ |
| Anion | 93.1 | $1.26 \times 10^{-3}$ | $-1.05 \times 10^{-3}$ |

**Cation:** $\mu_a = \frac{lL}{Vt} = \frac{(50)(58.5)}{(25{,}000)(38.4)} = 3.05 \times 10^{-3}$

**Neutral:** $\mu_{EOF} = \frac{(50)(58.5)}{(25{,}000)(50.7)} = 2.31 \times 10^{-3}$

$\mu_e = \mu_a - \mu_{EOF} = (3.05 \times 10^{-3}) - (2.31 \times 10^{-3}) = 7.40 \times 10^{-4}$

(note: $\mu_e$ will be negative for the anion)

**Figure 10**
**Calculation of electro-osmotic flow mobility and effective solute mobility**

The two different capillary lengths, effective and total, are described schematically in figure 11. The effective length is that from the point of injection to the point of detection. For on-capillary spectroscopic detection, this length is typically 5 to 10 cm shorter than the total length. For off-column

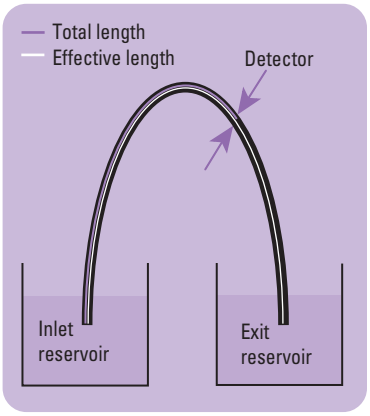



**Figure 11**
**Definition of effective and total capillary lengths**

detection (mass spectrometry, for example), the two lengths are equivalent. Knowledge of both lengths is important since the migration time and mobility are defined by the effective length, whereas the electric field is defined by the total length.

## 2.3.4 Dispersion

Separation in electrophoresis is based on differences in solute mobility. The difference necessary to resolve two zones is dependent on the length of the zones. Zone length is strongly dependent on the dispersive processes that act on it. Dispersion should be controlled because it increases zone length and the mobility difference necessary to achieve separation.

Dispersion, spreading of the solute zone, results from differences in solute velocity within that zone, and can be defined as the baseline peak width, $w_b$. For a Gaussian peak,

$$w_b = 4\,\sigma \qquad (10)$$

where $\sigma$ = standard deviation of the peak (in time, length, or volume).

The efficiency, expressed in number of theoretical plates, N, can be obtained by

$$N = \left(\frac{l}{\sigma}\right)^2 \qquad (11)$$

where $l$ = capillary effective length

and can be related to the HETP (height equivalent to a theoretical plate), H, by

$$H = \left(\frac{l}{N}\right) \qquad (12)$$

Under ideal conditions (that is, small injection plug length, no solute-wall interactions, and so on) the sole contribution to solute-zone broadening in CE can be considered to be longitudinal diffusion (along the capillary). Radial diffusion (across the capillary) is unimportant due to the plug flow profile. Similarly, convective broadening is unimportant due to the anticonvective properties of the capillary. Thus, the efficiency can be related to the molecular diffusion term in chromatography. That is:

$$\sigma^2 = 2\,Dt = \frac{2DlL}{\mu_e V} \qquad (13)$$

where D= diffusion coefficient of the solute.

Substituting equation (13) into equation (11) yields a fundamental electrophoretic expression for plate number

$$N = \frac{\mu_e Vl}{2DL} = \frac{\mu_e El}{2D} \qquad (14)$$

From equation (13), the reason for the application of high fields is evident. This follows simply because the solute spends less time in the capillary at high field and has less time to diffuse. In addition, this equation shows that large molecules such as proteins and DNA, which have low diffusion coefficients, will exhibit less dispersion than small molecules. The wide range of possible diffusion coefficients is illustrated in table 2.

| | D (× $10^{-5}$ $cm^2/s$) |
|---|---|
| HCl | 3.05 |
| NaCl | 1.48 |
| Glycine | 1.06 |
| Citrate | 0.66 |
| Cytochrome C | 0.11 |
| Hemoglobin (human) | 0.069 |
| Tobacco mosaic virus | 0.0046 |

**Table 2**
**Diffusion coefficients of selected molecules (in water, 25 °C)**

The theoretical plate number can be determined directly from an electropherogram, using, for example,

$$N = 5.54\left(\frac{t}{w_{1/2}}\right)^2 \qquad (15)$$

where: t = migration time
$w_{1/2}$ = temporal peak width at half height

Note that equation (15) should only be used for Gaussian peaks. Any asymmetry should be taken into account, for example, by use of central moments.

In practice, the measured efficiency, equation (15), is usually lower than the calculated efficiency, equation (14). This is because the theoretical calculation accounts only for zone broadening due to longitudinal diffusion. As described in the next section, other dispersive processes are often present.

### 2.3.4.1 Factors affecting efficiency

Dispersion in CE can have a number of contributors in addition to longitudinal diffusion. Among the most important are temperature gradients induced by Joule heating, injection plug length, and solute interactions with the capillary walls. Fortunately, these phenomena are usually controllable, as discussed below. These and other zone broadening mechanisms are described in table 3.

Dispersion, as described by equation (13), was derived with the assumption that the only contributor was molecular diffusion. The variance is better described by the total variance of the system, $\sigma^2_T$, which is given by the sum of the contributing variances

$$\sigma^2_T = \sigma^2_{DIF} + \sigma^2_{INJ} + \sigma^2_{TEMP} + \sigma^2_{ADS} + \sigma^2_{DET} + \sigma^2_{Electrodispersion} + \ldots \quad (16)$$

where the subscripts refer to diffusion, injection, temperature gradients, adsorption, detection, and electrodispersion, respectively. If any of the dispersion processes in equation (16) dominate the diffusion term, theoretical limits cannot be obtained and equation (14) will not be valid. In this case, only minimal improvements in efficiency and resolution can be obtained by increased voltage.

| Source | Comment |
|---|---|
| **Longitudinal diffusion** | • Defines the fundamental limit of efficiency<br>• Solutes with lower diffusion coefficients form narrower zones |
| **Joule heating** | • Leads to temperature gradients and laminar flow |
| **Injection length** | • Injection lengths should be less than the diffusion-controlled zone length<br>• Detection limit difficulties often necessitate longer than ideal injection lengths |
| **Sample adsorption** | • Interaction of solute with the capillary walls usually causes severe peak tailing |
| **Mismatched conductivities of sample and buffer (electrodispersion)** | • Solutes with higher conductivities than the running buffer result in fronted peaks<br>• Solutes with lower conductivities than the running buffer result in tailed peaks |
| **Unlevel buffer reservoirs** | • Generates laminar flow |
| **Detector cell size** | • Should be small relative to peak width |

**Table 3**
**Sources of zone broadening**

### 2.3.4.2 Joule heating and temperature gradients

The main advantage of performing electrophoresis in narrow-bore capillaries is reduction of the effects of heating which have traditionally limited electrophoretic techniques. Heating is problematic since it can cause nonuniform temperature gradients, local changes in viscosity, and subsequent zone broadening. While the theoretical equations for efficiency and resolution advocate the use of as high electric fields as possible, Joule heating ultimately limits the benefit of this approach, regardless of capillary dimensions and temperature control measures.

The heat generated by the passage of electrical current is called Joule heat. The temperature increase depends on the power (product of voltage and current) generated and is

determined by the capillary dimensions, conductivity of the buffer, and the applied voltage. Significantly elevated temperatures will result when the power generation exceeds dissipation. Typical power generation ranges from 0.5 to 5 W/m. Temperature increases of 10 °C are not uncommon, although 70 °C and higher can occur.

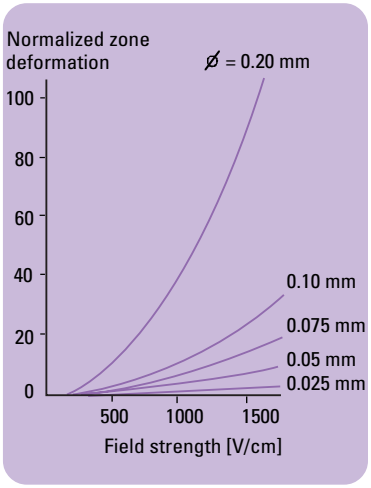



**Figure 12**
**Effect of Joule heating and temperature gradients on solute zone deformation[4]**
( ø = capillary id)

While the absolute rise in temperature is generally not detrimental (except possibly for sample degradation, and so on), temperature gradients are. Thermal dissipation of the heat through the capillary walls can result in higher temperatures in the center than at the walls. These temperature gradients cause viscosity differences of the running buffer and give rise to zone deformation. This is illustrated in figure 12 for a variety of inner diameter capillaries. Control of temperature differentials is critical since a one degree change in temperature results in a 2 to 3 % change in viscosity (and a 2 to 3 % change in mobility).

The thermal gradient between the center of the capillary and the surroundings is illustrated in figure 13. As shown, the temperature difference depends on the inner radius, the thickness of the wall, the thickness of the polyimide coating, and the heat transfer coefficient to the surroundings. Analytically this can be expressed by

$$\Delta T_T = \frac{Q r_1^2}{2}\left[\frac{1}{\kappa_1}\ln\left(\frac{r_2}{r_1}\right) + \frac{1}{\kappa_2}\ln\left(\frac{r_3}{r_2}\right) + \frac{1}{r_3}\left(\frac{1}{h}\right)\right] \quad (17)$$

where:
- $Q$ = power density
- $r$ = radius
- $\kappa$ = thermal conductivity
- $h$ = thermal transfer rate from the capillary to the surrounding

subscripts 1, 2, and 3 refer to the buffer,

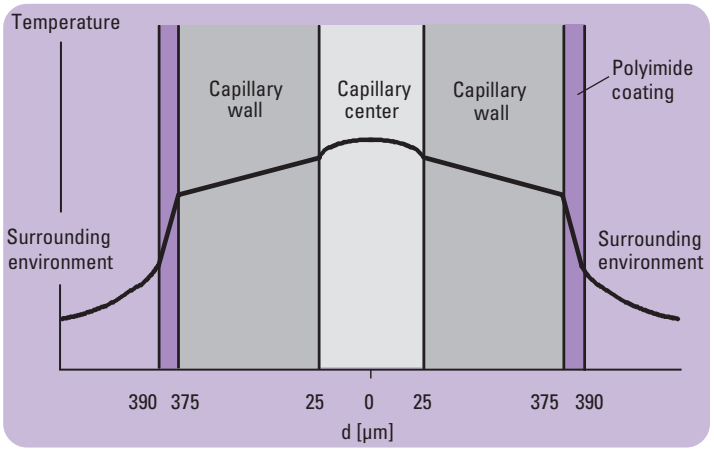



**Figure 13**
**Schematic of temperature gradients from capillary center to the surroundings**

An example of the calculated temperature difference between the internal capillary wall and the capillary center is given in table 4.

**Table 4**
**Capillary wall temperature and center-to-wall temperature difference[5]**

| Radius (µm) | Wall temperature, K | Temperature difference, K |
|---|---|---|
| 25 | 299.0 | 0.53 |
| 50 | 301.2 | 1.39 |
| 75 | 304.2 | 3.14 |
| 100 | 307.7 | 5.58 |
| 125 | 311.6 | 8.72 |

Equation (17) implies that it is advantageous to use narrow inner radii capillaries with large outer radii. As mentioned, the small volume limits the quantity of heat generated, even when several hundred volts per centimeter are applied. In addition, the high inner surface-to-volume ratio helps dissipate the generated heat through the capillary wall. The large outer diameter is advantageous due to a reduction in the insulating properties of the polyimide and improvement of heat transfer to the surroundings. Although the polyimide coating is only a few microns thick, its low thermal conductivity significantly limits heat transfer.

There are a number of methods that indicate excessive heat generation and possible temperature gradients. These phenomena may be indicated if efficiency is reduced as the

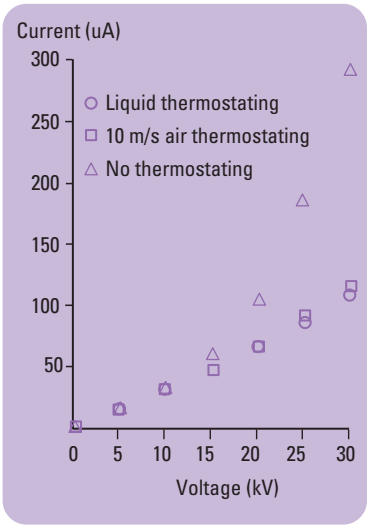



**Figure 14**
**Ohm's law plots to monitor Joule heating**

voltage is increased. Another indication is the disproportionate increase in EOF or solute mobility with increasing voltage. Similarly, a disproportionate increase in current with voltage (Ohm's law) indicates a temperature increase (figure 14).

A variety of methods to limit Joule heating are described in table 5. Equation (17) indicates that temperature gradients will be reduced linearly with a reduction in power. This can be accomplished by lowering either the applied voltage or decreasing the buffer conductivity by lowering the ionic strength or decreasing buffer ion mobility. The latter methods may be useful but have practical limitations. Reduced buffer concentration may decrease buffering capacity and also may lead to increased solute-wall interactions (see below).

**Table 5**
**Methods to control Joule heating and temperature gradients**

| Variable | Effect |
|---|---|
| **Decrease electric field** | • Proportional decrease in heat generated<br>• Reduces efficiency and resolution |
| **Reduce capillary inner diameter** | • Dramatic decrease in current ($i \alpha r^2$)<br>• Decreases sensitivity<br>• May cause increased sample adsorption |
| **Decrease buffer ionic strength or concentration** | • Proportional decrease in current<br>• May cause increased sample adsorption |
| **Active temperature control** | • Thermostats and removes heat from capillary |

An alternative is the use of low mobility buffers which contain large, minimally charged ions, such as tris, borate, histidine, and CAPS. Additional comments regarding buffer selection can be found in *section 3.1.1.1.*

A dramatic decrease in temperature differences can also be realized by reducing the capillary internal diameter due to the squared dependence of area (and thus electrical cur-

rent) with radius. Capillaries with internal diameters as small as 5 to 10 µm have been used. However, such small internal diameters are not practical for routine use due primarily to detection, sample loading, and capillary clogging difficulties. Internal diameters of 25 to 50 µm are more practical.

Removal of heat from the outer capillary wall is extremely important. This can most easily be accomplished by use of a fan to blow ambient temperature air around the capillary. Dramatic improvements can be made by use of a cooling system which thermostats the capillary with high velocity air or liquid. While liquid cooling is theoretically more efficient ($\kappa_{Liquid} > \kappa_{air}$), under typical conditions of less than 5 to 7 W/m power generation, high-velocity air cooling is sufficient. This is illustrated in the Ohm's law plots of figure 14.

Active temperature control is not only important for heat dissipation but also for maintaining constant capillary temperature. Even in the absence of Joule heating phenomena, variations in migration time can result from changes in ambient temperature and the resulting 2 to 3 % per °C changes in viscosity. Further, since sample loading is also dependent on the temperature, small variations can have profound effects on the quantity injected. Further details regarding temperature control and reproducibility can be found in the *chapter 4*.

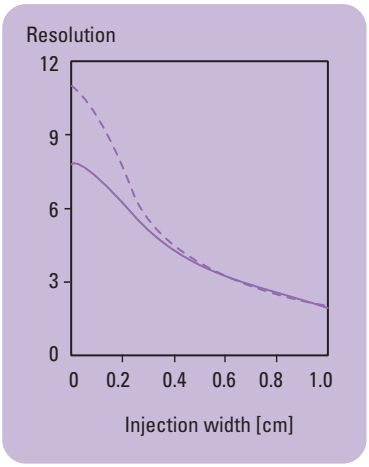



**Figure 15**
**Effect of injection plug length on resolution[6]**
Calculation using: Upper curve = 20 kV, lower curve = 10 kV, $\mu_{e1} = 3.0 \times 10^{-4}$ cm²/Vs, $\mu_{e2} = 3.15$ cm²/Vs, $\mu_{EOF} = 3.0 \times 10^{-4}$ cm²/Vs, diffusion coefficients = $7 \times 10^{-5}$ cm²/s

### 2.3.4.3 Injection plug length

During injection it is important that the sample plug length be minimized. If the length is longer than the dispersion caused by diffusion, efficiency and resolution will be sacrificed. This is illustrated in figure 15.

The contribution of injection to the total variance is given by

$$\sigma^2_{inj} = \frac{w_i^2}{12} \qquad (18)$$

where $w_i$ = injection plug length.

Ideally, the sample plug length should be less than the standard deviation due to diffusion, $(2Dt)^{1/2}$. The exact length depends both on the diffusion coefficient of the solutes and on the analysis time. Macromolecules can have diffusion coefficients 100 times lower than small solutes and will necessitate smaller sample plugs. The relationship between injection plug length and diffusion coefficient and their effect on efficiency is illustrated in table 6.

**Table 6**
**Effect of injection length and diffusion coefficient on efficiency**

| Injection length (mm) | N ($D=10^{-5}$ cm²/s) | N ($D=10^{-6}$ cm²/s) |
|---|---|---|
| 1 | 238,000 | 1,400,000 |
| 2 | 164,000 | 385,000 |
| 10 | 81,000 | 112,000 |

A practical limit of injection length is less than 1 to 2 % of the total capillary length. For a 70-cm capillary, a 1 % length corresponds to 7 mm (or 14 nl for a 50- mm id). While current instrumentation can load these small volumes reproducibly, under typical conditions detection limit difficulties often necessitate longer injection lengths. Methods to improve detection limits without degrading efficiency are discussed in *chapter 2, in section 4.1.3 and in section 4.3.*

### 2.3.4.4 Solute-wall interactions

Interaction between the solute and the capillary wall is detrimental to CE. Depending on the extent of interaction, peak tailing and even total adsorption of the solute can

occur. The primary causes of adsorption to the fused silica walls are ionic interactions between cationic solutes and the negatively charged wall, and hydrophobic interactions. The large surface area-to-volume ratio of the capillary, which is beneficial for heat transfer, in fact increases the likelihood of adsorption. Significant absorptive effects have been noted especially for large peptides and proteins primarily because these species posses numerous charges and hydrophobic moieties.

The variance due to adsorption can be given by

$$\sigma^2_{ads} = \frac{k' v_{EOF} l}{(1 + k')^2} \left( \frac{r^2 k'}{4D} + \frac{2}{K_d} \right) \qquad (19)$$

where
- $k'$ = capacity factor
- $v_{EOF}$ = electro-osmotic flow velocity
- $D$ = solute diffusion coefficient
- $l$ = capillary effective length
- $K_d$ = first order dissociation constant.

The capacity factor is defined as

$$k' = \frac{t_r - t_0}{t_0} \qquad (20)$$

where
- $t_r$ = elution time of a retained solute
- $t_0$ = elution time of an unretained solute.

Equation (19) describes both axial diffusion (that is, across the capillary) and adsorption-desorption kinetics ($K_d$). The variance is strongly dependent on the magnitude of the capacity factor. As shown in figure 16 and table 7, small interactions can have dramatic effects on efficiency. Capacity factors even less than 0.1 can be detrimental. In

| k' | H | N |
|---|---|---|
| 0.001 | 0.58 | $1.7 \times 10^6$ |
| 0.005 | 1.00 | $1.0 \times 10^5$ |
| 0.010 | 1.54 | $6.5 \times 10^5$ |
| 0.050 | 6.78 | $1.5 \times 10^5$ |
| 0.100 | 15.7 | $6.4 \times 10^4$ |

**Table 7**
**Impact of adsorption on efficiency[1]**
D = $5 \times 10^{-5}$ cm²/s $v_{EOF}$ = 1 mm/s
l = 50 cm L = 60 cm

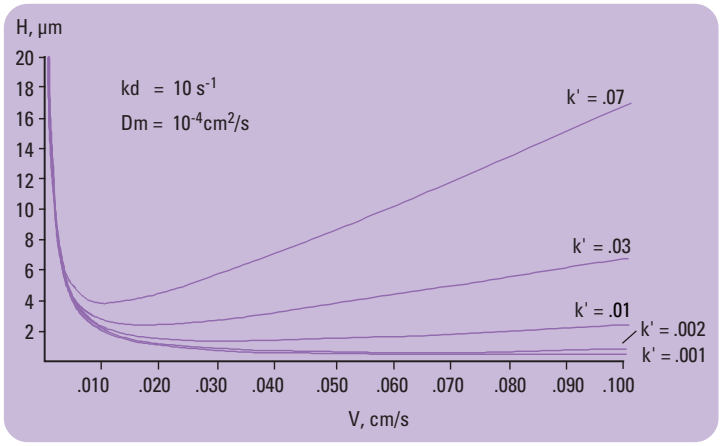



**Figure 16**
**Effect of protein-wall interactions on HETP[1]**

practice, protein separations typically exhibit capacity factors between 0.01 to 0.1. Thus, plate counts between 1 to $5 \times 10^5$ may be expected.

There are a variety of strategies employed to reduce solute-wall interactions. Among these, a few are very simple to implement and often yield exceptional results. Increasing the concentration of the buffer, for example, decreases solute interactions by reducing the effective surface charge. High ionic strength also decreases the EOF, thereby increasing the residence time of solutes in the capillary. The benefit of these effects is illustrated in the peptide digest separation of figure 17. Again, this approach is somewhat limited by the increased current and subsequent Joule heating. Use of zwitterionic buffer systems are an alternative.

Another approach to limit adsorption is to operate at the extremes of pH. At low pH (< 2 to 3) the silanol groups of fused silica will be essentially protonated and uncharged. While EOF will be nearly zero, since proteins (and other species) will also be protonated and positively charged, they will migrate toward the cathode. Conversely, at high pH (> 9

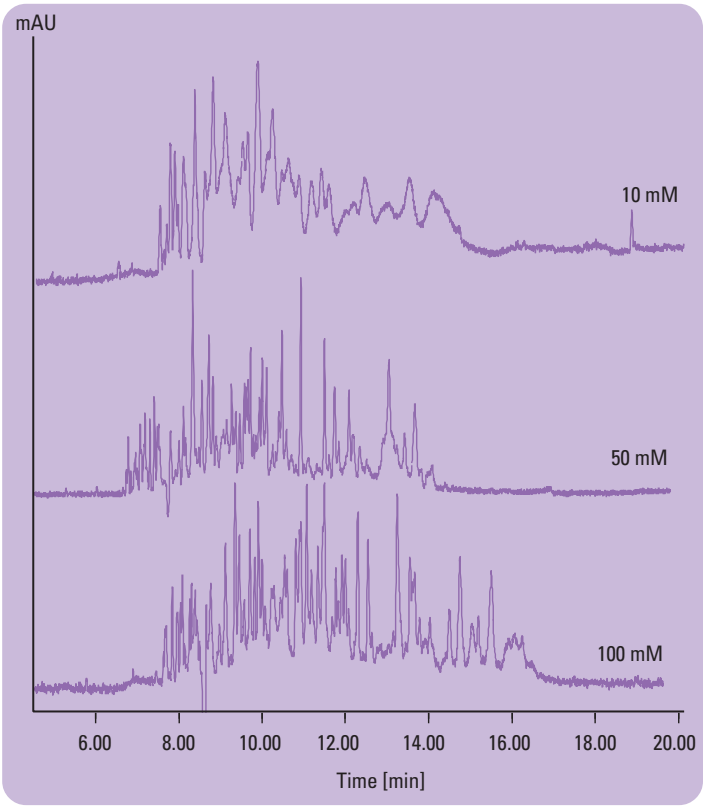



**Figure 17**
**Influence of buffer concentration on BSA tryptic digest separations**
Conditions: Phosphate buffer pH 7, V = 25kV, i = 9, 36, and 71 μA, respectively, l = 50 cm, L = 58.5 cm, id = 50 μm, od = 375 μm, BSA concentration = 2 mg/ml, injection = 100 mbar s

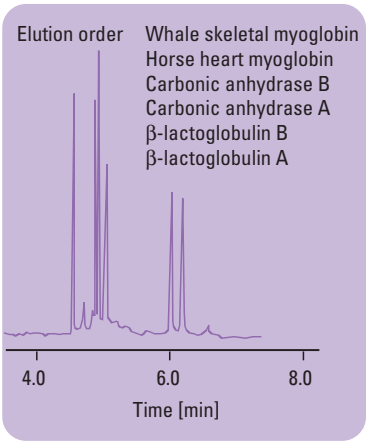



**Figure 18**
**CZE of proteins using buffer pH above solute pI[7]**
Conditions: 20 mM borate pH 8.25, V = 30 kV, l =55 cm, L = 101 cm, id = 52 μm

to 10), both the wall and the sample will be deprotonated and negatively charged, and solute-wall interactions will be limited by charge repulsion (figure 18).

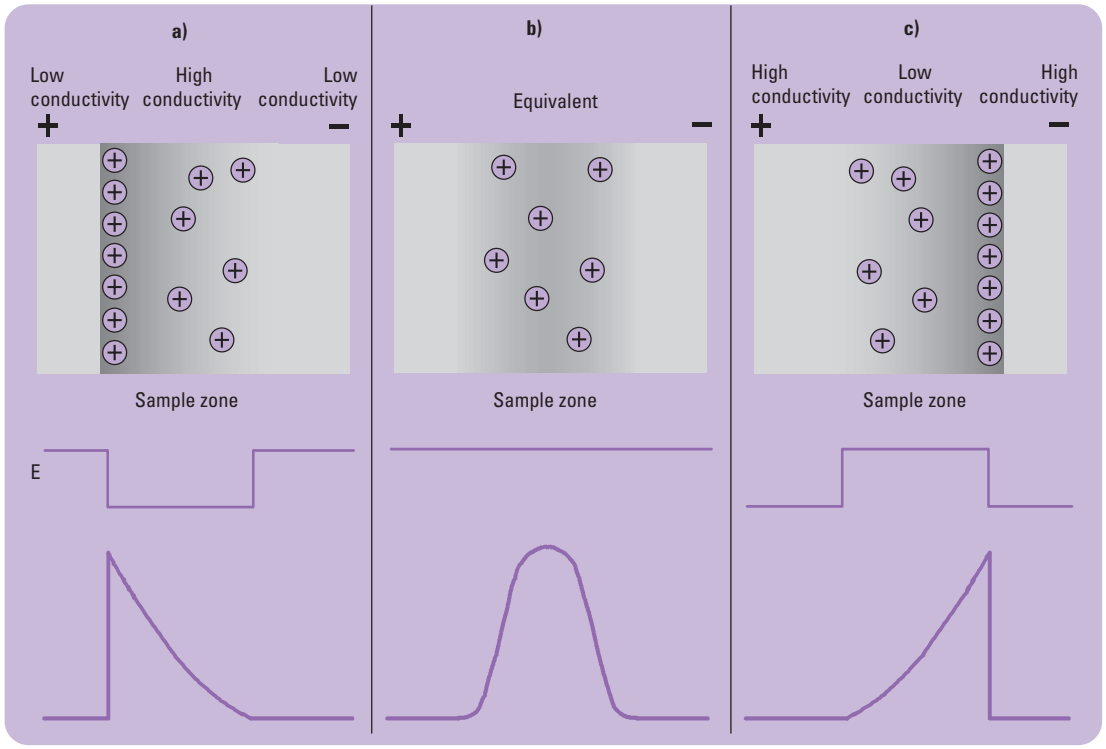
Coating the capillary wall is a useful method for decreasing solute adsorption by decreasing the free energy of interaction. Coatings can take various forms, including simple addition of dynamic deactivation with buffer additives (that is, hydrophilic polymers or detergents) or covalent modification of the wall. Both can be used to eliminate or reverse the charge on the wall, alter hydrophobicity and limit non-specific adsorption. More information regarding coatings and protein separations can be found in *section 3.1.2*.

### 2.3.4.5 Electrodispersion

Differences in sample zone and running buffer conductivities can have three major effects: 1) skewed peak shapes; 2) solute concentration or focusing (low conductivity sample), or solute defocusing (high conductivity sample); 3) temporary isotachophoretic states due to excess of a certain ion (for example, $Cl^-$).

As described by the Kohlrausch regulating function, when the solute zone has a higher mobility than the running buffer, the leading edge of the solute zone will be diffuse and the trailing edge sharp. Conversely, when the solute zone has a lower mobility than the running buffer, the leading edge will be sharp and the trailing edge diffuse. When the conductivities are equivalent, no such peak distortions will occur. Schematics of each case are shown in figure 19.

**Figure 19**
**Electrodispersion due to mismatched sample and buffer conductivities**



These peak shape distortions are caused by the differences in conductivity, and hence field, in each zone. When the solute zone has a higher mobility (that is, higher conductivity and lower resistance) than that of the running buffer, the front edge of the solute, which diffuses in the direction of migration, encounters a higher voltage drop when entering the buffer zone. This causes the diffusing solute (that is, anions when the EOF is toward the cathode) to accelerate away from the sample zone and results in zone fronting. As the solute at the trailing edge diffuses into the running buffer it also encounters an increase in voltage drop, but, in the same direction of migration, and accelerates back into the solute zone, keeping the trailing edge sharp. Similar reasoning accounts for the sharp leading and diffuse trailing edges of the reverse situation and for ions of opposite charge. Neutral species are unaffected by these conductivity differences.

Although these distortions always occur, they may be small relative to other dispersive effects, including diffusion. Distortions, however, are particularly evident with samples containing solutes with a wide range of mobilities. An example of the separation of inorganic ions and organic acids is illustrated in figure 20. Note the fronting of the high mobility,

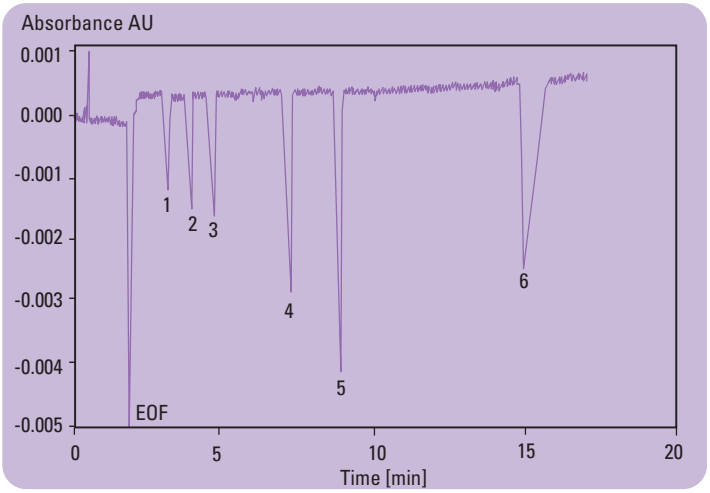



**Figure 20**
**Fronting and tailing of solutes due to electrodispersion[8]**
Peaks: 1) Chloride, 2) Chlorate, 3) Fluoride 4) Acetate, 5) Propionate, 6) MES
Conditions: Detection = Indirect UV, buffer = 0.01 M benzoic acid adjusted to pH 8 with Tris, detection wavelength = 254 nm, V = 25 kV, l = 50 cm, L = 57 cm, id = 75 μm,

rapidly eluting ions, the near-Gaussian peak shape of those of moderate mobility, and the tailing of the low mobility, late eluting ions.

Peak shape distortions are detrimental only if resolution is lost. Measures to decrease these phenomena include matching the mobilities of the buffer constituent to the sample mobility or by maintaining a running buffer concentration approximately two orders of magnitude higher than that of the sample.

## 2.3.5 Resolution

Resolution of sample components is the ultimate goal in separation science. Resolution is most simply defined as

$$R = \frac{2(t_2 - t_1)}{w_1 + w_2} = \frac{t_2 - t_1}{4\sigma} \qquad (21)$$

where
- $t$ = migration time
- $w$ = baseline peak width (in time)
- $\sigma$ = temporal standard deviation

subscripts 1 and 2 refer to the two solutes.

The numerator in equation (21) describes the separation process in terms of differential migration and the denominator the dispersive processes acting against it.

Separation in CE is primarily driven by efficiency, not selectivity. This is in contrast to chromatography in which the opposite is usually true. Due to very sharp solute zones, small differences in solute mobility (< 0.05 % in some cases) are often sufficient for complete resolution. Of course, the extent of dispersion is immaterial if sufficient mobility differences are realized.

The resolution of two components can also be expressed with respect to efficiency

$$R = \frac{1}{4} N^{1/2} \left( \frac{\Delta\mu}{\bar{\mu}} \right) \quad (22)$$

where $\Delta\mu = \mu_2 - \mu_1$

$$\bar{\mu} = \frac{\mu_2 + \mu_1}{2}$$

(note: time$^{-1}$ or velocity can be substituted for mobility).

Substituting equation (14) into equation (22) yields a commonly cited theoretical equation for resolution that does not require explicit calculation of efficiency. It also describes the effect of EOF on resolution.

$$R = \left( \frac{1}{4\sqrt{2}} \right) (\Delta\mu) \left( \frac{V}{D(\bar{\mu} + \mu_{EOF})} \right)^{1/2} \quad (23)$$

In contrast to efficiency, which increases linearly with applied voltage, a similar gain in resolution is not found, due to the square root relationship. The voltage must be quadrupled to double the resolution. The generation of Joule heat often limits the benefits gained from this action.

It is evident from equation (23) that infinite resolution will be obtained when $\bar{\mu}$ and $\mu_{EOF}$ are equal but opposite. That is, when the ion migrates in the opposite direction and at the same rate as the EOF. In this case, however, the analysis time approaches infinity. Clearly, the operational parameters should be controlled so as to balance resolution and analysis time.

Chapter 3

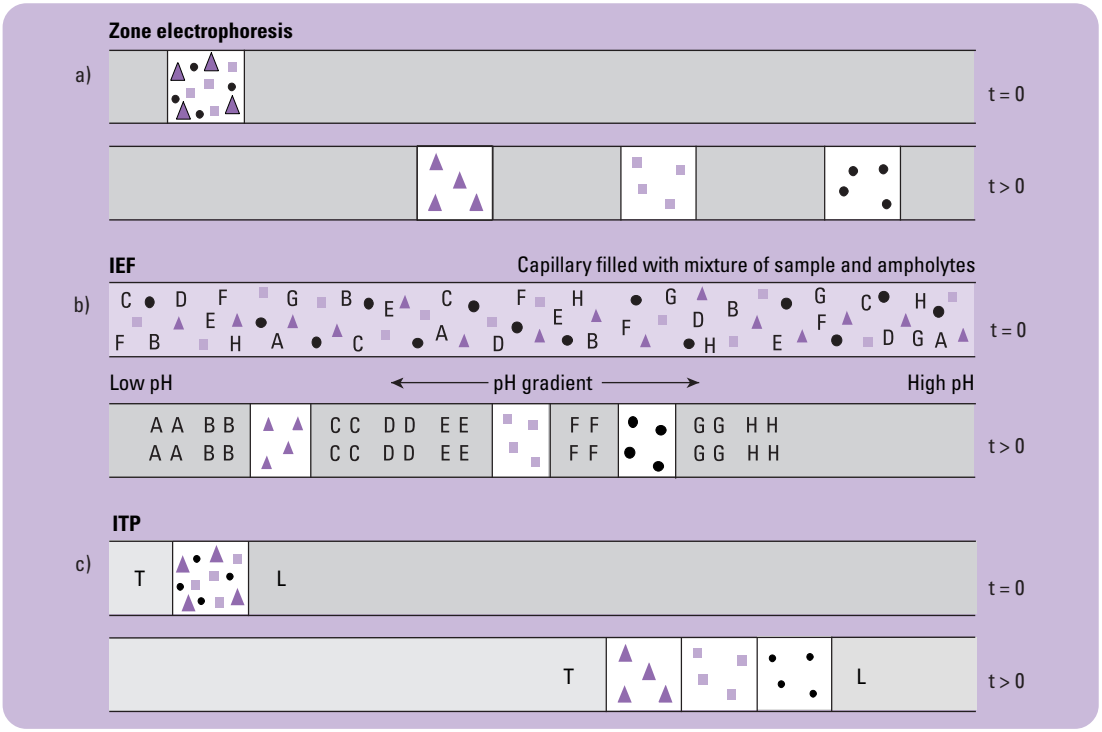
# Modes of operation

| Mode | Basis of separation |
|---|---|
| **Capillary zone electrophoresis** | Free solution mobility |
| **Micellar electrokinetic chromatography** | Hydrophobic/ ionic interactions with micelle |
| **Capillary gel electrophoresis** | Size and charge |
| **Isoelectric focusing** | Isoelectric point |
| **Isotachophoresis** | Moving boundaries |

**Table 8**
**Modes of CE**

The versatility of CE is partially derived from its numerous modes of operation. The separation mechanisms of each mode are different and thus can offer orthogonal and complementary information. The basic methods encompassed by CE include capillary zone electrophoresis (CZE), micellar electrokinetic chromatography (MEKC), capillary gel electrophoresis (CGE), capillary isoelectric focusing (CIEF), and capillary isotachophoresis (CITP).
The separation mechanisms of each mode are illustrated in figure 21 and described in table 8. For the most part, the different modes are accessed simply by altering the buffer composition. Each mode is described below, with emphases on basic operation and relevant applications.

**Figure 21**
**Illustration of zonal, IEF and ITP electrophoresis**



## 3.1 Capillary zone electrophoresis

Capillary zone electrophoresis (CZE) is the most widely used mode due to its simplicity of operation and its versatility. The application range of CZE is diverse. Application areas include the analysis of amino acids, peptides, ions, a wide range of enantiomers, and numerous other ionic species. In the area of protein analysis it has been used for purity validation, screening protein variants, and conformational studies, for example.

CZE is fundamentally the simplest form of CE, mainly because the capillary is only filled with buffer. As shown in figure 8 and 21a, separation occurs because solutes migrate in discrete zones and at different velocities. Separation of both anionic and cationic solutes is possible by CZE due to electro-osmotic flow (EOF). Neutral solutes do not migrate and all coelute with the EOF.

The name CZE is somewhat confusing in that it implies that it is the only mode in which "zonal" electrophoresis occurs. However, MEKC and CGE are also zonal techniques. In contrast, CIEF is defined as a "focusing" technique and ITP as a "moving boundary" technique. More suitable names for CZE might be open-tube or free-solution electrophoresis. Despite the ambiguity, the term CZE will be used here to maintain consistent nomenclature with most published literature.

Many of the theoretical aspects of CZE have been considered in *section 2.3*. The following sections discuss methods of altering selectivity, the use of additives, and describe coatings that alter the charge and hydrophobicity of the capillary wall.

### 3.1.1 Selectivity and the use of additives

Selectivity, the relative order of solute migration, is determined by the mechanism that effects the separation. The capability to control selectivity can improve resolution and also yield complementary information that is useful for validation of a separation. In essence, the different modes of CE (that is, CZE, MEKC, CGE, and CIEF) yield different selectivities due to different separation mechanisms. While the focus in this section is on selectivity within CZE, mechanisms of separation in CZE performed with additives sometimes overlap with MEKC.

In CZE, selectivity can most readily be altered through changes in running buffer pH or by use of buffer additives such as surfactants or chiral selectors. A more complete list is given in table 9. Note that while these and other methods also result in changes in EOF, the EOF is itself not responsible for the changes in selectivity, only in migration time and resolution.

#### 3.1.1.1 Buffer selection

The running buffer selection is extremely important to the success of any CE separation. The sensitivity of EOF to pH requires the use of buffers that maintain constant pH. Effective buffer systems have a range of approximately two pH units centered around the $pK_a$ value. Polybasic buffers such as phosphate and citrate have more than one useful $pK_a$ and thus can be used in more than one pH range. A buffer for use in CE should possess the following properties:

- good buffering capacity in the pH range of choice;
- low absorbance at the wavelength of detection;
- low mobility (that is, large, minimally-charged ions) to minimize current generation.

| Additive | Example | Use |
|---|---|---|
| **Surfactants (anionic, cationic, or neutral)** | SDS, CTAB, BRIJ, TWEEN | • EOF modification<br>• Solubilize hydrophobic solutes<br>• Ion pairing<br>• MEKC above CMC |
| **Zwitterionic substances** | MES, Tris, CHAPS, CHAPSO | • Increase ionic strength without increasing conductivity<br>• Affect selectivity of proteins |
| **Linear hydrophilic polymers** | Methyl cellulose, polyacrylamide, PEG, PVA | • Reduce EOF<br>• Minimize sample adsorption at low concentrations<br>• CGE at high concentrations |
| **Organic modifiers** | Methanol, acetonitrile, TFA | • Alter EOF (generally reduce)<br>• Change selectivity in MEKC and chiral analyses |
| **Chiral selectors** | Cyclodextrins, crown ethers, bile salts | • Chiral separations<br>• Solubilization of hydrophobic solutes |
| **Metal ions** | $K^+$, $Na^+$, $Cu^{2+}$, $Li^+$ | • Alter selectivity in MEKC and CGE |
| **Hydrogen bonding /solubilizing agents** | Urea | • Melt double stranded DNA in CGE<br>• Solubilize proteins<br>• Alter selectivity in MEKC |
| **Complexing buffers** | Borate | • Carbohydrate and catechol separations |
| **Quaternary amines** | Diaminopropane | • Ion pairing<br>• EOF reversal |

**Table 9**
**Additives in CE**

| Name | p$K_a$ |
|---|---|
| Phosphate | 2.12 (p$K_{a1}$) |
| Citrate | 3.06 (p$K_{a1}$) |
| Formate | 3.75 |
| Succinate | 4.19 (p$K_{a1}$) |
| Citrate | 4.74 (p$K_{a2}$) |
| Acetate | 4.75 |
| Citrate | 5.40 (p$K_{a3}$) |
| Succinate | 5.57 (p$K_{a2}$) |
| MES | 6.15 |
| ADA | 6.60 |
| BIS-TRIS propane | 6.80 |
| PIPES | 6.80 |
| ACES | 6.90 |
| MOPSO | 6.90 |
| Imidazole | 7.00 |
| MOPS | 7.20 |
| Phosphate | 7.21 (p$K_{a2}$) |
| TES | 7.50 |
| HEPES | 7.55 |
| HEPPS | 8.00 |
| TRICINE | 8.15 |
| Glycine amide, hydrochloride | 8.20 |
| Glycylglycine | 8.25 |
| TRIS | 8.30 |
| BICINE | 8.35 |
| Morpholine | 8.49 |
| Borate | 9.24 |
| CHES | 9.50 |
| CHAPSO | 9.60 |
| CAPS | 10.40 |
| Phosphate | 12.32 (p$K_{a3}$) |

**Table 10**
**Commonly used buffers**

A number of commonly used buffers and their useful pH ranges are given in table 10. The so-called biological 'Good buffers' (that is Tris, borate, histidine, CAPS,...) are especially useful. These buffer ions are generally large and can be used in high concentrations without generating significant currents. A potential disadvantage of these large buffer ions is their strong UV absorbance characteristics.

Matching buffer ion mobility to solute mobility is important for minimizing peak shape distortions, as mentioned *in section 2.3.4.5*. Furthermore, it is necessary to select leading and trailing buffer ions for on-capillary sample focusing by isotachophoresis, as described in this *section 3.5*.

Buffer ions can also be used to complex with solutes and alter selectivity. Tetraborate is one notable example. This ion has been used to improve separations of catechols and carbohydrates.

### 3.1.1.2 Buffer pH

Alterations in pH are particularly useful when solutes have accessible pI values, such as peptides and proteins. Working above and below the pI value will change the solute charge and cause the solute to migrate either before or after the EOF. Below its pI a solute possesses a net positive charge and migrates toward the cathode, ahead of the EOF. Above the pI the opposite occurs. Due to the high chemical stability of the fused silica capillary, the accessible pH range can vary from below 2 to more than 12, but is usually limited by the pH stability of the solute.

In addition to affecting solute charge, changing the pH will also cause a concomitant change in EOF. This may necessitate re-optimization of a separation. For instance, adequate resolution may be obtained at low pH, but when increased

| | pH 2.6 | pH 8.2 |
|---|---|---|
| | 32 | 115 |
| | 98 | 104 |
| $t_m$ | 39 | 32 |
| | 104 | 39, 98 |
| | 115 | 16 |

**Table 11**
**CZE elution order of HPLC-collected peptides**
Number corresponds to reversed phase LC retention time

to alter solute charge, the EOF may be too high so that solutes elute before resolution is achieved. In this case it would be necessary either to increase the effective length of the capillary or reduce the EOF by one of the methods described in table 1 of the previous chapter.

An example of selectivity differences as a result of pH change is illustrated for peptide mapping (table 11). Here peptides were separated and collected by reversed phase LC and then analyzed by CZE. The elution orders were different not only for LC and CE, but within CZE itself upon change in pH from 2.6 to 8.2.

### 3.1.1.3 Surfactants

Surfactants are among the most widely used buffer additives in CE. Numerous types of surfactants can be used in CZE (that is, anionic, cationic, zwitterionic, or non-ionic). At concentrations below the critical micelle concentration (CMC) monomer ionic surfactant molecules can act as solubilizing agents for hydrophobic solutes, as ion-pairing reagents, or as wall modifiers. The interaction of the monomer surfactant with the solute can occur via two mechanisms; ionic interactions with the charged end of the surfactant and/or through hydrophobic interactions between the alkyl chain and hydrophobic moieties of the solute.

In addition to interacting with the solute, many surfactants adsorb to the capillary wall, modifying EOF and also limiting potential solute adsorption. Depending on surfactant charge, EOF can be increased, reduced, or reversed. EOF reversal, for example, can be obtained by addition of cationic surfactants such as CTAB to the buffer. As depicted in figure 22, CTAB monomers adhere to the wall through ionic interactions. The positive charge results from hydrophobic interaction of free CTAB molecules with those bound to the wall.

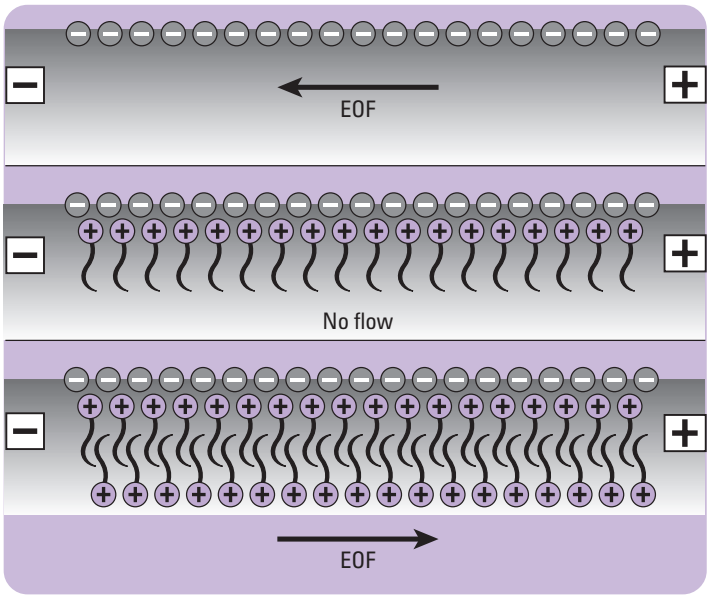



**Figure 22**
**Elimination and reversal of electro-osmotic flow using a cationic surfactant**

Surfactant concentrations above the CMC significantly alter the mechanism of separation. This leads to another mode of CE, micellar electrokinetic chromatography, which is discussed in *section 3.2*.

### 3.1.1.4 Chiral selectors

Chiral analysis is becoming increasingly important in both the drug and food industries. Currently, chiral separations are primarily performed by HPLC and GC. These analyses can be complicated and difficult to optimize. In addition, chiral stationary phases are usually expensive.

In contrast to the use of chiral stationary phases, chiral analysis by CZE usually involves the addition of a chiral selector to the running buffer. These selectors can be cyclodextrins, crown ethers, bile salts, copper (II)-aspartate complexes, for example. Compared with chiral chromatography, which uses a vast array of chiral phases to alter selectivity, the high efficiency of CE results in the use of a relatively small number of chiral selectors.

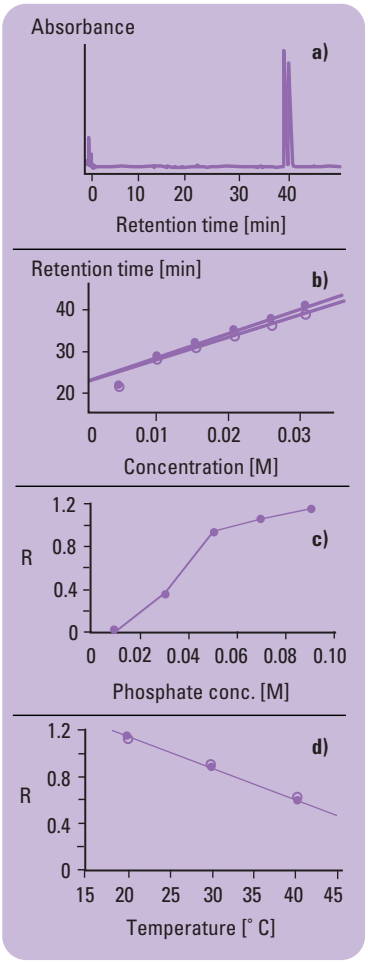



**Figure 23**
**Chiral analysis using β-cyclo-dextrin[9]**
**a) Separation of Quinagolide enantiomers**
**b) Effect of CD concentration on migration time**
**c) Effect of buffer concentration on resolution**
**d) Effect of temperature on resolution.**
Conditions: 30 mM β-CD in 50 mM phosphate pH 2.5

Selectivity can be tuned by adjusting the type and concentration of the chiral additive, and also by the addition of modifiers such as alcohols, surfactants, urea, and metal ions. CZE has been used successfully for the separation of D,L amino acids and chiral drugs, as well as positional isomers, among others. CDs have also been used to separate hydrophobic, but non-chiral specifiers such as polycyclic aromatic hydrocarbons.

Cyclodextrins (CDs) are the most widely used chiral selectors. CDs are nonionic cyclic oligosaccharides consisting of six, seven, or eight glucose units, and are called α, β, and γ, CDs, respectively. CDs have the shape of a hollow truncated cone with a cavity diameter determined by the number of glucose units. The cavity is relatively hydrophobic while the external surface is hydrophilic. The circumference contains chiral secondary hydroxyl groups. Chiral selectivity results from inclusion of a hydrophobic portion of the solute in the cavity and also from the hydrogen bonding to the chiral hydroxyl moieties. Various CD derivatives, such as carboxyalkylated, succinylated, and soluble ionized polymers, can be used to both alter selectivity and improve detection properties.

The use of β-CDs for the separation of racemic mixtures of Quinagolide, a dopamine agonist, is shown in figure 23. As is typical, resolution is influenced by the CD concentration, buffer concentration, and temperature, as shown in the figure. From the migration time versus CD concentration plot, the complex equilibrium constant, K, can be determined.

In this example, the migration time increases with increasing buffer concentration due to both the decreased EOF and the increased hydrophobic interactions between the solute and the CD. At high buffer concentration, the slope of the migration time versus buffer concentration curve levels out due to excessive Joule heating.

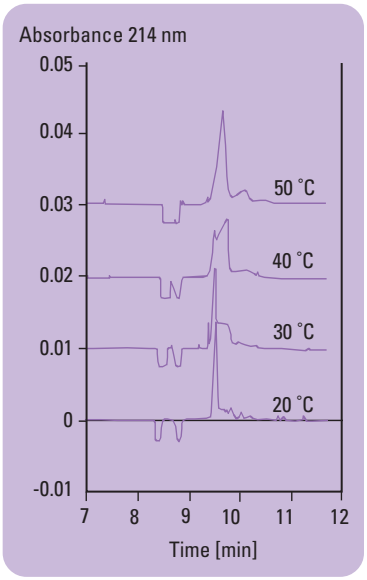



**Figure 24**
**Temperature-induced structural changes of horse heart myoglobin[10]**
Conditions: 0.1 M Tris-25 mM boric acid, pH 8.6, constant current = 9.8 μA, l = 50 cm, L = 57 cm, id = 75 μm, λ = 214 nm

In addition, as the temperature is increased, resolution is reduced, presumably due to decreased equilibrium constant and limited solute-CD interaction.

#### 3.1.1.5 Temperature

Although the primary purposes of thermostatically controlling capillary temperature are to maintain constant temperature and to remove Joule heat, temperature control can also be used as a parameter in optimizing a CZE separation. Elevated or reduced temperatures alter viscosity, EOF, and analysis time. As shown in the cyclodextrin example (figure 23d), it can also be used to affect chemical equilibria and kinetics. Temperature can also be used to affect protein conformation or protein-DNA interactions, for example. To this end, the temperature-induced structural changes of myoglobin are shown in figure 24. This behavior has been attributed to the possible reduction of the iron coordinated to the heme group.

### 3.1.2 Capillary wall modifications

CZE is an important separation technique for both small and macromolecular solutes. From basic theory it is expected that macromolecules such as proteins would yield very high efficiencies ($N > 10^6$) due to their low diffusion coefficients. It has been found, however, especially for proteins, that interaction with the capillary surface greatly reduces efficiency. These interactions can be ionic and/or hydrophobic in nature. Such difficulties are not surprising considering the variability of proteins with regard to charge, hydrophobicity, size, and dynamic nature.

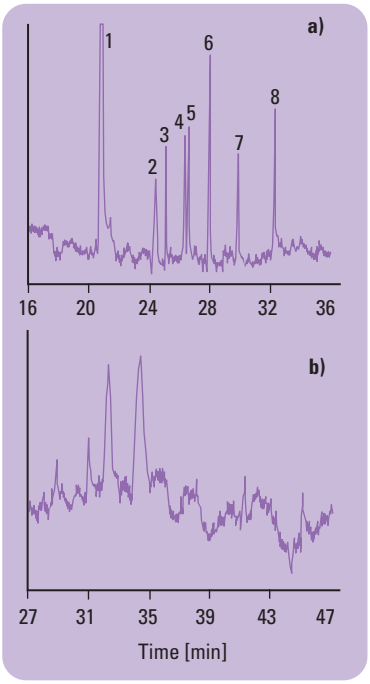



**Figure 25**
**Aryl pentafluro-coated capillaries to improve protein separations. Coated capillary above, bare fused silica below[11]**
Peaks: 1 = lysozyme, 2 = DMSO (EOF marker), 3 = bovine pan- creatic trypsinogen, 5 = whale myoglobin, 6 = horse myoglobin, 7 = human carbonic anhydrase, 8 = bovine carbonic anhydrase B.
Conditions: 200 mM phosphate, 100 mM KCl, pH 7, E = 250 V/cm, id = 20 μm, λ = 219 nm

As described in *section 2.3*, studies have shown that protein-wall interactions with k' values that would be considered of no significance in LC can have an appreciable effect in CE. Such interactions often result in peak tailing or even total retention in the capillary.

Without explicit wall modification, use of pH extremes is very effective in reducing ionic interactions. A possible limitation of this approach is the alteration of protein structure at non-biological pH values. High ionic strength buffers can limit ionic interactions, although ultimately limited by Joule heating. While narrow-bore capillaries can be beneficial with respect to heating, protein-wall interactions are exacerbated by the high surface area-to-volume ratio capillary.

Capillary wall modification is an alternative to limit solute adsorption. Two fundamental approaches have been taken: a) permanent modification by covalently bonded or physically adhered phases; and b) dynamic deactivation using running buffer additives. Both approaches have been somewhat successful, although no single method is clearly superior.

### 3.1.2.1 Bonded or adhered phases

A number of permanent wall modifications are described in table 12. Notably, silylation followed by deactivation with a suitable functional group has been the most widely used approach. Deactivation can be accomplished with such varied species as polyacrylamide, aryl pentafluoryl groups, or polysaccharides. The electropherograms in figure 25 show the type of improvement that can be expected for the separation of proteins using coated capillaries. Unfortunately, the siloxane bond (Si-O-Si) is stable only between pH ≅ 4 and 7 and hydrolysis usually limits long term stability.

| Type | Comment |
|---|---|
| **Silylation coupling (Si-O-Si-R)**<br>R = Polyacrylamide<br>Aryl penta fluoro<br>Protein or amino acid<br>Sulfonic acids<br>Maltose<br>PEG<br>Polyvinyl pyrrolidinone | • Numerous usable functional groups<br>• Generally simple to prepare<br>• Slioxane bond stable between pH 4 and 7<br>• Limited long term stability |
| **Direct Si-C coupling**<br>Polyacrylamide via Grignard | • Si-C binding eliminates need for silylation<br>• pH stable between 2 and 10<br>• Difficult to prepare |
| **Adsorbed polymers**<br>Cellulose, PEG, PVA | • Poor long-term stability<br>• Low pH range (2 to 4)<br>• Relatively hydrophobic |
| **Adsorbed, crosslinked polymers**<br>Polyethyleneimine | • Reverses EOF<br>• Useful for basic proteins<br>• Stable at physiological pH |
| **GC phases**<br>PEG<br>Phenylmethyl silicone | • Hydrolytically unstable |
| **LC phases**<br>$C_2$, $C_8$, $C_{18}$ | • Can increase protein adsorption |

**Table 12**
**Bonded or adhered phases**

Depending on the deactivation, the EOF can be eliminated or reversed. Neutral deactivation with polyacrylamide or polyethylene glycol, for example, eliminates EOF. This results from both decreased effective wall charge and increased viscosity at the wall. Deactivation with cationic groups reverses the EOF. Deactivation with amphoteric species, such as proteins or amino acid, yield reversible EOF depending on the pI of the coating and pH of the buffer (figure 26).

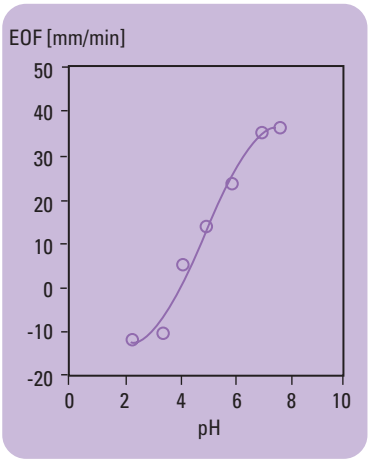



**Figure 26**
**Reversible electroosmotic flow in a α-lactalbumin-coated capillary[12]**

These covalent modifications are intended to be permanent and to require little or no maintenance. Since the capillaries are usually washed after use (adsorption may occur even with the coating), they must be stable to washing solutions and to hydrodynamic flow. Unfortunately, the stability of most coatings is limited. It is anticipated that numerous types of stable coatings will soon be purchasable, similar to LC and GC columns.

### 3.1.2.2 Dynamic deactivation

Addition of modifiers to the running buffer is an alternative to the bonded or adhered phases. An advantage of dynamic coatings is stability. Since the modifier is in the buffer, the coating is continuously regenerated and permanent stability is not required.

As with covalent coatings, additives can interact with the wall and alter charge and hydrophobicity. These modifiers are both easier to implement and optimize since they are prepared by simple dissolution of the modifier in the buffer. Several dynamic deactivation methods are listed in table 13 and an illustration of the use of cationic surfactants to reverse the EOF was shown in figure 22.

A potential disadvantage of the dynamic modification approach is that solutes as well as the capillary surface are affected. Biological-type conditions will be sacrificed by the use of pH extremes and addition of surfactants. Another limitation can be the equilibration time needed to obtain a reproducible surface and constant EOF. Furthermore, post-column analyses such as mass spectrometry and enzymatic assays are sensitive to additives, especially those in high concentrations.

| Type | Result | Comment |
|---|---|---|
| **Extremes of pH** | • Reduce coulombic interactions by eliminating wall and solute charge differences | • pH range from 2 to 12<br>• EOF nearly eliminated at low pH<br>• EOF very fast at high pH<br>• May denature protein<br>• Can decrease peak capacity by decreasing charge differences |
| **High buffer ionic strength/ concentration** | • Reduce coulombic interactions | • Decreases EOF<br>• Often limited by Joule heating |
| **Hydrophilic polymers** (Alkyl celluloses, polyvinyl alcohol, dextrans, polyacrylamide) | • Mask wall charge and reduce EOF | • Increases viscosity<br>• Can separate by size if used at high concentrations (CGE) |
| **Surfactants**<br>Anionic (SDS)<br>Cationic (CTAB)<br>Non-ionic (BRIS)<br>Zwitterionic (CHAPS)<br>(see table 14) | • Deactivate capillary surface through hydrophobic and/ or ionic interaction | • Wide variety of surfactants<br>• Easy to use<br>• MEKC above CMC<br>• Can decrease or reverse EOF<br>• May irreversibly denature protein<br>• Can be used in conjunction with reversed phase LC surface |
| **Quaternary amines** | • Decrease or reverse EOF | • Also act as ion-pairing reagents |

**Table 13**
**Dynamic deactivation methods**

## 3.1.3 Applications of CZE

Many of the applications of CZE have been in the bioscience area, specifically for peptides and proteins. One example, in addition to those presented earlier in this chapter, is glycoprotein separations. Glycoproteins are typically

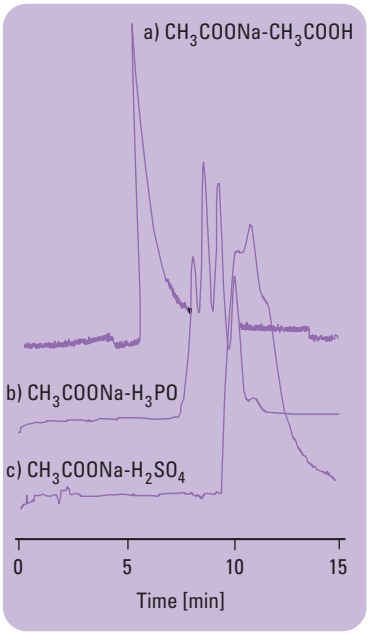



**Figure 27**
**Influence of buffer composition on separation of erythropoietin glycoforms[13]**
Conditions: Buffer concentration = 100 mM, pH 4, V = 10 kV, i = 10, 120, 200 µA in a, b, and c, respectively, l = 20 cm, L = 27 cm, id = 75 µm, λ = 214 nm

difficult to analyze by the traditional techniques of slab gel electrophoresis, isoelectric focusing, or liquid chromatography. In some cases CZE is advantageous. Shown in figure 27 is the separation of glycoforms of the human recombinant protein hormone, erythropoietin. The different species result from heterogeneity after post-translational modification. CZE is well suited for such analyses since many post translational modifications have an impact on protein charge (that is, N- or C- terminal modifications, phosphorylation, carboxylation, or N-glycosylation).

Significant success has been realized for peptide mapping by CZE. In peptide mapping a protein is enzymatically or chemically cleaved into smaller peptide fragments and subsequently separated. The analysis is primarily qualitative and is used to detect subtle differences in proteins. A typical CZE peptide map is shown in figure 28. CZE is also useful as a second-dimension analysis of HPLC-purified peptides (figure 29).

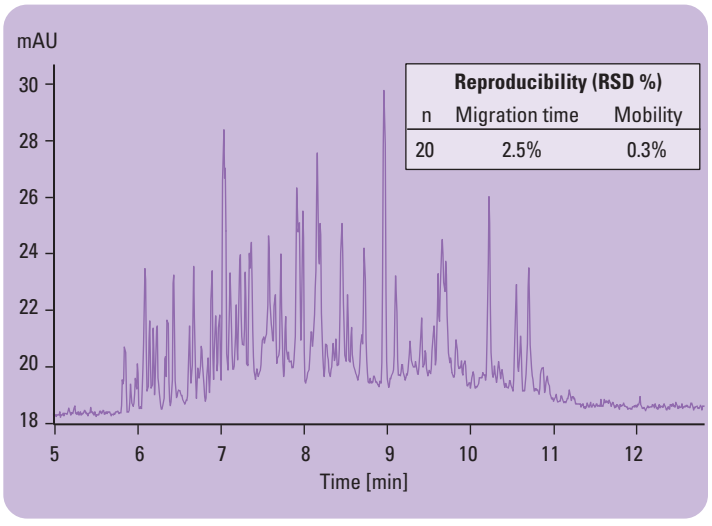



**Figure 28**
**Rapid BSA peptide map**
Conditions: 20 mM phosphate, pH 7, V = 25 kV, i = 16 µA, l = 50 cm, L = 57 cm, id = 50 µm with 3X extended pathlength detection cell, λ = 200 nm

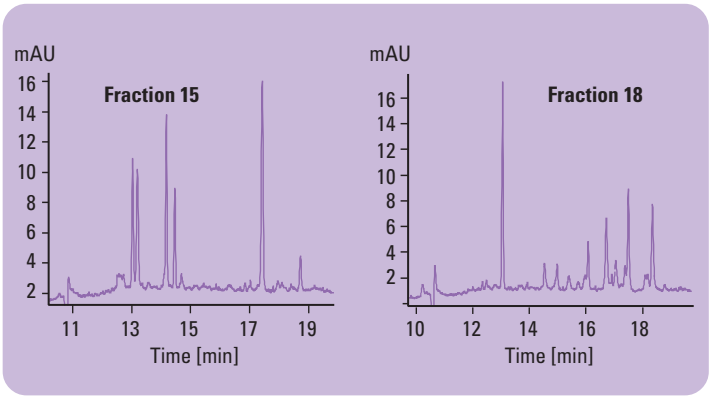



**Figure 29**
**CZE of reversed phase HPLC collected peptides**
Conditions: 30 mM, 17 mM phosphate, pH 8.2, V = 25 kV, i = 36 μA, l = 70 cm, L = 78.5 cm, id = 50 μm with 3X extended pathlength detection cell, λ = 200 nm

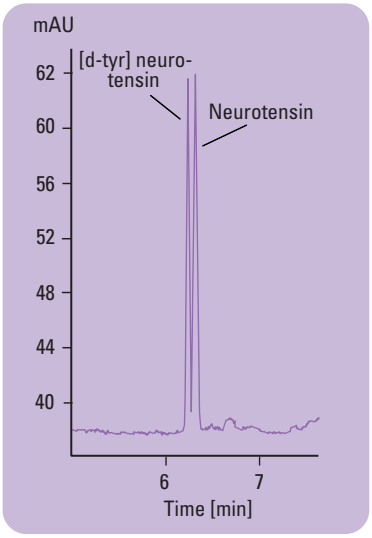



**Figure 30**
**CZE separation of neurotensin isomers**
Neurotensin amino sequence: pGlu-Leu-Try-Glu-Asn-Lys-Pro-Arg-Arg-Pro-**Tyr**-Ile-Leu-and pGlu-Leu-Try-Glu-Asn-Lys-Pro-Arg-Arg-Pro-**D-Tyr**-Ile-Leu.
Conditions: 30 mM tetraborate, pH 8.3, V = 18 kV, i = 42 μA, l = 50 cm, L = 65 cm, id = 50 μm with 3X extended pathlength detection cell, λ = 200 nm, peptide concentration $2.5 \times 10^{-5}$ M each

Under optimized conditions, the high efficiency is sufficient to resolve small differences in solute structure. As shown in figure 30, two 8-amino acid long peptides which differ only by the substitution of D-tyrosine for L-tyrosine can be resolved. This example illustrates the effect of conformational differences on mobility, since CZE cannot separate isomers without chiral additives.

Determination of drugs and drug metabolites has also been successful by CZE. Figure 31a shows the analysis of Cefixime (CX), an oral cephalosporin antibiotic, and its metabolites. The strong hydrophilicity of these metabolites makes extraction from biological fluids difficult. For the same reason, separation by reversed phase liquid chromatography is insufficient, even with ion-pairing agents. Identification of the drug in human urine is shown in figure 31b. Quantitative analysis within about 3 % RSD was obtained.

An example of the use of CZE for environmental analysis is shown in figure 32. In this study, conditions were optimized to separate aromatic sulfonic acids and related compounds. Here, 4-chlorobenzesulfonate is identified as a leachate at an environmental clean-up site in the U.S. Peak identification was confirmed by mass spectrometry. Along with

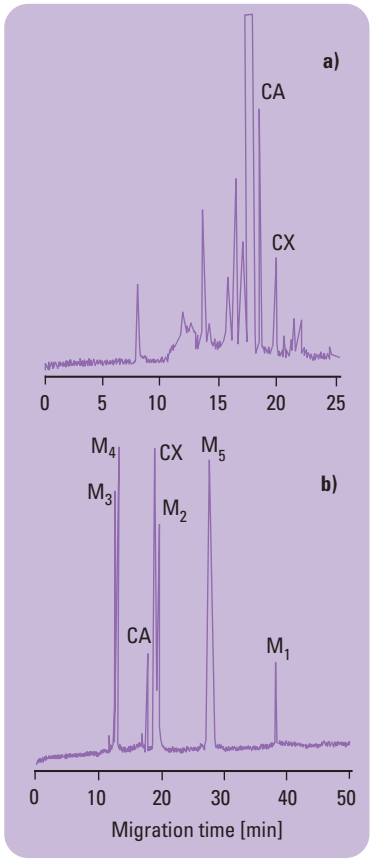



**Figure 31**
**a) CZE of Cefixime (CX) and its metabolites. b) Analysis of human urine spike with CX and cinnamic acid (CA)[14]**
Conditions: 50 mM phosphate, pH 6.8, V = 15 kV, l = 50 cm, L = 72 cm, id = 50 µm, λ = 280 nm

liquid chromatography, CE was suggested to hold great potential for the separation and characterization of polar, non-volatile environmental samples.

CZE has also been used for the separation of inorganic ions and organic acids, which has been traditionally performed by ion chromatography (figure 33). Indirect UV detection is necessary due to the lack of chromophores in these species. Indirect detection is performed using a highly absorbing species such as chromate or imidazole in the running buffer and monitoring at the absorbance maximum of that species (for example, 254 nm for chromate). Solute ions displace the chromate ions and a decrease in absorbance occurs when the zones pass the detector region. In order to decrease the analysis time for the cations shown here, a cationic surfactant (CTAB) was added to the running buffer to reverse the EOF toward the cathode. Due to the high mobility of small ions, however, the EOF is not strong enough to carry ions migrating in the opposite direction and thus anions cannot be analyzed simultaneously. In these small ion determinations, peak shapes are often skewed due to the conductivity differences between the running buffer and the ions.

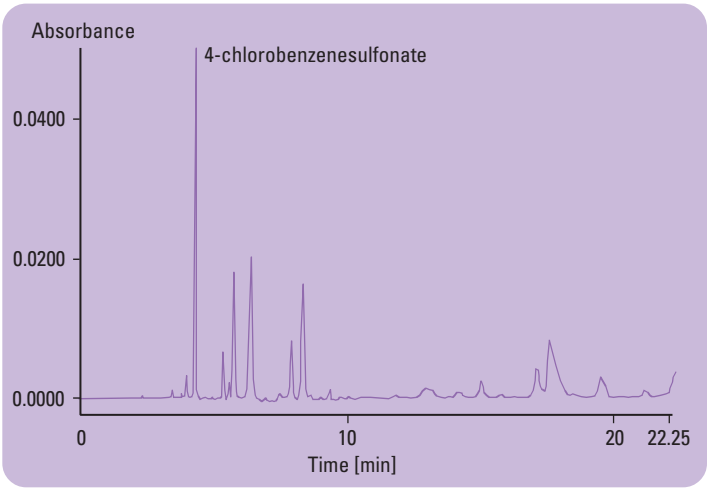
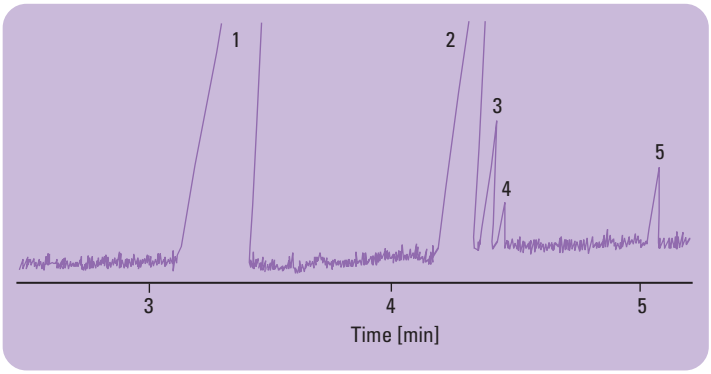



**Figure 32**
**Analysis of environmentally hazardous leachates by CZE[15]**
Conditions: 50 mM borate-boric acid, pH 8.3, V = 30 kV, i = 33 µA, l = 50 cm, L = 75 cm, id = 50 µm, λ = 280 nm

**Figure 33**
**Ion analysis of fermentation broth using indirect-UV detection[16]**
Peaks: 1 = $K^+$, 2 = $Na^+$, 3 = $Mg^{2+}$, 4 = $Mn^{2+}$, 5 = $Zn^{2+}$.
Conditions: 5 mM Waters UVCat-1, 6.5 mM α-hydroxyisobutyric acid, pH 4.2, v = 20 kV, i = 33 μA, l = 52 cm, L = 60 cm, id = 75 μm, λ = 214 nm, siphoning injection = 30 s/10 cm




## 3.2 Micellar electrokinetic chromatography

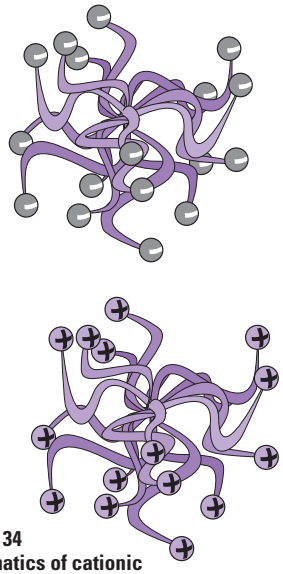


**Figure 34**
**Schematics of cationic and anionic micelles**

Micellar electrokinetic chromatography (MEKC or MECC) is a hybrid of electrophoresis and chromatography. Introduced by Terabe in 1984, MEKC is one of the most widely used CE modes. Its main strength is that it is the only electrophoretic phoretic technique that can be used for the separation of neutral solutes as well as charged ones.

The separation of neutral species by MEKC is accomplished by the use of surfactants in the running buffer. At concentrations above the critical micelle concentration (8 to 9 mM for SDS, for example), aggregates of individual surfactant molecules, micelles, are formed. Micelles are essentially spherical with the hydrophobic tails of the surfactant molecules oriented towards the center to avoid interaction with the hydrophilic buffer, and the charged heads oriented toward the buffer. A representation of micelles is depicted in figure 34. It is the differential interaction between the micelle and the neutral solutes that causes the separation.

The surfactant and thus the micelles are usually charged and migrate either with or against the EOF (depending on the charge). Anionic surfactants such as SDS migrate toward the anode, that is, in the opposite direction to the

EOF. Since the EOF is generally faster than the migration velocity of the micelles at neutral or basic pH, the net movement is in the direction of the EOF. During migration, the micelles can interact with solutes in a chromatographic manner through both hydrophobic and electrostatic interactions.

For neutral species, it is only partitioning in and out of the micelle that effects the separation. The more the solute interacts with the micelle the longer is its migration time since the micelle carries it against the EOF. When the solute is not in contact with the micelle it is simply carried with the EOF. The more hydrophobic compounds interact more strongly with the micelle and are "retained" longer. The overall MEKC separation process is depicted schematically in figure 35.

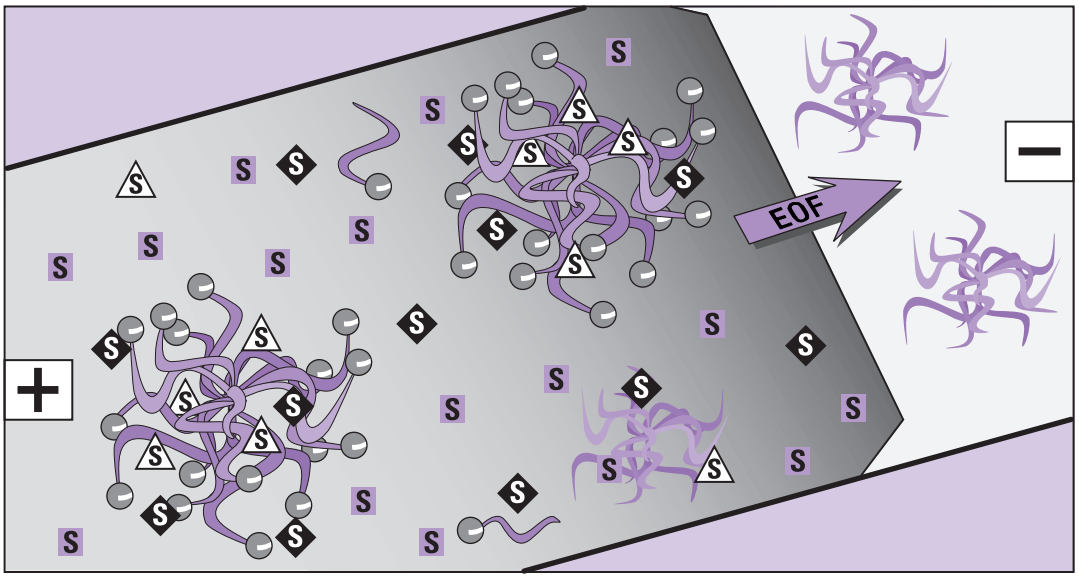



**Figure 35**
**Separation in MEKC**
s = solute

The separation mechanism of neutral solutes in MEKC is essentially chromatographic and can be described using modified chromatographic relationships. The ratio of the total moles of solute in the micelle (that is, the pseudo-stationary phase) to those in the mobile phase, the capacity factor, k', is given by:

$$k' = \frac{(t_r - t_0)}{t_0\left(1 - \frac{t_r}{t_m}\right)} = K\left(\frac{V_S}{V_M}\right) \qquad (24)$$

where
- $t_r$ = retention time of the solute,
- $t_0$ = retention time of unretained solute moving at the EOF rate (or "dead time")
- $t_m$ = micelle retention time
- $K$ = partition coefficient
- $V_S$ = volume of the micellar phase
- $V_M$ = volume of the mobile phase.

This equation is modified from the normal chromatographic description of k' to account for movement of the pseudo-stationary phase. Note that as $t_m$ becomes infinite (that is, the micelle becomes truly stationary) the equation reduces to its conventional form.

Resolution of two species in MEKC can be described by

$$R = \left(\frac{N^{1/2}}{4}\right)\left(\frac{\alpha - 1}{\alpha}\right)\left(\frac{k'_2}{k'_2 + 1}\right)\left(\frac{1 - \left(t_0/t_m\right)}{1 - \left(t_0/t_m\right)k_1}\right) \qquad (25)$$

Efficiency | Selectivity | Retention

where $\alpha = k_2' / k_1'$

From equation (25), resolution can be improved by optimizing efficiency, selectivity, and/or the capacity factor. With regard to the capacity factor, this can be most easily ad justed by varying the concentration of the surfactant. Generally, the capacity factor increases linearly with concentration. A potential problem with the use of ionic surfactants, especially at high concentrations, is the increase in generated current. Power generation exceeding 5 to 10 W/m at moderate field strengths can develop. Even with narrow-bore capillaries (25 to 50 mm) the use of extremely high electric fields is often avoided and efficient capillary thermostating is necessary.

Resolution is improved by extending the elution range or time window. In the separation of neutral solutes, all solutes elute between $t_0$ and $t_m$ (figure 36). Hydrophilic solutes that do not interact with the micelle elute with the EOF and those that are totally retained by the micelles (Sudan III, for example) elute with the micelles. While the time window is often fairly small, the peak capacity can be very high due to the high efficiency. It is therefore desirable to employ conditions that open the time window, that is, moderate EOF and micelles exhibiting high mobility.

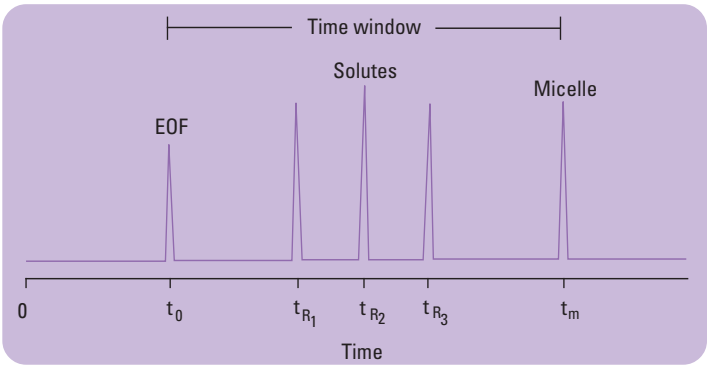



**Figure 36**
**Eluton time window for neutral solutes in MEKC**

The selectivity can easily be manipulated in MEKC. Varying the physical nature (that is, size, charge, geometry) of the micelle by using different surfactants can yield dramatic changes in selectivity, similar to those obtained by changing

the stationary phase in LC. Surfactants can be anionic, cationic, non-ionic, zwitterionic, or mixtures of each (table 14). In each category, alkyl chain length or structure can also be varied. In addition, MEKC can be performed using bile salts or microemulsions. In all cases, variations in buffer concentration, pH, temperature, or use of additives such as urea, metal ions, or chiral selectors can also be used to affect selectivity.

| | Biological detergents | CMC (mM) | Aggregation number |
|---|---|---|---|
| **Anionic** | SDS | 8.2 | 62 |
| **Cationic** | DTAB | 14 | 50 |
| | CTAB | 1.3 | 78 |
| **Non Ionic** | Octylglucoside | — | — |
| | n-Dodecyl-β-D-maltoside | 0.16 | — |
| | Triton X-100 | 0.24 | 140 |
| **Zwitterionic** | CHAPS | 8 | 10 |
| | CHAPSO | 8 | 11 |
| **Bile Salt** | Cholic acid | 14 | 2– 4 |
| | Deoxycholic acid | 5 | 4 –10 |
| | Taurocholic acid | 10 –15 | 4 |

**Table 14**
**Surfactants**

As in chromatography, organic modifiers can be added to manipulate solute-micelle interaction. Modifiers such as methanol, acetonitrile, and 2-propanol have all been used successfully. Added to the running buffer in concentrations from a few percent up to 50 % (v/v) can lessen hydrophobic interactions between the solute and micelle. They can also decrease the hydrophobic interactions which maintain micellar structure, allowing more rapid chromatographic kinetics.

Surfactants used for MEKC can also interact with the capillary wall and have dramatic effects on the EOF as well as solute-wall interactions. The direction of solute and

micelle migration varies and depends on the micelle charge and the rate of EOF. Generally, high pH buffers are used to maintain reasonable EOF and ensure migration direction.

## 3.2.1 Applications of MEKC

MEKC is a dynamic mode of CE since it can be used for charged and uncharged analytes and for a wide range of substances with hydrophilic or hydrophobic characteristics. Applications include amino acids, nucleotides, vitamins, a wide range of pharmaceuticals, aromatic hydrocarbons, and explosive constituents, to name a few.

For pharmaceuticals, MEKC has been used for the determination of active drugs in tablets, creams, and injectable formulations. The separation of the active ingredients of a cold-relief preparation using SDS micelles is illustrated in figure 37. The migration order is determined by solute lipophilicity and polarity. Only cationic, and to a certain extent, neutral species become incorporated with the micelle. In this work, the effect of SDS concentration and type, pH, and organic modifier were studied. In addition, quantitative analysis of Novapon granules was performed

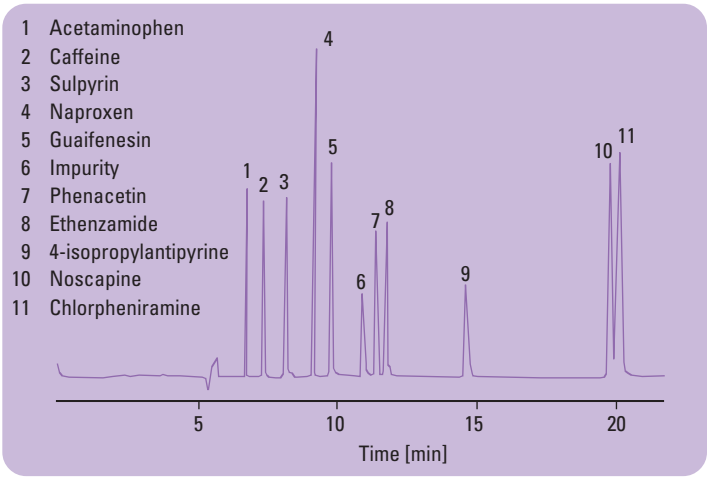



**Figure 37**
**MEKC separation of cold-relief medicine constituents[17]**
Conditions: 20 mM phosphate-borate, 100 mM SDS, pH 9, V= 20 kV, L = 65 cm, id = 50 µm, λ = 210 nm

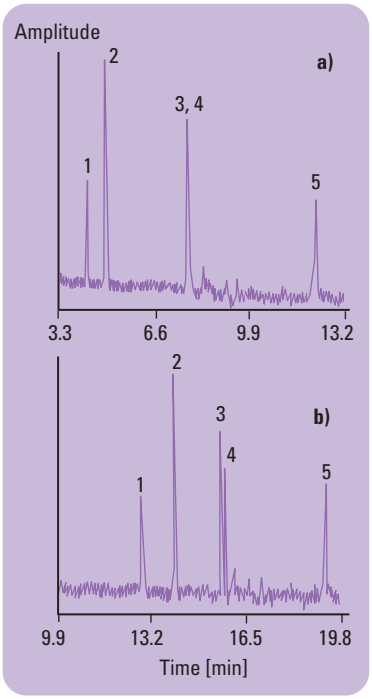



**Figure 38**
**Separation of closely related peptides using non-ionic surfactants[18]**
Peaks: 1 = bradykinin, 2 = luteinizing hormone releasing hormone, 3 = [val$^2$]-angiotensin III, 4 = angiotensin III, 5 = angiotensin II
Conditions: 250 mM phosphate, pH 7, 80 mM octyl glucoside (only in B), E = 250 V/cm, i = 33 μA, λ = 70cm, id = 17 μm, l = 210 nm, aryl pentafluoro coated capillaries

with a migration time reproducibility of 0.8 % RSD and a peak area reproducibility of 2.2 % RSD.

The use of non-ionic surfactants to enhance selectivity is illustrated in figure 38. Peaks 3 and 4 are angiotensin III neuropeptides which differ by a single methyl substitution. As shown, no resolution of peaks 3 and 4 was obtained by CZE (figure 38a). Upon addition of 80 mM octyl glucoside the pair was resolved. Non-ionic (and zwitterionic) surfactants are advantageous in that they do not dramatically change the EOF, do not increase the conductivity of the buffer, and can have little impact on protein structure or activity.

An example of the use of MEKC for the analysis of illicit drugs for forensic purposes is shown in figure 39. Here, a phosphate-borate buffer containing SDS and acetonitrile was employed. This analysis shows that MEKC is applicable to a large variety of forensic samples. It was especially useful for those samples that were difficult to analyze by GC, including phenethylamines, benzodiazapines, ergot alkalids, psilcybin, and PCP. For LSD and LAMPA, MEKC was thought to be superior to HPLC, which was usually employed.

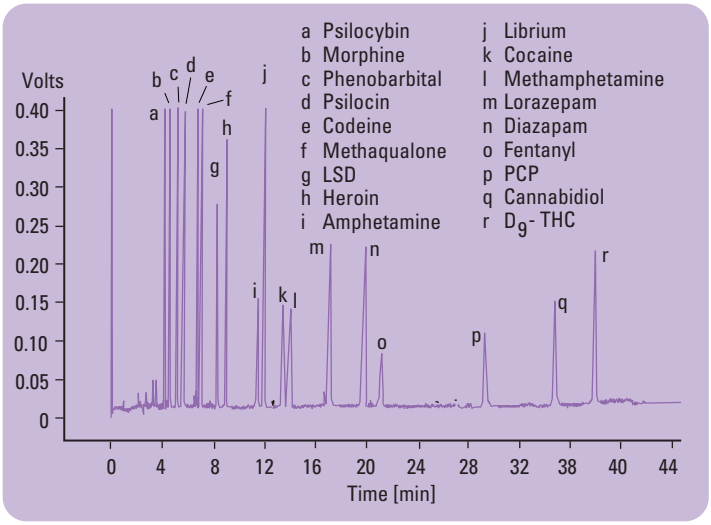



**Figure 39**
**MEKC forensic drug screen[19]**
Conditions: 8.5 mM borate, 8.5 mM phosphate, 85 mM SDS, 15 % acetonitrile, pH 8.5, V = 20 kV, l = 25 cm, L = 47 cm, id = 50 μm, λ = 210 nm

The versatility of MEKC is further exemplified by the determination of organic gunshot and explosive constituents. A separation of gunshot and explosive standards is shown in figure 40. Here, qualitative characterization of six reloading powders is made. Differences can be seen in each manufacturer's product reflecting the amounts of propellants, stabilizers, and plasticizers. Ageing of powders also gives rise to compositional changes. Overall, MEKC was considered to be a superior technique for such analyses due to excellent mass detection limits, low cost, rapid analysis time, superior resolution, and extremely small sample requirements. Further advantages included limited consumption of expensive and hazardous reagents.

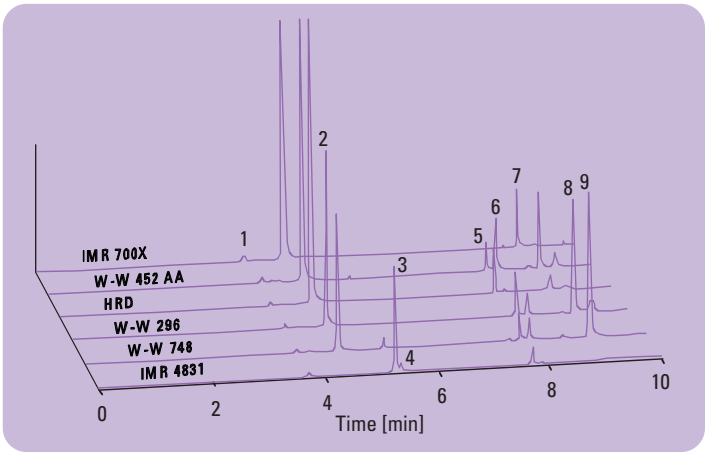



**Figure 40**
**MEKC of extracts from different reloading powders[20]**
Peaks: 1 = EtOH, 2 = nitroglycerin, 3 = 2,4-DNT, 4 = 2,6-DNT, 5 = diphenylamine, 6 = N-nitroso-diphenylamine, 7 = 2-nitrodiphenylamine, 8 = ethylcentralite, 9 = dibutylphthalate
Conditions: 2.5 mM borate, 25 mM SDS, pH 8.9, V = 20 kV, I = 50 cm, L = 67 cm, id = 100 μm, λ = 200 nm

## 3.3 Capillary gel electrophoresis

Gel electrophoresis has principally been employed in the biological sciences for the size-based separation of macromolecules such as proteins and nucleic acids. The size separation is obtained by electrophoresis of the solutes through a suitable polymer which acts as a "molecular sieve". This form of zonal electrophoresis is illustrated in figure 41. As charged solutes migrate through the polymer network they become hindered, with larger solutes

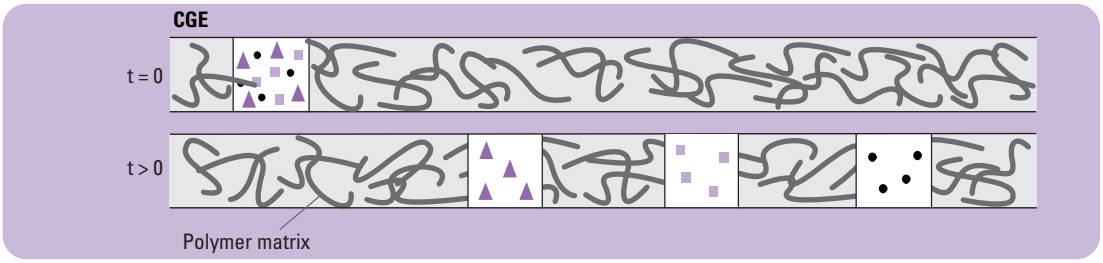



**Figure 41**
**Size-separation in CGE**

hindered more than smaller ones. Macromolecules such as DNA and SDS-saturated proteins cannot be separated without a gel since they contain mass-to-charge ratios that do not vary with size. That is, with DNA for example, each additional nucleotide added to a DNA chain adds an equivalent unit of mass and charge and does not affect the mobility in free solution.

Capillary gel electrophoresis (CGE) is directly comparable to traditional slab or tube gel electrophoresis since the separation mechanisms are identical. The CE format can offer a number of advantages over traditional slab gel electrophoresis, including the use of 10 to 100 times higher electric fields without the deleterious effects of Joule heating (although ultra-thin slab gels have recently been used to limit heating), on-capillary detection, and instrumental automation. In addition, due to the anticonvective nature of the capillary it is not necessary to use a gel that is itself anticonvective. The capacity to run preparative separations, considered a major advantage of the slab, can also be accomplished to a certain extent in HPCE by the use of wide-bore capillaries (internal diameters >100 to 200 μm) and low electric fields. The multi-lane capacity of a slab, however, is difficult to reproduce in capillary format, although the rapid analysis times in CGE compensate.

Traditionally, crosslinked polyacrylamide and agarose have been used in the slab or tube format. The former usually has

smaller mesh spacings (or pore sizes) and is used for protein separations. The larger mesh spacings of the latter are more suitable for DNA. In the slab or tube, these media are not only used to effect a size separation, but also because they are anticonvective and can hold their own shape. This latter requirement limits the minimum concentration and also the possible compositions of gels that can be used. As mentioned, this limitation is relaxed by the anticonvective nature of the narrow-bore capillary.

Use of the term "gel" in CGE is somewhat ambiguous. A gel usually implies a solid-like structure as used in slab-gel electrophoresis. Since many of the "gels" used in CGE do not (and need not) possess this property, a more suitable term may be "polymer network". Polymers in CGE can be covalently crosslinked (such as bis-polyacrylamide), hydrogen bonded (such as agarose), or linear polymer solutions (such as polyacrylamide or methylcellulose). Although the polymer structure of the uncrosslinked gel is radically different from that of crosslinked gels, the mechanism of separation is identical. Subsequently, macromolecules can be size-separated using either gel type (table 15).

**Table 15**
**Polymer matrices for CGE**

| Polymer | Concentration | Application |
|---|---|---|
| **Crosslinked polymers** | | |
| Polyacrylamide/bis-acrylamide | 2– 6 % T, 3 – 6 % C | • Oligonucleotides, DNA sequencing,<br>• Native and SDS-bound proteins |
| **Linear polymers** | | |
| Polyacrylamide | < 0.1– 6 % | • Restriction fragments |
| Hydroxylalkyl cellulose, polyvinyl alcohol, dextran | 6 –15 % | • Oligonucleotides, DNA sequencing, proteins |
| **Agarose** | 0.05 –1.2 % | • Restriction fragments<br>• Proteins |

Crosslinked polyacrylamide, a widely used matrix, is usually polymerized in situ and not removed from the capil- lary. Preparation of these gels requires extreme care. Too rapid polymerization, use of non-degassed solutions, or impure chemicals often lead to bubble formation or unstable gels. A potential disadvantage of crosslinked polyacrylamide is its rigid nature. Dirty samples, clogging of the ends, or bubble formation during use may make the capillary unusable. Care must be exercised to prevent these from occurring. With proper use, numerous injections on a single capillary are possible. In this regard, the rigidity of the gel precludes the use of hydrodynamic sample injection.

Linear polymers offer an alternative to the crosslinked gels. Since they are essentially polymer solutions, they are much more flexible. The linear polymer solutions may also be polymerized in situ, but it is not necessary. Pre-polymerized polymer can be dissolved in buffer and hydrodynamically loaded into the capillary. For polyacrylamide, a wide range of gel concentrations can be used (that is, below 1 % to more than 20 %). Generally, the polymer concentration necessary is inversely proportional to the size of the analyte.

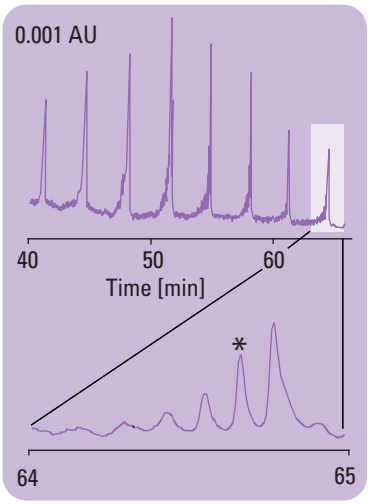



**Figure 42**
**CGE separation of polydeoxythyamidylic acid mixture, $p(dT)_{20-160}$ using crosslinked polyacrylamide[21]**

Conditions: Bis-crosslinked polyacrylamide (6 % T, 5 % C), 100 mM Tris, 25 mM borate, 7 mM urea, pH 7.6, E = 200 V/cm, i = 8.2 µA, l = 100 cm, id = 75 µm, λ = 260 nm, polyacrylamide coated capillary

With low viscosity polymer solutions, pressure can be used for sample injection and they can be repeatedly filled into the capillary (depending on concentration and viscosity). They are also less susceptible to bubble formation and other failures. While they possess more stability in these respects, the less viscous the gel the more dependent the system is on the integrity of the wall coating (if used). With either type, the capillary wall is usually coated to eliminate EOF.

Resolution and efficiency in CGE are identical to that in CZE since they are both "zonal" electrophoretic techniques. One notable difference is the ultra high efficiency achievable for DNA separations. Figure 42 illustrates that over $10^7$ plates/m (*) can be realized for single-stranded oligonucleotides using crosslinked polyacrylamide. As in CZE, selectivity in

CGE can be altered by the addition of chiral selectors, ion-pairing reagents, or another complexing agent (such as ethidium bromide for DNA and SDS for proteins). These species can be covalently bound to the gel or simply added to the running buffer.

## 3.3.1 Applications of CGE

CGE has been employed for both molecular biology and protein chemistry applications. The former includes oligonucleotide purity analysis, anti-sense gene therapy, DNA sequencing, PCR product analysis, and DNA forensics. The latter includes native and SDS-complexed protein separations.

Separation of double-stranded DNA restriction fragments, produced by the enzymatic digest of large DNA, have also been successful. Generally, the more open structure of linear or minimally crosslinked gels are better suited for these large molecules, which can range in size from a few base pairs to more than $10^6$ base pairs. The separation of a 1-kbp ladder, for example, is shown in figure 43.

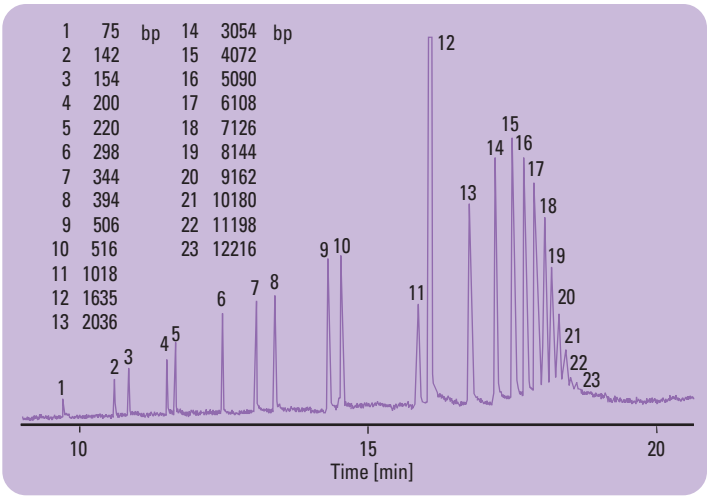



**Figure 43**
**CGE of 1 kbp ladder using minimally crosslinked polyacryl amide[22]**
Conditions: Bis-crosslinked polyacrylamide (3 % T, 0.5 % C), 100 mM Tris-borate, pH 8.3, E = 250 V/cm, i = 12.5 µA, l = 30 cm, L = 40 cm, id = 75 µm, λ = 260 nm, polyacrylamide coated capillary

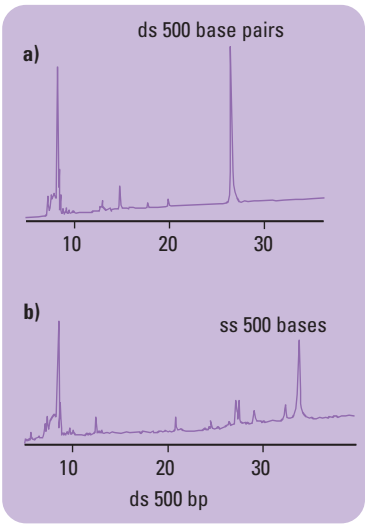



**Figure 44**
**PCR analysis of single and double stranded DNA**
Conditions: Uncrosslinked polyacrylamide (9 % T, 0 % C), 100 mM Tris-borate, pH 8.3, E = 300 V/cm, i = 9 μA, l = 20 cm, L = 40 cm, id = 75 μm, λ = 260 nm, poly-acrylmide coated capillary

This sample contains a ladder of 1-kbp DNA extending from 1 to 12-kbp and numerous smaller fragments which result from the enzymatic nature of the sample preparation. The resolution of fragments from 75 bp to 12 kbp in a single analysis illustrates the wide sample range of CGE.

An example of PCR product analysis is shown in figure 44. Here, the analysis of a single-stranded DNA prepared by asymmetric PCR is shown. The peaks were identified by a calibration curve obtained using DNA size standards. Note that the single-stranded DNA migrates slower than the double-stranded DNA of the same size due to increased random three-dimensional structure. In addition, the lack of specificity of asymmetric PCR yields more by-products than normal PCR.

In the area of protein separations, much research has gone into SDS-gels for size-based separation. Both standard SDS-PAGE gels and linear gels with SDS have been employed. The separation of size standards is shown in figure 45 using crosslinked polyacrylamide. Alternatively, linear dextran polymers have been used since these offer higher gel stability and lower background absorbance at low wave-lengths.

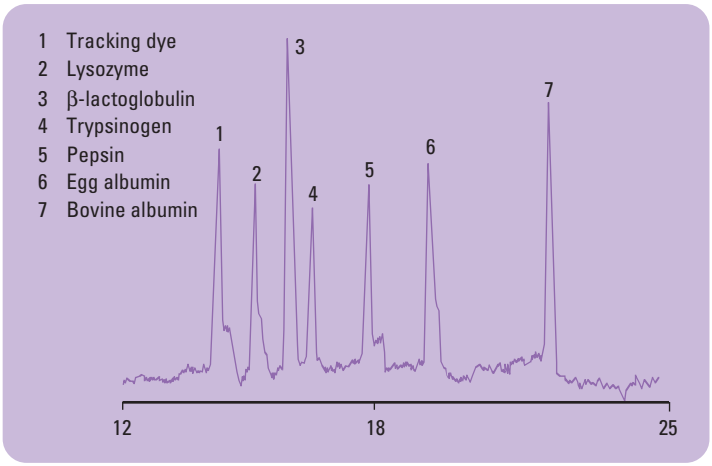



**Figure 45**
**SDS-PAGE separation of protein standards[23]**
Conditions: Bis-crosslinked polyacrylamide (7.5 % T, 5 % C), 100 mM tris-borate, 0.1 % SDS, 8 M urea, pH 7.3, E = 300 V/cm, i = 12 μA, l = 15 cm, id = 75 μm, λ = 280 nm, polyacrylamide coated capillary

## 3.4 Capillary isoelectric focusing

Capillary isoelectric focusing (CIEF) is a "high resolution" electrophoretic technique used to separate peptides and proteins on the basis of pI. CIEF can be used to separate proteins that differ by 0.005 pI units and less. Similar to CGE, this is a well-established gel electrophoretic technique recently adapted to the CE format.

In CIEF a pH gradient is formed within the capillary using ampholytes. Ampholytes are molecules that contain both an acidic and a basic moiety (that is, they are zwitterionic) and can have pI values that span the desired pH range of the CIEF experiment (pH 3 to 9, for example). After filling the capillary with a mixture of solute and ampholytes, the gradient is formed. With a basic solution at the cathode and an acidic solution at the anode, upon application of the electric field the charged ampholytes and proteins migrate through the medium until they reach a region where they become uncharged (at their pI). This process is known as "focusing". The protein zones remain narrow since a protein which enters a zone of different pH will become charged and migrate back. The overall separation process was depicted in figure 21b.

The status of the focusing process is indicated by the current. Once complete, a steady-state is reached and current no longer flows. After focusing, the solutes and ampholytes are mobilized and the zones passed through the detector. Mobilization can be accomplished by either application of pressure to the capillary or by addition of salt to one of the reservoirs.

The zone width, $\sigma$ (standard deviation), in CIEF is given by

$$\sigma = \left[ \frac{D}{\left( d\mu / d\mathrm{pH} \right) \left( d\mathrm{pH} / dx \right)} \right]^{1/2} \qquad (26)$$

and resolution with respect to pI difference is given by:

$$\Delta pI = 3 \left[ \frac{\left( dpH / dx \right)}{E \left( -d\mu / dpH \right)} \right]^{1/2} \quad (27)$$

where
- D = diffusion coefficient ($cm^2/s$)
- E = electric field (V/cm)
- $dpH/dx$ = pH gradient at the zone
- $d\mu/dpH$ = mobility slope at the pI (which depends on the charge of the protein near its pI).

As described by equation (27) high resolution is obtained with high electric fields, a high mobility slope at the isoelectric point, and a shallow rate of change of pH.

EOF needs to be reduced or eliminated in CIEF since the flow could flush the ampholytes from the capillary before focusing is complete. Reduction of EOF can be accomplished by the use of dynamic or covalent coatings. The dynamic coatings have the advantage of simplicity, but obtaining reproducible EOF is often difficult. The coating, either dynamic or covalent, is also helpful in limiting protein adsorption to the capillary walls.

Since the solute is loaded on to the capillary during filling with the amphoteric solution, it is possible to load significantly larger volumes than in most other CE modes. Precipitation, which results from very high protein concentrations within the zones, is usually the limiting factor in sample loading.

## 3.4.1 Applications of CIEF

CIEF has been used successfully to measure protein pI and for the separation of isoforms, and other protein species problematic by other methods, including immunoglobulins and hemoglobins, and analysis of dilute biological solutions. CIEF of protein standards is shown in figure 46. An example of the use of CIEF for the separation of transferrin isoforms is shown in figure 47.

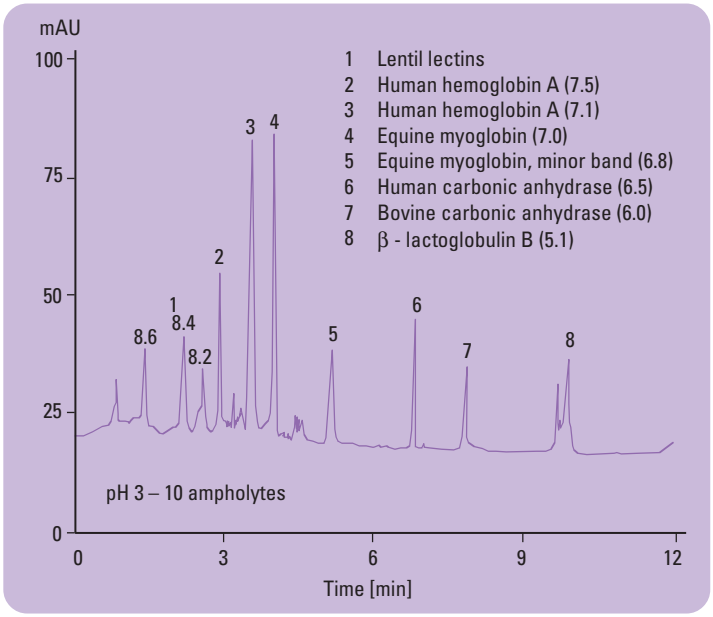



**Figure 46**
**CIEF of standard protein mixture[24]**
Polyacrylamide coated capillary

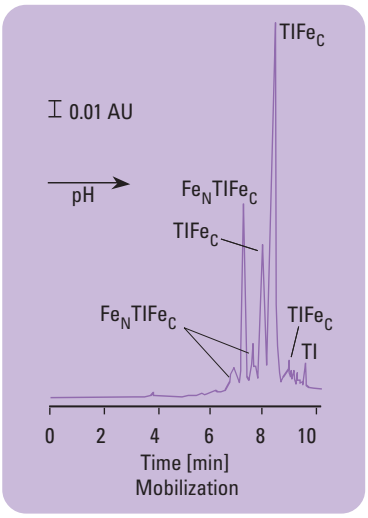



**Figure 47**
**CIEF of transferrin isoforms[25]**
Conditions: V = 4kV for focusing and mobilization, 2 % Bio-Lyte 5/7 (containing 1 mg of transferrin), L = 14 cm, id =100 μm, λ = 280 nm for protein and 460 for iron

## 3.5 Capillary isotachophoresis

Capillary isotachophoresis (CITP) is a "moving boundary" electrophoretic technique. In ITP, a combination of two buffer systems is used to create a state in which the separated zones all move at the same velocity. The zones remain sandwiched between so-called leading and terminating electrolytes. In a single ITP experiment either cations or anions can be analyzed.

For anion analyses, for example, the buffer must be selected so that the leading electrolyte contains an anion with an effective mobility that is higher than that of the solutes. Similarly, the terminating anion must have a lower mobility than that of the solutes. When the electric field is applied the anions start to migrate towards the anode. Since the leading anion has the highest mobility it moves fastest, followed by the anion with the next highest mobility, and so on. In ITP the individual anions migrate in discrete zones, but all move at the same velocity, as defined by the velocity of the leading anion. The separation process was illustrated in figure 21c.

The steady-state velocity in ITP occurs since the electric field varies in each zone. The field is self-adjusting to maintain constant velocity (that is, velocity = mobility x field), with the lowest field across the zone with highest mobility. This phenomenon maintains very sharp boundaries between the zones. If an ion diffuses into a neighboring zone its velocity changes and it immediately returns to its own zone.

Another interesting feature of ITP is the constant concentration in each zone, determined by the concentration of the leading electrolyte. Since ITP is usually performed in constant current mode, a constant ratio must exist between the concentration and the mobility of the ions in each zone. Zones that are less (or more) concentrated than the leading electrolyte are sharpened (or broadened) to adapt to the proper concentration. The solute-concentrating principle of

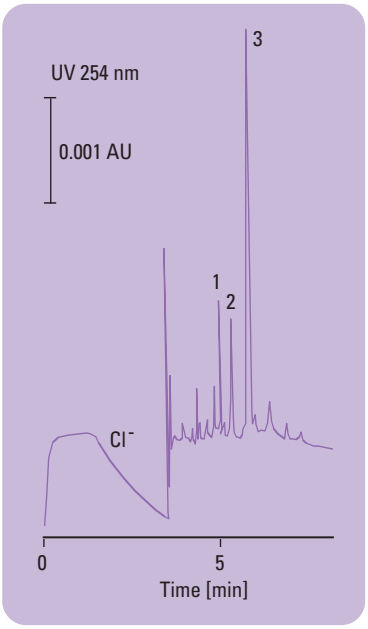



**Figure 48**
**CITP-stacked uraemic sera separation by CZE[26]**
Peaks (tentative): 1 = huppuric acid, 2 = p-hydroyhippuric acid, 3 = uric acid
Conditions: 10 mM Mes, histidine, pH 6.05, 0.05 % methyl hydroxyethyl cellulose, constant current = 35 μA, l = 25 cm, id = 200 μm, PTFE capillaries

ITP has been used as a preconcentration step prior to CZE, MEKC, or CGE. In most cases, a true ITP steady-state is not obtained since the modes are mixed. Nonetheless, up to 30 to 50 % of the capillary can be filled with sample while maintaining good separation quality. A difficulty often arises with finding buffer systems that contain both leading and trailing ions and also form the desired pH. An additional limitation is that only cations or anions can be sharpened, not both simultaneously.

Zone sharpening can occur upon the addition of a high concentration of leading and/or trailing electrolytes to the sample (that is, addition of salt to the sample). An example of such improvements is illustrated in figure 48. Here, the sample contains a high concentration of chloride (≅110 mM) in addition to the solutes of interest. The earliest eluting peaks exhibit over $10^6$ plates, significantly more than described by simple theory. This example also illustrates how ITP effects can inadvertently occur, especially with complex samples.

Chapter 4

# Instrumental and operational aspects of CE

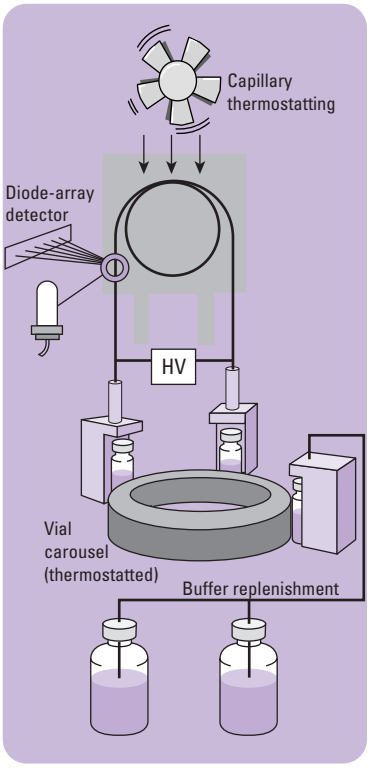



**Figure 49**
**Schematic of CE instrumentation**

This chapter describes the instrumental and experimental aspects of CE. In order to facilitate the discussion, the Agilent CE system will be used as an example. However, most instrumental aspects are described generically and need not be specific to this instrument.

The basic instrumental design is shown schematically in figure 49. A typical CE experiment, performed by these Integrated components, involves a series of steps: 1) removal of the inlet buffer reservoir and replacing it with sample vial; 2) loading the sample by applying either low pressure or voltage across the capillary; 3) replacing the inlet buffer reservoir; 4) applying the separation voltage. After a period of time, the separated sample zones reach the region of the optical window where spectrophotometric detection takes lace.

The individual components for injection, separation, detection, and liquid handling are described in the following sections. Discussions include the different types of sample injection and quantitative aspects of injection, capillary thermostating, high voltage power supply considerations, and UV-Visible and diode-array detection. Aspects of liquid handling include buffer replenishment, buffer leveling, autosampling, and fraction collection. Basic instrument features designed to simplify method development and automated analysis are also considered.

## 4.1 Sample injection

In CE only minute volumes of sample are loaded into the capillary in order to maintain high efficiency. These small volumes are, of course, proportional to the small volumes of the capillaries. With respect to sample overloading, the injection plug length is a more critical parameter than

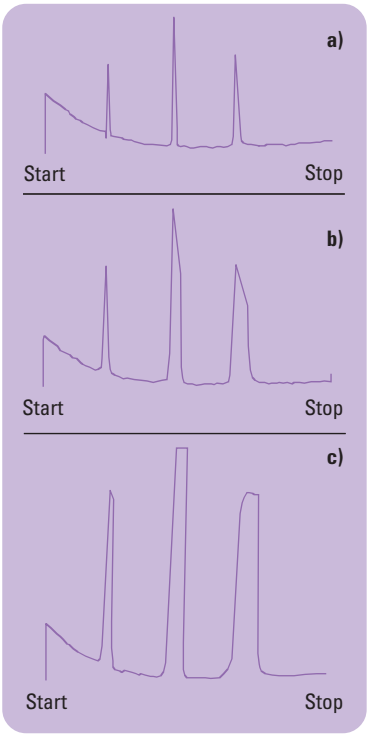



**Figure 50**
**Effect of sample overloading on peak shape 27**
Starting zone widths: a = 0.6 cm,
b = 2.0 cm,
c = 3.0 cm

volume. As a rule of thumb, the sample plug length should be less than 1 to 2 % of the total length of the capillary. This corresponds to an injection length of a few millimeters (or 1 to 50 nl), depending on the length and inner diameter. This is an advantage when sample volumes are limited since 5 µl of sample is sufficient to perform numerous injections. Conversely, the small volumes seriously increase sensitivity difficulties for dilute samples.

Sample overloading can have two significant effects, both detrimental to resolution (figure 50). First, injection lengths longer than the diffusion controlled zone width will proportionally broaden peak width. Secondly, it can exacerbate field inhomogeneities and distorted peak shapes caused by mismatched conductivity between the running buffer and the sample zone.

Quantitative sample injection can be accomplished by a number of methods. The two most common are hydrodynamic and electrokinetic (figure 51). In either case, the sample volume loaded is generally not a known quantity, although it can be calculated. Instead of volume, the quantifiable parameters are pressure/time for hydrodynamic injection, or voltage/time for electroldnetic injection, as described in detail in the next two sub-sections.

## 4.1.1 Hydrodynamic injection

Hydrodynamic sample injection is the most widely used method. It can be accomplished by application of pressure at the injection end of the capillary, vacuum at the exit end of the capillary, or by siphoning action obtained by elevating the injection reservoir relative to the exit reservoir (figure 51a,b,c). With hydrodynamic injection, the quantity of sample loaded is nearly independent of the sample matrix.

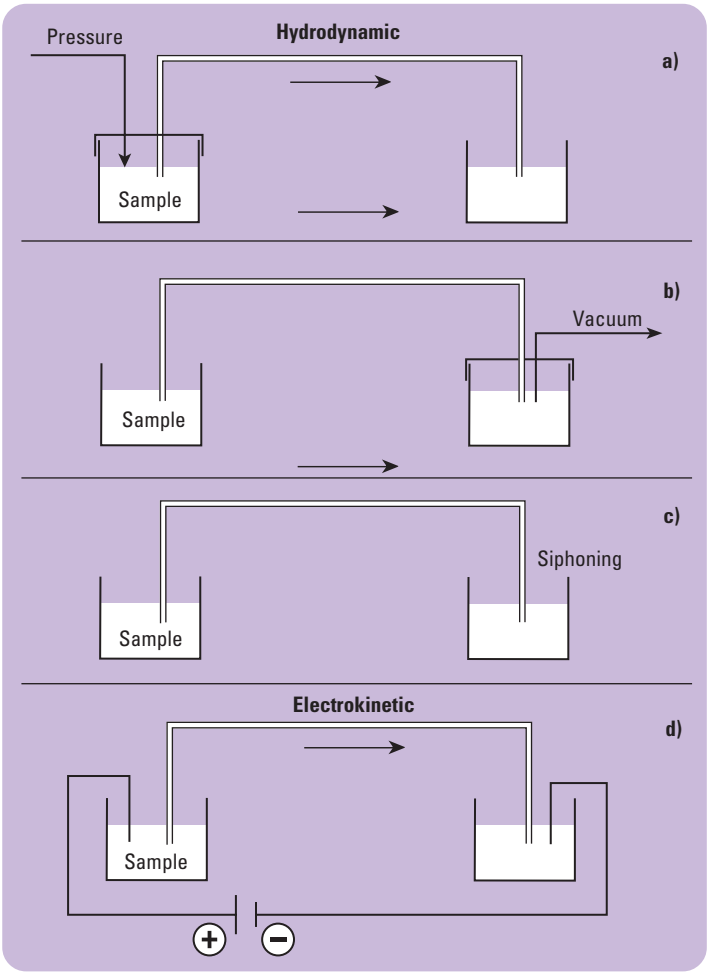



**Figure 51**
**Methods of sample injection**

The volume of sample loaded will be a function of the capillary dimensions, the viscosity of the buffer in the capillary, the applied pressure, and the time. This volume can be using the Hagen-Poiseuille equation.

$$\text{Volume} = \frac{\Delta P d^4 \pi t}{128 \eta L} \qquad (28)$$

where $\Delta P$ = pressure difference across the capillary
$d$ = capillary inside diameter
$t$ = time
$\eta$ = buffer viscosity
$L$ = total capillary length

Typical injection pressures and times range from 25 to 100 mbar and 0.5 to 5 s, respectively. Using equation (28), the sample plug volume and length loaded, as a function of applied pressure, time, and capillary dimensions, is given in table 16.

| Injection pressure x time, mbars | 10 µm $V_p$ (nl) | 10 µm $l_p$ (mm) | 25 µm $V_p$ (nl) | 25 µm $l_p$ (mm) | 50 µm $V_p$ (nl) | 50 µm $l_p$ (mm) | 75 µm $V_p$ (nl) | 75 µm $l_p$ (mm) | 100 µm $V_p$ (nl) | 100 µm $l_p$ (mm) |
|---|---|---|---|---|---|---|---|---|---|---|
| 25 | 0.0008 | 0.01 | 0.03 | 0.06 | 0.5 | 0.25 | 2.6 | 0.59 | 8.2 | 1.04 |
| 50 | 0.0016 | 0.02 | 0.06 | 0.12 | 1.0 | 0.50 | 5.2 | 1.18 | 16.4 | 2.08 |
| 75 | 0.0024 | 0.03 | 0.09 | 0.18 | 1.5 | 0.75 | 7.8 | 1.77 | 24.6 | 3.13 |
| 100 | 0.0032 | 0.04 | 0.12 | 0.24 | 2.0 | 1.00 | 10.4 | 2.26 | 32.8 | 4.16 |
| 150 | 0.0048 | 0.06 | 0.18 | 0.36 | 3.0 | 1.50 | 15.6 | 3.54 | 49.2 | 6.26 |

$V_p$ = plug volume L = 75 cm $\eta$ = 1
$l_p$ = plug lengths T = 25 °C

**Table 16**
**Injection volume and plug length as a function of pressure, time and capillary id**

For siphoning injection, the pressure differential for use in equation (28) is given in

$$\Delta P = \rho g \Delta H \qquad (29)$$

where $p$ = buffer density
$g$ = gravitional constant
$\Delta h$ = height differential of the reservoirs.

A typical siphoning injection is obtained by raising the sample reservoir 5 to 10 cm relative to the exit reservoir for 10 to 30 seconds, depending on the conditions. Siphoning is typically used in systems without pressure injection capabilities.

If sensitivity is not limiting, the smallest injection lengths possible should be used. However, injection reproducibility is usually diminished with short injection times due to instrumental limitations. This is especially true when short and/or wide-bore capillaries are employed or when concentrated samples are used. Reproducibility can be improved significantly by use of an integrated pressure/time profile with active feedback control to compensate for system risetime effects and variations in the applied pressure.

From an instrumental standpoint, injection reproducibility can be better than 1 to 2 % RSD. Reproducibility in peak area, however, can be reduced by other phenomena, including sample interaction with the variations in capillary temperature, capillary walls, integration of peaks with low signal-to-noise ratios, and so on (see table 19).

Precise temperature control (± 0.1°C) of the capillary is necessary to maintain constant injection volume. As with migration time, viscosity of the buffer in the capillary and thus the injected quantity varies 2 to 3 % per °C. Note that sample viscosity does not significantly affect injection volume since the sample plug is only a very small volume relative to the total liquid volume in the capillary.

Finally, to avoid unwanted sample injection by siphoning it is important that the duration of the injection be as short as possible. In addition, the liquid levels of the sample and buffer reservoirs should be equal. Siphoning can cause poor peak area reproducibility and even overloading. It has also been found that simply placing the capillary in a sample reservoir will cause an injection due to capillary action. This phenomenon has been called a zero-injection effect. While often insignificant, with concentrated samples the injected amount can be quantifiable and should be considered during quantitative analysis.

## 4.1.2 Electrokinetic injection

Electrokinetic, or electromigration, injection is performed by replacing the injection-end reservoir with the sample vial and applying the voltage (figure 51d). Usually a field strength 3 to 5 times lower than that used for separation is applied. In electroldnetic injection, analyte enters the capillary by both migration and by the pumping action of the EOF. A unique property of electrokinetic injection is that the quantity loaded is dependent on the electrophoretic mobility of the individual solutes. Discrimination occurs for ionic species since the more mobile ions are loaded to a greater extent than those that are less mobile.

The quantity injected, Q (g or moles), can be calculated by

$$Q = \frac{(\mu_e + \mu_{EOF})\, V\pi r_2 C t}{L} \qquad (30)$$

where
- $\mu$ = electrophoretic mobility of the analyte
- $\mu_{EOF}$ = EOF mobility
- V = voltage
- r = capillary radius
- C = analyte concentration
- t = time
- L = capillary total length

As described by equation (30), sample loading is dependent on the EOF, sample concentration, and sample mobility. Variations in conductivity, which can be due to matrix effects such as a large quantity of an undetected ion such as sodium or chloride, result in differences in voltage drop and quantity loaded (figure 52). Due to these phenomena electroldnetic injection is generally not as reproducible as its hydrodynamic counterpart.

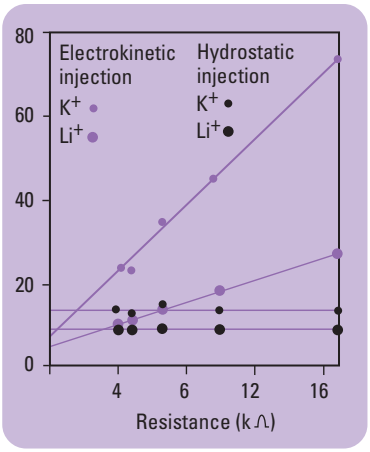



**Figure 52**
**Quantity of sample loaded as a function of sample resisteance for hydrodynamic and electrokinetic injection[28]**

Despite quantitative limitations, electrokinetic injection is very simple, requires no additional instrumentation, and is advantageous when viscous media or gels are employed in the capillary and when hydrodynamic injection is ineffective.

### 4.1.3 On-capillary sample concentration

Several techniques have been described to enhance sensitivity by on-capillary sample concentration during or just after sample injection. These methods are based on the field strength differences between the sample zone and the running buffer, and are called “stacking”. Generating an isotachophoretic system is one method. As described in section 3.5, in ITP the concentrations of each migrating analyte will adopt the concentration of the leading electrolyte. Theoretically, orders of magnitude concentration can be obtained by ITP. Despite often not attaining true steady-state ITP, the properties can be used to increase sample concentration upon injection simply by proper choice of running buffers.

Another method of stacking is obtained when the conductivity of the sample is significantly lower than that of the running buffer. Upon application of the voltage, a proportionally greater field will develop across the sample zone causing the ions to migrate faster. Once the ions reach the running buffer boundary, the field decreases and they migrate slower. This continues until all of the ions in the sample zone reach the boundary and cause the sample to become concentrated into a smaller zone. At this point, the field becomes homogeneous in the zone and normal electrophoresis begins.

The simplest way to perform a stacking experiment is to dissolve the sample in water or low conductivity buffer (for example, 100 to 1000 times lower than that of the running buffer) and inject normally either hydrodynamically or electrokinetically. Stacking will occur automatically. More than a 10-fold sample enrichment can be obtained (figure 53). If the conductivity of the sample and running buffer are equivalent, stacking can be induced by injecting a short plug of water before sample introduction.

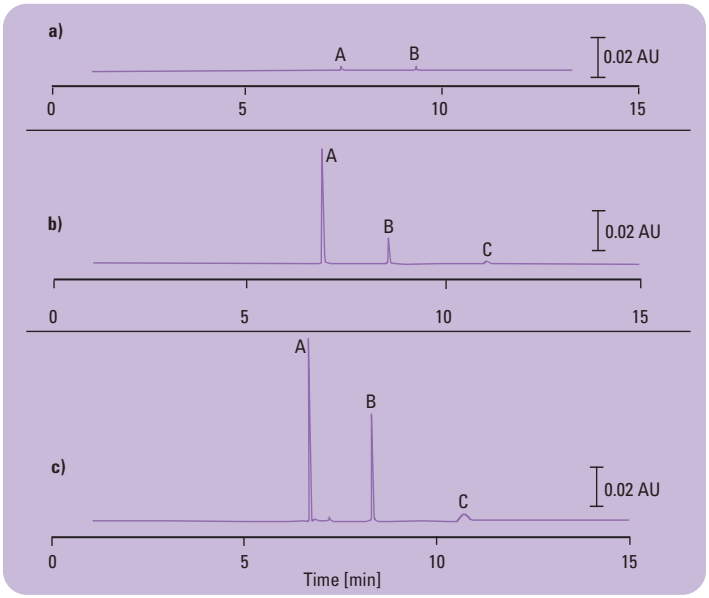



**Figure 53**
**Field amplified sample injection[29]**
a) sample dissolved in buffer
b) sample dissolved in water
c) short plug of water injected before sample in (b)

Other stacking methods have been described in which up to 50 % of the capillary can be filled with sample, the buffer removed by the EOF, and the sample stacked in a small zone at the head of the capillary (called field amplified injection). Effective use of these methods is limited, however, by the electro-osmotic pressure developed at the boundary between the water and buffer zones. This pressure difference causes generation of laminar flow and

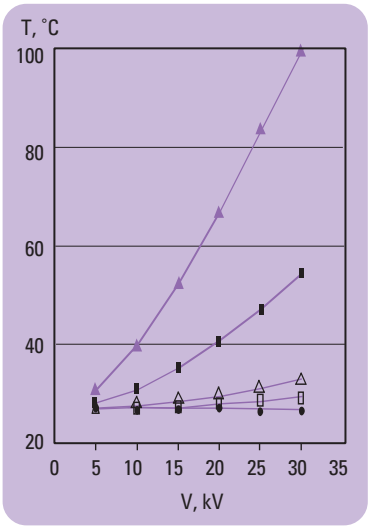



**Figure 54**
**Estimated temperature of sample zone and running buffer under stacking conditions[30]**
closed symbols = sample zone
open symbols = buffer
Conditions: 10 mM tricine, pH 8.0,
containing 0 mM NaCl (circles)
25 mM NaCl (squares)
50 mM NaCl (triangles)

results in zone broadening. Optimal stacking is obtained when the running buffer concentration is about 10 times of the sample and when the plug length is up to 10 times the diffusion-limited peak width.

One further consideration in the use of stacking during injection is the generation of heat in the sample zone. Under typical stacking conditions most of the voltage drop occurs in the stacking zone. The corresponding power generation can result in significantly elevated temperatures. In fact, temperatures exceeding 90 °C in the sample zone, even with capillary thermostating, have been reported (figure 54). This can be of particular concern for thermally labile samples.

## 4.2 Separation

The separation step in the CE experiment includes the following components: the capillary, capillary thermostating system, and power supply. Each is described in the following sub-sections, with emphasis on aspects of migration time/mobility reproducibility.

### 4.2.1 Capillary

Ideal properties of the capillary material include being chemically and electrically inert, UV-Visible transparent flexible and robust, and inexpensive. Meeting most of these requirements, fused silica is the primary material employed today. Fused silica has been used in applications such

as optic cells and GC columns. Similar to GC columns, the capillaries are coated with a protective layer of polyimide to make them strong and easy to handle. For detection, an optical window can easily be placed in the capillary by removal of a small section of the protective polyimide coating. This is accomplished by burning off a few millimeters of polyimide using an electrical arc or electrically-heated wire, or by scraping with a razor blade. Care must be exercised when handling a capillary with a window since it is very brittle after the polyimide is removed.

Teflon is another material used in CE, although not to the extent of fused silica. Teflon is transparent to UV and thus requires no special optical window. Although unchanged, it also exhibits significant EOF. Unfortunately, it is difficult to obtain with homogeneous inner diameters, exhibits sample adsorption problems similar to fused silica, and has poor heat transfer properties. These disadvantages have limited its use.

Fused silica capillaries with intemal diameters ranging from 10- to 200-µm with a range of outer diameters are available, however, 25- to 75-µm id and 350- to 400-µm od are typical. From an analysis time perspective, capillaries as short as possible should be used. Effective lengths range from as short as 10 cm for gel-filled capillaries and as long as 80 to 100 cm for complex-sample CZE separations. Most commonly, 50- to 75-cm effective lengths are employed. Ideally the effective length should be as large a percentage of the total length as possible in order to be able to apply very high electric fields and to decrease the time necessary for capillary conditioning, fraction collection, and so on. The total length is generally 5 to 15 cm longer, depending on the dimensions of the instrument (that is, the distance from the detector to the exit reservoir).

### 4.2.1.1 Capillary conditioning

One of the most important factors leading to good reproducibility is capillary conditioning. Maintaining a reproducible capillary surface is one of the most significant problems in CE. The most reproducible conditions are encountered when no conditioning other than with buffer is employed. However, adsorption of sample to the surface and changes in EOF often do not allow this.

Base conditioning to remove adsorbates and refresh the surface by deprotonation of the silanol groups is most commonly employed. A typical wash method includes flushing a new capillary with 1N NaOH, followed by 0. 1 N NaOH and then buffer. Before each analysis only the last two steps are performed. Other washing procedures can employ strong acids, organics such as methanol or DMSO, or detergents.

A potential problem with base conditioning, especially when employing low pH running buffers, is a hysteresis of the wall charge (figure 55). This hysteresis can cause non-reproducible EOF and necessitate long equilibration times. Unless migration time reproducibility is unacceptable or significant solute adsorption occurs, it may be advisable to avoid conditioning with basic solutions, especially when using low pH running buffers. Equilibration of the surface at neutral or high pH is rapid and generally not problematic.

An additional factor in maintaining constant surface charge is adsorption of buffer components. Phosphate, for example, is known to adsorb to the surface and to require long equilibration times. In addition, surfactants can render permanent changes to the capillary surface. It has been suggested that once a capillary is exposed to a particular detergent it should be dedicated for use only for buffers containing that surfactant if high reproducibility is required.

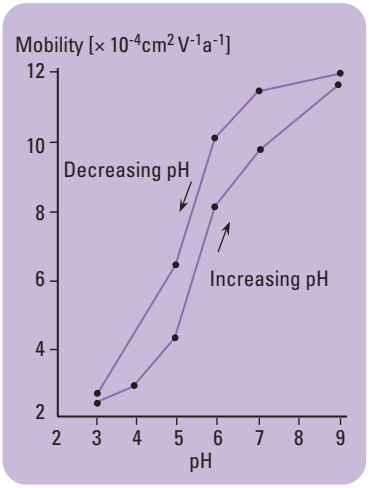



**Figure 55**
**pH hysteresis of EOF in fused silica capillaries[31]**
Conditions: 10 mM phosphate, benzyl-alcohol neutral marker, 25 kV, 25 °C, L = 80 cm, id = 75 µm

Batch-to-batch reproducibility is highly dependent on the nature of the fused silica itself. Surface charge and EOF can vary 5 % RSD or more between capillary batches. Comparison of inter-capillary data often requires normalization of EOF.

### 4.2.2 Capillary thermostating

Effective control of capillary temperature is important for reproducible operation. Temperature regulation to ± 0.1 °C is beneficial due to the strong viscosity dependence of sample injection and migration time. Further, the system should isolate the capillary from changes in ambient temperature. The two approaches generally used are to bath the capillary in a high velocity air stream or in a liquid. While liquid thermostating is theoretically more efficient, forced air thermostating at ≡ 10 m/s air velocity is usually sufficient for the quantity of heat generated in CE. As shown in the Ohm's law plots of figure 14, efficiency of the two systems is similar up to about 5 W/m. Although the liquid system is more effective at higher power generation, CE experiments are not usually performed under such conditions. A benefit of the air thermostating system is instrumental simplicity and ease of use.

### 4.2.3 High voltage power supply

In CE a DC power supply is used to apply up to about 30 kV and current levels of 200 to 300 mA. Stable regulation of the voltage (± 0.1 %) is required to maintain high migration time reproducibility.

The power supply should have the capability to switch polarity. Under normal conditions the EOF is in the direction of the cathode. In this case, injection is made at the anode.

However, if EOF is reduced, reversed, or if gels are used, it may be necessary to reverse the polarity of the electrodes; that is, to switch the cathode to the injection end. Since the inlet and outlet ends of the capillary are usually predetermined by the detector geometry, polarity switching must be performed at the power supply. This can best be accomplished by use of a dual polarity power supply. With such a power supply it is important to realize that the high voltage electrode and the ground electrode remain fixed. That is, the high voltage electrode is driven either positive or negative with respect to the ground electrode. It is also beneficial if polarity switching is software controlled, especially if switching is desired during an analysis.

While constant voltage analyses are most common, it is often beneficial to use either constant current or constant power modes. Constant current or power mode is particularly useful for isotachophoretic experiments or when capillary temperature is not adequately controlled. With regard to the latter, temperature changes alter buffer viscosity and migration time in constant voltage mode. In constant current mode, these viscosity changes are compensated by proportional changes in the applied voltage, maintaining constant migration time.

Another power supply feature is the ability to run voltage, current, or power gradients (also called field programming) during an analysis. Field programming can be used to ramp the voltage at the beginning of an analysis to avoid rapid heating, thermal expansion of buffer, an expulsion of sample from the capillary. Field programming is also particularly useful for decreasing the analysis time of complex samples and is often necessary for fraction collection. Since manipulation of narrow, closely spaced solute zones (for example, 5 to10 s) is difficult under high field conditions, reduction of the field immediately prior to collection increases the time window and relaxes the stringent timing problems associated with precise collection *(see section 4.4.2)*.

## 4.2.4 Migration time/mobility reproducibility

Run-to-run migration time and mobility reproducibility in CE can often be better than 0.5 % RSD. This value is dependent on the condition of capillary wall, the composition, pH, and viscosity of the buffer, and the nature of the sample, and quality of instrumentation. Additional detail is given in table 17.

| Factor | Causeleffect | Solution |
|---|---|---|
| **Temperature change** | • Changes viscosity and EOF | • Thermostat capillary |
| **Adsorption to capillary walls** | • Changes EOF<br>• Caused by buffer, additive, or sample adsorption | • Condition capillary and allow sufficient equilibration time |
| **Hysteresis of wall charge** | • Caused by conditioning capillary at high (or low) pH and employing a low (or high) pH running buffer | • Avoid pH differences<br>• Allow sufficient equilibration time |
| **Changes in buffe composition** | • pH changes due to electrolysis<br>• Buffer evaporation<br>• Conditioning waste flushed into outlet reservoir<br>• Carrying sodium hydroxide from conditioning vial into buffer vial, for example | • Replenish buffer<br>• Cap buffer vials and cool carousel<br>• Use separate reservoir to collect wash solutions<br>• First dip capillary in separate buffer or water vial |
| **Buffer reservoirs not level** | • Non-reproducible laminar flow | • Level liquid in reservoirs<br>• If not replenishing, do not use inlet vial for washing capillary |
| **Different silanol content of silica batches** | • Different wall charge and variations in EOF | • Measure EOF and normalize if necessary |
| **Variations in applied voltage** | • Proportional changes in migration time | • Not user accessible |

**Table 17**
**Factors affecting migration time reproducibility**

Calculation of mobility or use of an internal standard can greatly improve relative migration time reproducibility. Since mobility is calculated relative to the EOF, it is in effect an internal standard. Variations in temperature due to changes in ambient or from Joule heating, interaction of the running buffer with the surface (that is, equilibration), or by slight changes in buffer composition or concentration can alter EOF but generally do not affect selectivity. In these cases an internal standard can improve reproducibility, as exemplified in table 18 for a peptide mapping separation. The internal standard can either be added to the sample mixture or can be a component in the original sample. The EOF, often present as a negative-going peak due to displacement of buffer, can be a useful reference for calculating mobility or for use as an internal standard.

| | Migration time [min] | | | Relative migration |
|---|---|---|---|---|
| Run# | Peak 1 | Peak 2 | Peak 3 | Peak 3 / Peak 1 |
| 1 | 15,193 | 17.965 | 18.421 | 1.212 |
| 3 | 14,971 | 17.672 | 18.12 | 1.210 |
| 5 | 15,107 | 17.914 | 18.391 | 1.217 |
| 7 | 15,303 | 18.119 | 18.597 | 1.215 |
| 9 | 15,237 | 18.04 | 18.512 | 1.215 |
| 11 | 14,713 | 17.337 | 17.79 | 1.209 |
| 13 | 14,575 | 17.145 | 17.58 | 1.206 |
| 15 | 14,401 | 16.95 | 17.402 | 1.208 |
| 17 | 14,522 | 17.137 | 17.61 | 1.213 |
| 19 | 14,869 | 17.584 | 18.073 | 1.215 |
| (n = 20) | | | | |
| Average | 14.942 | 17.654 | 18.119 | 1.213 |
| σ | 0.342 | 0.45 | 0.462 | 0.004 |
| **% RSD** | **2.29** | **2.55** | **2.55** | **0.32** |

**Table 18**
**Migration time reproducibility — absolute and internal standard**

## 4.3 Detection

Detection in CE is a significant challenge as a result of the small dimensions of the capillary. Although CE requires only nanoliter volumes of sample, it is not a “trace” analysis technique since relatively concentrated analyte solutions or

pre-concentration methods are often necessary. A number of detection methods have been used in CE to meet this challenge, many of which are similar to those employed in liquid column chromatography. As in HPLC, UV-Visible detection is by-far the most common. Table 19 contains a list of many of the detection methods investigated, along with detection limits, and advantages/disadvantages.

### 4.3.1 UV-Visible absorption

UV-Visible absorption is the most widely used detection method primarily due to its nearly universal detection nature. With fused-silica capillaries, detection below 200 nm up through the visible spectrum can be used. The high efficiency observed in CE is due in part to on-capillary detection. Since the optical window is directly in the capillary there is no zone broadening as a result of dead-volume or component mixing. In fact the separation is still occurring while in the detection window. As with all optical detectors, the width of the detection region should be small relative to the solute zone width to maintain high resolution. This is best accomplished with a slit designed for specific capillary dimensions. Since peaks in CE are typically 2 to 5 mm wide, slit lengths should be maximally one third this amount.

Detector design is critical due to the short optical path length. The optical beam should be tightly focused directly into the capillary to obtain maximum throughput at the slit and to minimize stray light reaching the detector. These aspects are important to both sensitivity and linear detection range.

### 4.3.2 Sensitivity

Sensitivity is defined as the slope of the calibration curve (detector signal versus sample concentration). A steeper slope indicates better sensitivity. For absorptive

| Method | Mass detection limit (moles) | Concentration detection limit (molar)* | Advantages/ disadvantages |
|---|---|---|---|
| UV-Vis absorption information | $10^{-13}$-$10^{-15}$ | $10^{-5}$-$10^{-8}$ | • Universal<br>• Diode array offers spectral |
| Fluorescence | $10^{-15}10^{-11}$ | $10^{-7}$-$10^{-9}$ | • Sensitive<br>• Usually requires sample derivatization |
| Laser-induced fluorescence | $10^{-18}$-$10^{-20}$ | $10^{-14}$-$10^{-16}$ | • Extremely sensitive<br>• Usually requires sample derivatization<br>• Expensive |
| Amperometry | $10^{-18}$-$10^{-19}$ | $10^{-10}$-$10^{-11}$ | • Sensitive<br>• Selective but useful only for electroactive analyses<br>• Requires special electronics and capillary modification |
| Conductivity | $10^{-15}$-$10^{-16}$ | $10^{-7}$-$10^{-8}$ | • Universal<br>• Requires special electronics and capillary modification |
| Mass spectrometry | $10^{-16}$-$10^{-17}$ | $10^{-8}$-$10^{-9}$ | • Sensitive and offers structural information<br>• Interface between CE and MS complicated |
| Indirect UV, fluorescence, amperometry | 10-100 times less than direct method | – | • Universal<br>• lower sensitivity than direct methods |

Others:
Radioactivity, thermal lens, refractive index, circular dichroism, Raman

*assume10 nl injection volume

**Table 19**
**Methods of detection[32]**

detectors the absorbance of a solute is dependent on path length, b, concentration, C, and molar absorptivity, ε, as defined by Beer's law

$$\mathbf{A = bC\varepsilon} \qquad (31)$$

The short pathlength is the factor that mainly limits sensitivity in CE. Due to the curvature of the capillary, the actual pathlength in the capillary is less than the inner diameter since only a fraction of the light passes directly through the center. The actual pathlength can be determined by filling the capillary with a solute of known concentration and molar absorptivity.

High sensitivity can often be realized by use of low-UV detection wavelengths. Peptides and carbohydrates, for example, have no strong chromophores but can be adequately detected at 200 nm or below (figure 56). Detection at these low wavelengths necessitates the use of minimally-absorbing running buffers since high background absorbance increases baseline noise and decreases signal. Phosphate and borate are useful in this respect. Many biological buffers such as HEPES, CAPS, and Tris are inappropriate for use below about 215 nm.

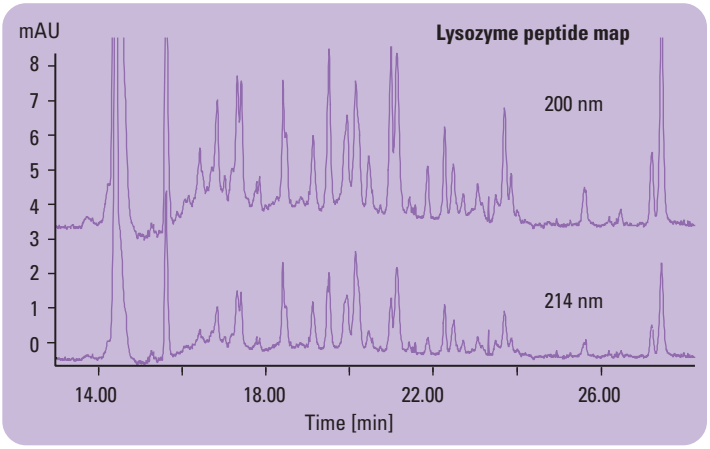



**Figure 56**
**Use of low detection wavelengths to increase signal-to-noise ratio**

## 4.3.3 Linear detection range

Instrumental deviations from Beer's law ultimately limit quantitative analysis. At high analyte concentrations, the deviation usually takes the form of decreased slope of the calibration curve (figure 57). The linear detection range is mainly limited by stray light reaching the detector. Ideally, all light should pass through the center of the capillary, not through the wall. Note that the linear detection range is significantly lower than that observed in LC (0.4 to 0.7 AU in CE versus 1.2 to 1.5 AU in LC) due to the small size and curvature of the capillary.

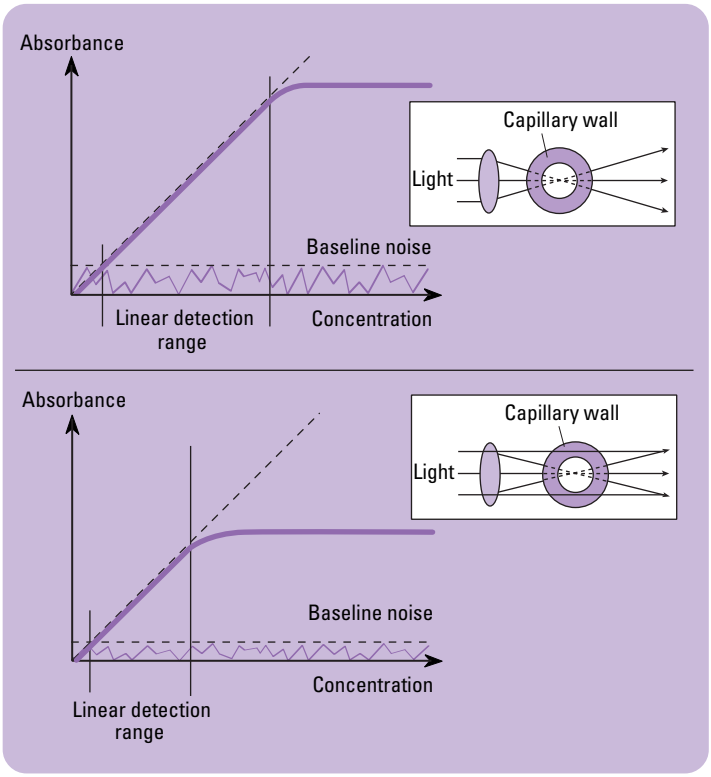



**Figure 57**
**Dependence of linear detection range and baseline noise on light path through the capillary**

There is an interdependent relationship between sensitivity and linear detection range. For a given design, increasing the light throughput, by increasing the slit size for example, can improve detection limits by increasing the light level to the photodiode and thereby decreasing the baseline noise. However, if the slit allows stray light through the capillary walls, the linear detection range will be compromised. If the slit is increased along the length of the capillary, resolution will be reduced. Depending on the analysis, slit size can be chosen to optimize either sensitivity, or resolution and linear detection range.

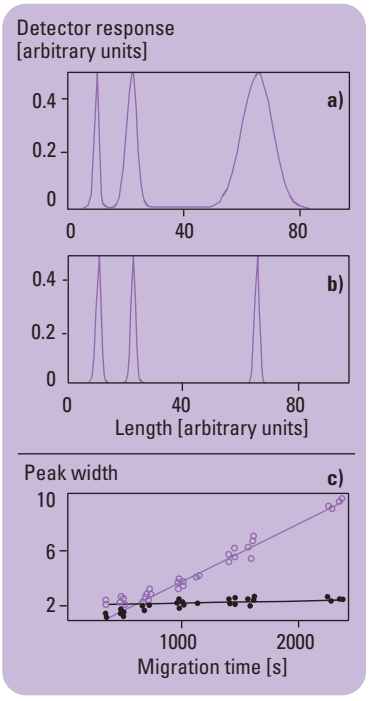



**Figure 58**
**detector response as a) a function of time; b) as corrected for differences in zone velocity; c) peak width corrected (•) and uncorrected (o)[6]**
Conditions in c): dansylated lysine run at different voltages to produce different migration times

## 4.3.4 Aspects of quantitative analysis

Peak area reproducibility is critical for quantitative analysis. Typically, better than 2 % RSD can be obtained under wellcontrolled conditions. The major factors affecting peak area are temperature variations, sample adsorption, precise injection of small sample plugs, and integration of signals with low signal-to-noise ratios. A more complete list is given in table 20. Many of these factors can be directly affected by the user, while a few are completely instrument dependent. As with migration time reproducibility, use of an internal standard can often be useful.

An interesting aspect of quantitative analysis of peak area results from different migration velocities of the solutes. This is in contrast to chromatographic techniques in which all solutes travel at the same rate when in the mobile phase. One must correct for velocities since different residence times in the detection region artificially affect peak area. Solutes of low mobility remain in the detection window for a longer time than those of higher mobility, and thus have increased peak area. This phenomenon can be corrected simply by dividing integrated peak area by migration time.This is illustrated in figure 58.

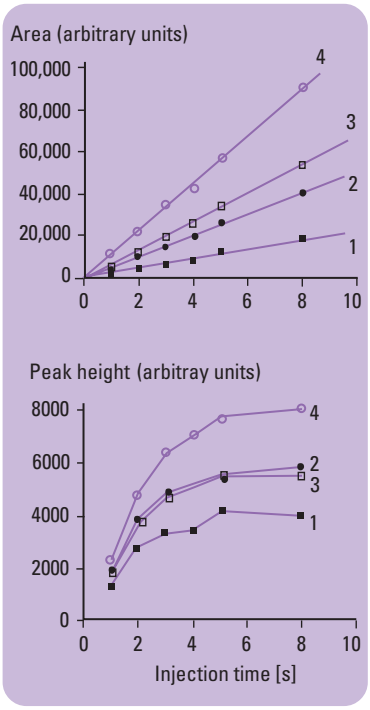



**Figure 59**
**Linearity of peak area and peak height as a function of injection time for different solutes[33]**
1 = mesityl oxide, 2 = phenylglycine, 3 = mandelic acid, 4 = benzoic acid

| Factor | Cause/effect | Solution |
|---|---|---|
| **Temperature changes** | •Changes in viscosity and injection amount | •Thermostat capillary |
| **Sample evaporation** | •Increasing sample autosampler | •Cap vials and/or cool |
| **Instrumental limitations** | •System rise time significant proportion of injection time | •Increase injection time |
| **Sample carry-over** | •Extraneous injection | •Use capillary with flat, smooth injection end<br>•Remove polyimide from end of capillary<br>•Dip capillary in buffer or water after injection |
| **Zero-injection caused by simply dipping the capillary in the sample** | •Extraneous injection can be quantified | •Cannot be eliminated but |
| **Sample adsorption to capillary walls** | •Distorted peak shape<br>•Non-eluting sample concentration<br>•Use additive | •Change buffer pH<br>•Increase buffer |
| **Low signal-to-noise ratio** | •Integration errors | •Optimize integration parameters<br>•Increase sample concentration<br>•Use peak height |
| **Sudden application of high voltage** | •Heating, thermal expansion of buffer, and expulsion of sample | •Ramp separation voltage |
| **Electrokinetic injection** | •Variations in sample matrix | •Use hydrodynamic injection |

**Table 20**
**Factors affecting peak area reproducibility**

Peak height is generally not as useful for quantitative analysis as peak area (figure 59). The major problem is that peak height is dependent on stacking which occurs during injection. As discussed, stacking can result simply from differences in sample and running buffer composition (for example, matrix effects) that are often difficult to define. Differences in solute and buffer conductivity can cause peak shape distortions that affect peak height but not peak area. In addition, peak height should only be used quantitatively if the solute zone is less than about 5 % of the bandwidth at the detector. Peak height may be useful when signal-to-noise is too low for precise integration of peak area.

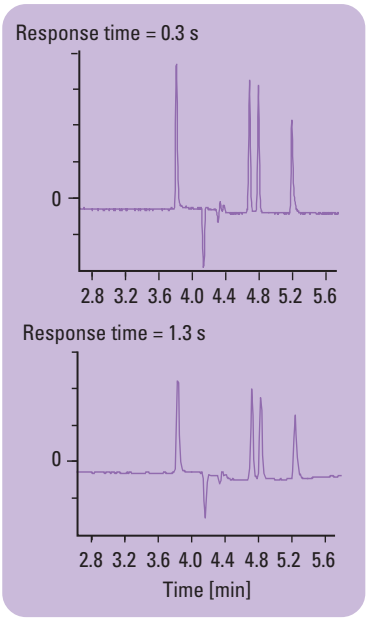



**Figure 60**
**Effect of detector response time on peak shape**

## 4.3.5 Detector response time and data collection rate

The high efficiency often realized in CE results in very narrow solute zones and sharp peaks. Peak widths of 5 seconds or less are not uncommon, especially when isotachophoretic effects occur. Data collection rates must be rapid enough to adequately describe the peak. Generally, at least 20 data points are needed. Data rates should be 5 to 10 Hz.

The response or rise time of the detector should also be sufficient to respond to the slope of the peak. Response times that are too slow can result in broadened, distorted peak shapes (figure 60). Values between 0.1 s and 0.5 s are usually sufficient. Since high data acquisition rates and rapid reponse times also increase noise, these should be adjusted to the peak widths of the specific analysis.

## 4.3.6 Extended light path capillaries

Sensitivity and linear detection range can usually be improved by increasing the inner diameter of the capillary. This approach is limited, however, by the increase in current and subsequent heating within the capillary. For example, a two-fold increase in diameter will yield a twofold increase in absorbance but a four-fold increase in current. Special capillary designs can be used to extend the optical pathway without increasing the overall capillary area. One such design is the “bubble cell” (figure 61).

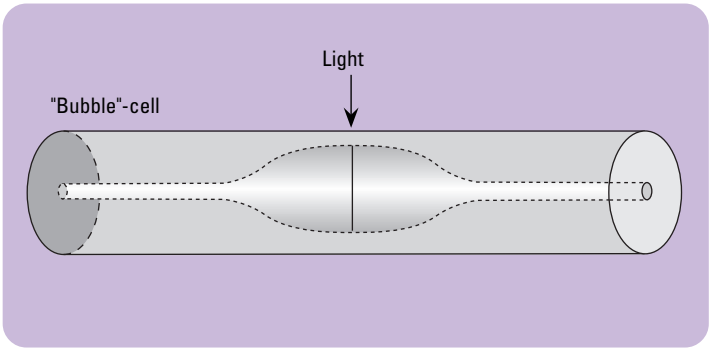



**Figure 61**
**Schematic of extended light path capillary**

The bubble cell offers a unique method to extend the pathway with nearly no degradation of separation efficiency and resolution. It is made by forming an expanded region, a bubble, directly within the capillary. Since the bubble is located only in the detection region no increase in current occurs. In the region of the bubble the electrical resistance is reduced and thus the field is decreased. Concomitant to this is a proportional decrease in flow velocity due to the expanded volume of the bubble. When the zone front enters the bubble its velocity decreases and the zone concentrates or “stacks” in a manner similar to electrophoretic stacking during injection. As the sample zone expands radially

(across the capillary) to fill the increased volume, it contracts longitudinally (along the capillary). Thus the sample concentration remains constant but the path length increases. Figure 62 shows a photograph of the zone front of a dye in the expanded region of the bubble cell.

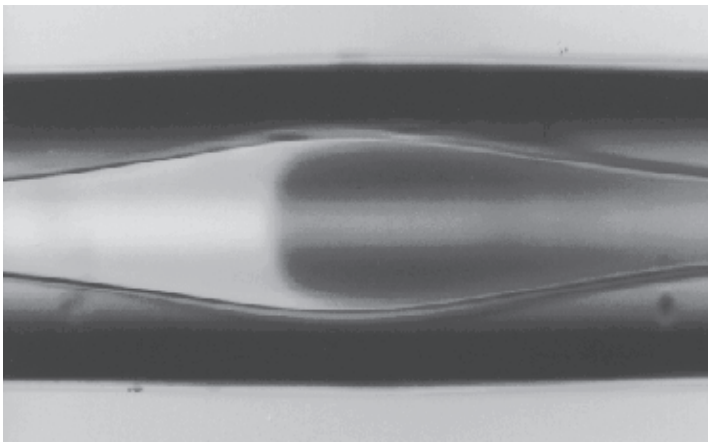


**Figure 62**
**Photograph of a dye front passing through the extended light path capillary detection region**

The use of the bubble cell is illustrated in figure 63 for the separation of neuropeptides. Here the bubble is three times larger than the inner diameter (150-µm id bubble in a 50-µm id capillary). This yields nearly a 3-fold increase in signal relative to a straight capillary. Importantly there is nearly no measurable band broadening. In addition, the lower limit of detection and linear detection range are also improved due to the increased light throughput of the bubble cell.

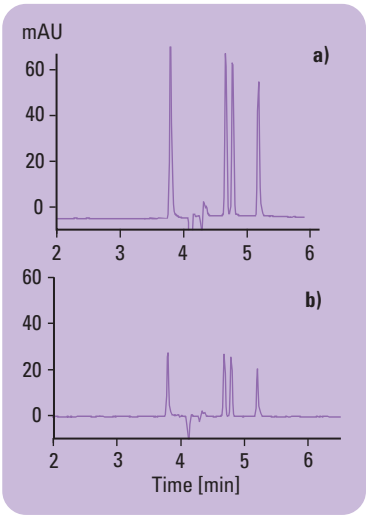



**Figure 63**
**Peptide analysis using: a) the extended light path capillary, and b) a normal straight capillary**
Conditions: a) id = 50 µm with 150 µm detection cell; b) id = 50 µm

## 4.3.7 Diode-array detection

Diode-array detection (DAD) is an alternative to single or multiple wavelength detection. Instrumentally a DAD consists of an achromatic lens system to focus

light into the capillary. The beam is then dispersed by a diffraction grating and falls on the photodiode-array. An array consists of numerous diodes (211, for example), each of which is dedicated to measuring a narrow-band spectrum. The number of wavelengths falling on a photodiode is called the bandwidth.

Importantly, DAD optics can yield detection limits, sensitivity; and linear detection range that equal or exceed that of single or multiple wavelength detectors. With respect to spectral analysis, DAD also has significant advantages over rapid scanning detectors. Unlike in the scanning design, the signal-to-noise ratio of spectral data is independent of the number of wavelengths acquired, the bandwidth of individual wavelengths is not predetermined, and on-line spectra are available at all times.

DAD can greatly simplify analysis of electrophoretic data. When developing a new electrophoretic method there is usually little or no information regarding the required detector conditions, and in particular no optimal wavelength. Using a conventional variable wavelength detector, the sample must be injected repeatedly, changing the detector wavelength each time to make sure that all solutes are detected. With a diode-array a whole wavelength range can be selected, for example from 190 to 600 nm with a bandwidth of 400 nm. In a single analysis, all solutes absorbing within this range will be detected.

Once all peaks have been detected, the diode-array can be used to determine the wavelength for the absorbance maximum for all analytes. Appropriate software can calculate the absorbance maxima automatically (figure 64). Alternatively, the data can be presented in three-dimensional form— either as analysis time versus wavelength

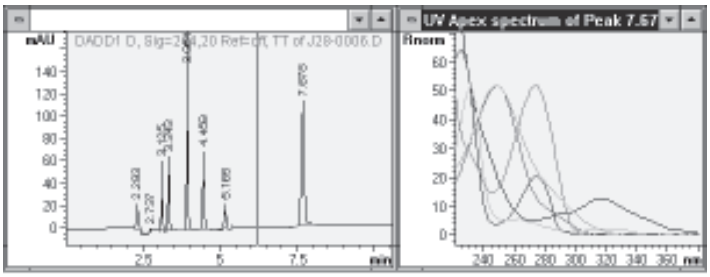



**Figure 64**
**Electropherogram and corresponding peak spectra**

and absorbance (figure 65) or as an isoabsorbance plot which displays analysis time, wavelength, and absorption intensity. The isoabsorbance plot is especially useful to determine wavelength maxima for complex mixtures.

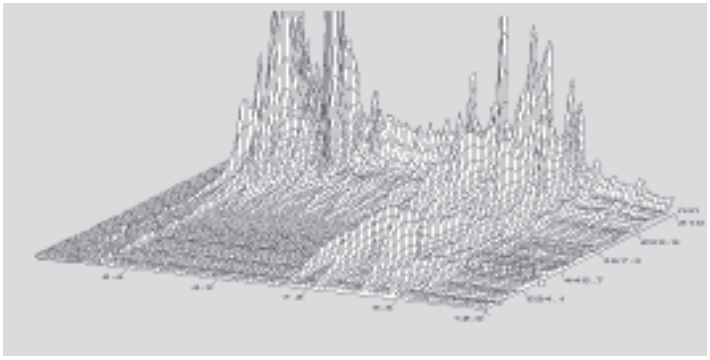


**Figure 65**
**Diode-array detection: 3-D data analysis**

### 4.3.7.1 Multisignal detection

The diode-array can monitor a sample at more than one wavelength. This is useful when the wavelength maxima of the analytes are different. Absorption of non relevant peaks can therefore be minimized. In addition, the quantity of collected data can be greatly reduced if only a few wavelengths are stored.

#### 4.3.7.2 Quantifying non-separated peaks

If two solutes are electrophoretically not separated, neither qualitative nor quantitative analysis can be performed with a single wavelength detector. With the diode-array, such analysis can be performed even if the spectra overlap across all wavelengths. This is accomplished through *peak suppression or* signal subtraction using an extra wavelength, known as the reference wavelength.

#### 4.3.7.3 Validation of peak purity

It is important to determine whether electrophoretic peaks are pure or if they consist of more than one solute. With a diode-array detector, peak purity can be examined if the spectra of the solute and the impurity are different. The most common method involves acquiring several spectra during the peak's elution. By normalizing and overlaying the spectra, the plots can be compared. When the spectra match over all wavelengths, the peak can be considered pure.

Other validation methods include examining the absorbance ratio at two wavelengths with the data plotted in the time domain, spectral suppression, three-dimensional plotting of the data, spectral deconvolution as a function of elution time, and principal component analysis.

The peak purity function can be used when analysing non-Gaussian shaped peaks. This is especially beneficial in CE where peaks can be skewed as a result of conductivity differences between the solutes and the running buffer. Without this function, peak shape distortions can easily be misconstrued as impurities. Significant effort in optimization of buffer systems can be avoided by use of this spectral information.

#### 4.3.7.4 Confirming peak identity

Migration time or mobility measurements are often insufficient to confirm peak identity. Spectral analysis and library searching is a useful way to accomplish this. Spectra at the peak apex can be acquired automatically with the diode-array detector. The spectra can then be compared with those stored in a library. With automated comparison a match factor is calculated to give the statistical probability of peak identity. The spectral libraries can easily be built up by the user. In addition, the vast collection of libraries compiled from liquid chromatography can be used.

Additional detail regarding the use of DAD can be found in the Agilent Technologies primer entitled *Applications of diode-array Detection in HPLC*.

## 4.4 Liquid handling

Liquid handling in CE is important both to maintain high separation efficiency, automation, and overall experimental flexibility. Such a system can be considered to include an autosampler, fraction collector, buffer replenishment system and buffer leveling system.

### 4.4.1 Autosampler

In CE an autosampler has two functions. It should transport both the sample vials and buffer vials to the capillary ends. For automated method development, usually a single sample vial and numerous buffer vials (each pair containing a different buffer composition) are used. Conversely, for routine analysis, numerous samples but only a single buffer is used. Thus it is important to have a high vial capacity and

the capability of random access to the vials. In either case, it is important that the anodic and cathodic reservoirs contain the same buffer composition. Differences in the reservoir contents can result in absorbance fluctuations, current changes, and variations in EOF.

Cooling of the autosampler is required if thermally labile samples are used. Thermostating is also beneficial in reducing sample evaporation which can increase sample concentration and limit quantitative analysis. Similarly, tightly capped sample vials also limit evaporation.

### 4.4.2 Fraction collector

Although minute quantities are injected into an CE system, fraction collection is often desirable. While only picogram quantities of material are usually collected, this may be sufficient for a number of further analyses, including re-injection with different running buffers or using different CE modes, or sequencing, mass spectrometry or enzymatic assay. If sufficient material is not obtained, multiple collections can easily be automated.

Elution of the solute into the collection vial may best be accomplished by pressure since the solute may migrate to the electrode and adsorb or react. This is especially relevant when the collection vial contains water or other low conductivity medium. As described for the autosampler, cooling of the fraction collector is desirable.

Collection of narrow, closely spaced peaks can be simplified by voltage programming. As previously described, reduction of the field immediately prior to collection increases the time-window and relaxes the stringent timing problems associated with precise collection. This is illustrated in figure 66.

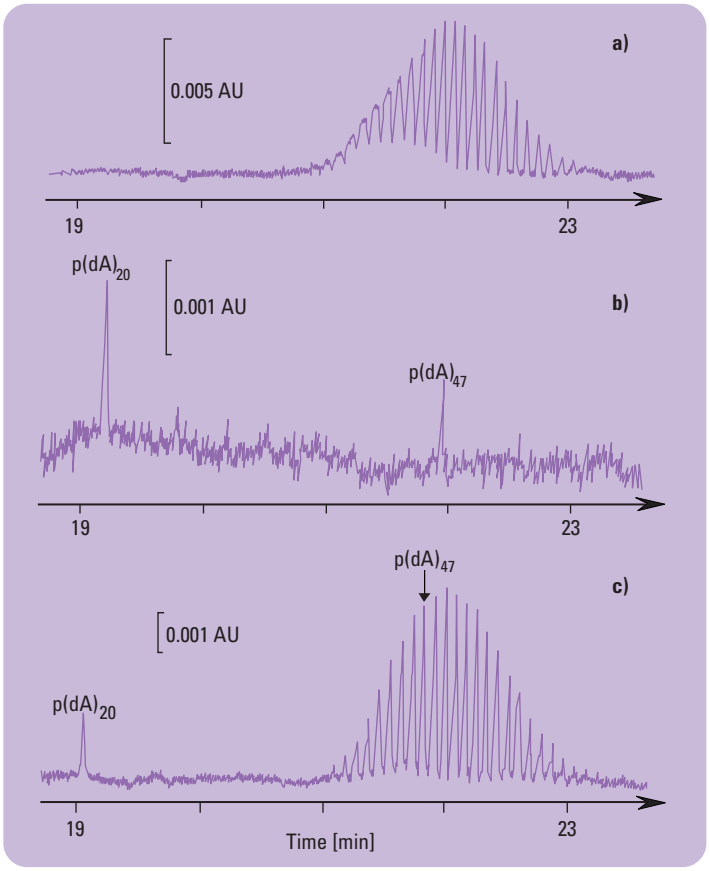



**Figure 66**
**Fraction collection of oligonucleotides in CGE[21]**
a) preparative injection of $p(dA)_{40-60}$ sample,
b) re-injection of collected oligonucleotide with $p(dA)_{20}$ as internal standard
c) tentative peak identification by relative migration time

## 4.4.3 Buffer replenishment

Buffer replenishment is also an integral part of maintaining high reproducibility. Electrolysis of solution can alter running buffer pH and subsequently change EOF In aqueous solution, electrolysis of water produces soluble protons at the anode and hydroxide ions at the cathode. In combination with ion migration, this phenomenon has been called buffer depletion. The extent of electrolysis is dependent on the current generated and total run time. The extent of the pH change is dependent on the buffering capacity of the buffer

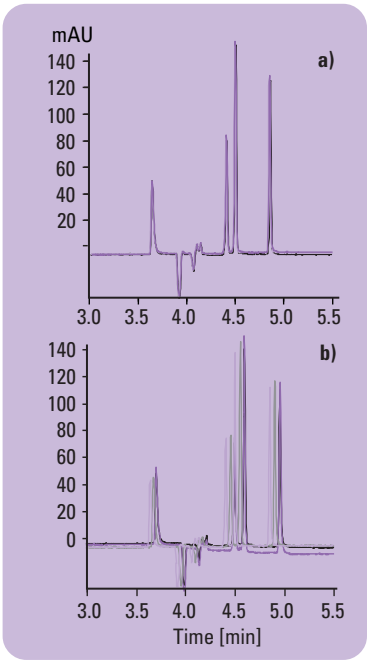



**Figure 67**
**Improved migration time reproducibility using buffer replenishment**
Overlay of runs 5, 10, and 15
a) replenish after every 5 runs
b) no replenishment

system, the volume of the reservoirs, and whether the capillary conditioning washes are flushed into the exit reservoir (not recommended). For these reasons, frequent replacement of buffer is recommended. An example of the benefit of frequent buffer replenishment is shown in figure 67.

The replenishment system operates by emptying the contents of the reservoirs into a waste bottle and then refilling them with fresh buffer. With a large buffer replenishment system minimal vial locations in the autosampler are needed for buffer, leaving more locations for samples. In addition, a large reservoir affords the capability to run automated analysis for a long period of time (over the weekend, for example).

## 4.4.4 Buffer leveling

As described in chapter 2, maintaining equal liquid levels is important to both efficiency and migration time reproducibility. Siphoning from one reservoir to the other will superimpose laminar flow into the system. This is not only detrimental to reproducibility but also to the efficiency. The influence of non-level reservoirs is dependent on the diameter and length of the capillary and the viscosity of the buffer. Clearly, wide-bore, short capillaries run at elevated temperatures would exhibit the most deleterious effects. It has been shown that a height difference of 2 mm in a 50-µm id capillary can change migration time 2 to 3 %. This increases to about 10 % in a 100-µm id capillary.

An automated buffer leveling system would accurately deliver liquid to a user-specified level during replenishment. It can also be used to re-level liquids in the running reservoirs caused by EOF, without emptying them first.

Chapter 5

# List of abbreviations

| Abbreviation | Full Name | Unit (SI) | Unit (common) |
|---|---|---|---|
| A | Absorbance | | |
| $\alpha$ | Selectivity (k'2/k'1) | | |
| ACES | 2-[(2-amino-2-oxoethyl)amino] ethanesulfonic acid | | |
| ADA | N-[2-acetamido]-2-iminodiaetic acid | | |
| b | Pathlength | m | cm |
| BES | 2-[bis-(2-hydroxyethyl)amino] ethanesulfonic acid | | |
| BICINE | N, N-bis[2-hydroxyethyl] glycine | | |
| BIS TRIS Propane | 1,3-bis[tris(hydroxymethyl]-methylamino]propane | | |
| BRIJ | Polyoxyethylene ethers | | |
| C | Concentration | mole/$m^3$ | |
| C % | Percentage of total polyacrylamide/crossliker due to crosslinker | | |
| CAPS | 3-[cyclohexylamino]-1-propane-sulfonic acid | | |
| CD | Cyclodextrin | | |
| CGE | Capillary gel electrophoresis | | |
| CHAPS | 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate | | |
| CHAPSO | 3-[(3-cholamidopropyl)-dimethylammonio]-2-hydroxy-1-propane sulfonate | | |
| CHES | 2-[N-cyclohexylamino]ethane-sulfonic acid | | |
| CIEF | Capillary isoelectric focusing | | |

| Abbreviation | Full Name | Unit (SI) | Unit (common) |
|---|---|---|---|
| CITP | Capillary isotachophoresis | | |
| CMC | Critical micelle concentration | | |
| CTAB | Cetyl trimethyl ammonium bromide | | |
| CZE | Capillary zone electrophoresis | | |
| D | Diffusion coeficient | $m^2/s$ | $cm^2/s$ |
| $\Delta$ | Difference | | |
| d | Diameter | m | µm |
| DAD | Diode-array detection | | |
| DMSO | Dimethylsulfoxide | | |
| DNA | Deoxyribonucleic acid | | |
| DTAB | Dodecyltrimethylammonium bromide | | |
| E | Electric field | V/m | V/cm |
| $\varepsilon$ | Dielectric constant | $C^2/J\ m$ | |
| EOF | Electro-osmotic flow | | |
| g | Gravitational constant | $kg/m^2$ | |
| GC | Gas chromatography | | |
| H | Reduced plate height | m | µm |
| h | Thermal transfer coefficient | W/m K | |
| $\eta$ | Viscosity | $N\ s/m^2$ | cpoise |
| HEPES | N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid | | |
| HEPPSO | N-[2-hydroxyethyl]piperazine-N′-[2-hydroxypropanesulfonic acid | | |

| Abbreviation | Full Name | Unit (SI) | Unit (common) |
|---|---|---|---|
| CE | High perfomance capillary electrophoresis | | |
| HPLC | High perfomance liquid chromatography | | |
| i | Current | A | µA |
| id | Internal diameter | m | µm |
| IEF | Isoelectric focusing | | |
| ITP | Isotachophoresis | | |
| K | Partition coefficient | | |
| $\kappa$ | Thermal conductivity | W/m K | |
| k' | Capacity factor | | |
| kbp | Kilo base pair | | |
| $K_D$ | First-order dissociation constant | $s^{-1}$ | |
| L | Total capillary length | m | cm |
| l | Effective capillary length | m | cm |
| l | Detection wavelength | m | nm |
| LDR | Linear dynamic range | | |
| $\mu_a$ | Apparent mobility | $m^2/Vs$ | $cm^2/Vs$ |
| $\mu_e$ | Effective mobility | $m^2/Vs$ | $cm^2/Vs$ |
| MECC | Micellar electrokinetic capillary chromatography | | |
| MEKC | Micellar electrokinetic chromatography | | |
| MES | 2-[N-morpholino]-ethanesulfonic acid | | |
| MOPS | 3-[N-morholino]-propanesulfonic acid | | |

| Abbreviation | Full Name | Unit (SI) | Unit (common) |
|---|---|---|---|
| MOPSO | 3-[N-morpholino]-2-hydroxypropanesulfonic acid | | |
| N | Number of theoretical plates | | |
| od | Outer diameter | m | µm |
| P | Pressure | Pa | mbar |
| PAGE | Polyacrylamide gel electrophoresis | | |
| PCR | Polymerase chain reaction | | |
| PEG | Polyethylene glycol | | |
| pI | Isoelectric point | | |
| PIPES | Piperazine-N,N'-bis-[ethanesulfonic acid] | | |
| $pk_a$ | pH at which concentrations of acid and base forms are equal | | |
| PVA | Polyvinyl alcohol | | |
| Q | Power density | $W/m^3$ | |
| $Q_i$ | Quantity | mole or g | |
| q | Charge | C | |
| R | Resolution | | |
| r | Radius | m | m or mm |
| r | Density | kg/m | g/cm |
| RSD | Relative standard deviation | | |
| $\sigma$ | Standard deviation | | |
| $\sigma^2$ | Peak variance | | |
| SDS | Sodium dodecylsulfate | | |
| SFC | Supercritical fluid chromatography | | |

| Abbreviation | Full Name | Unit (SI) | Unit (common) |
|---|---|---|---|
| T | Temperature | K | °C |
| T % | Total percentage of poly-acrylamide and crosslinker | | |
| TES | N-tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid | | |
| TFA | Trifluoroacetic acid | | |
| $t_m$ | Migration time | s | s or min |
| TRICINE | N-[2-hydroxymethyl)ethyl]-glycine | | |
| TRIS | Tris(hydroxymethyl)amino-methane | | |
| TWEEN | Polyoxyethylenesorbitan | | |
| UV-Vis | Ultra violet-visible | | |
| V | Voltage | V | kV |
| v | Velocity | m/s | mm/s or cm/s |
| $V_m$ | Volume of mobile phase | | |
| $V_s$ | Volume of micellar phase | | |
| $w_{1/2}$ | Peak width at half-height | s or min | |
| $w_b$ | Peak width at baseline | s or min | |
| $w_i$ | Injection plug length | m | mm |
| $\zeta$ | Zeta potential | V | V |

Chapter 6

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