Preface

Since the advent of the clinical laboratory in the 20th century, the need to report more accurate results, faster, and at a lower cost has driven technology. One area that has lagged behind the rest of the laboratory is electrophoretic separations of analytes that are clinically relevant. Because of this, electrophoresis has been relegated to the very specialized sections of the laboratory, limiting its use in patient care.

Electrophoresis, as we use it today, was first described by Tiselius in his PhD thesis in 1937. In pioneering experiments that have led to the methods used today, he used a U-shaped quartz tube to show the zonal separation of serum in free solution using Schlieren optics to monitor the migration of the protein bands. Driven by the desire to make electrophoresis easier, a number of matrixes—such as paper, cellulose acetate, agarose, starch gel, and polyacrylamide—were investigated and, in one form or another, are still used today. From the basic method described by Tiselius a number of innovative electrophoretic methods have now been developed, including immunoelectrophoresis, isoelectric focusing (IEF), isotachophoresis (ITP), and size separation by gradient electrophoresis.

Tiselius's basic concept of using a tube for electrophoretic separation received little notice until the late 1960s when Hjerten described the first capillary electrophoresis (CE) apparatus. In spite of the pioneering work by Hjerten, CE remained relatively unknown until 1981 when Jorgenson and Lukacs described the separation and fluorescent detection of amino acids, peptides, and urine proteins by capillary zone electrophoresis. Since then, all of the classical separation techniques—IEF, ITP, zone electrophoresis, and micellar electrokinetic chromatography (MEKC)—have allowed CE to rival the versatility of high pressure liquid chromatography (HPLC). MEKC, which in its simplest form is the addition of detergent to the buffer, has enabled CE to be used in an area once thought impossible for electrophoresis techniques, the separation of small, electrically neutral molecules.

CE has come a long way since it was first described. Current methods are capable of being automated, and, because it is a microtechnique, the method conserves precious samples and minimizes the use of hazardous organic chemicals. Although CE has not made inroads into the clinical

laboratory that many anticipated, we expect that, in the future, it will find its "proper" place. Because this "proper" place may surprise everyone involved in the clinical applications of CE, this book is not meant to give an in-depth methodological description of the use of CE in the clinical laboratory, but to give an overview of its current use.

We arranged *Clinical and Forensic Applications of Capillary Electrophoresis* into six main sections. Section I covers the history and some of the potential applications of CE. This section also covers the principles necessary for the clinical laboratory scientist to understand the basics of CE. Section II covers the separation of proteins, probably the first use of CE in the clinical laboratory. The section describes the potential problems and solutions when using CE to separate proteins, along with outlining how CE has been used to separate serum and CSF proteins, detect serum and urine paraproteins, and separate lipoproteins and hemoglobin variants. Section III covers metabolic diseases, which are usually detected by abnormalities in small molecules, such as amino acids, organic acids, or steroids. Section IV covers the use of CE in immunoassay, where CE is used as a separation method. Although this may seem trivial at first glance, it opens up the possibility of simple, yet highly sensitive, analysis at the point of care. Section V describes what may be the future of CE in the clinical laboratory, the use of CE in molecular diagnostics, both for the detection of diseases and quantiation of viral loads and its use in the forensic DNA identification laboratory. Finally, Section VI describes how CE can be used in conjunction with mass spectrometry, its potential use in detection of heavy metal poisoning, therapeutic drug monitoring, and clinical and forensic toxicology.

John R. Petersen
Amin A. Mohammad

2

Basic Principles and Modes of Capillary Electrophoresis

Harry Whatley

1. BASIC PRINCIPLES OF CAPILLARY ELECTROPHORESIS

1.1. Fundamentals of Electrophoresis

Capillary electrophoresis (CE) is a special case of using an electrical field to separate the components of a mixture. Electrophoresis in a capillary is differentiated from other forms of electrophoresis in that it is carried out within the confines of a narrow tube. To understand the behavior of molecules under the influence of an electrical field inside a capillary it is essential to understand the phenomena that result from the geometry of a capillary.

1.1.1. Basic Principles

It has long been known that molecules can be either positively or negatively electrically charged. When the numbers of positive and negative charges are the same, the charges cancel, creating a neutral (uncharged) molecule. If given the freedom to move, charged particles will seek regions, such as an electrode, having an opposing charge; in other words, opposites attract. Figure 1 illustrates a simple example of electrophoresis. In this example a mixture of ionic substances is dissolved in a suitable solvent, such as water. In the absence of an electrical field, the motion of these ions is essentially random. When the electrical field is applied, charged species begin to move. A crude separation occurs, resulting in a less random distribution of charged particles. Cations (positively charged ions) move toward the cathode (negatively charged electrode), and anions (negatively charged ions) move toward the anode (positively charged electrode).

Figure 1 also illustrates another aspect of electrophoresis in solution, the significance of the mass charge ratio (m/z). In this figure there are actually four types of charged particles: large and small positively charged and large

From: *Clinical and Forensic Applications of Capillary Electrophoresis*
Edited by: J. R. Petersen and A. A. Mohammad

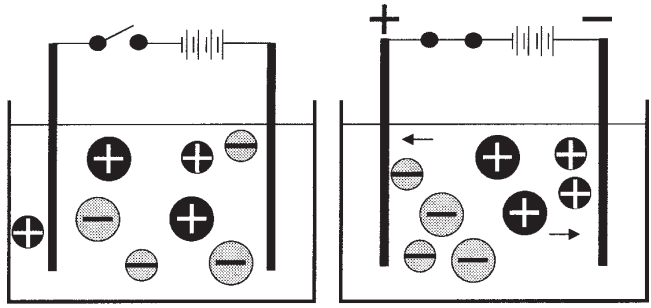

Fig. 1. Simple electrophoresis.

and small negatively charged. If each particle has only a single charge, then the absolute value of the force on each particle will be the same. The acceleration created by this force can be determined by the relationship Force = mass × acceleration (F = ma). The viscosity of the separation medium opposes the acceleration with the result that a steady velocity is achieved under constant conditions. This means that the system can not only separate particles having opposite charges, but can also separate particles of the same charge if there are other differences between them. The science of electrophoresis is largely concerned with creating systems that exploit the differences between the molecules. Alternatively, the analyst may wish to develop a system that creates differences between molecules. Varying the pH of the separation method is an example. At pH = 10.0, glycine and acetic acid will have the same charge (–1). At pH = 7.0, glycine will have a very small net charge whereas acetic acid will still have a charge of –1. Separation of these two molecules would therefore be different at pH = 7.0 and at pH = 10.0.

Numerous other factors besides pH affect electrophoretic separations. These include the hydrodynamic radius of the molecules, the viscosity of the separation medium, and temperature. In real systems there are other forces, in addition to the electrical field, acting on the charged molecules, e.g., the entire fluid mass may be moving relative to the vessel in which it is contained. Some of these factors can affect the electrophoresis in a very complex manner; for example, the passage of current through a liquid can raise the temperature of that liquid. This change in temperature can influence the electrical resistance of the system (and hence the current), the viscosity, and the velocity of the molecules moving in the field. These factors will be discussed later in the specific context of CE. Janini and Issaq presented a detailed discussion of the theoretical underpinnings of these factors *(1)*.

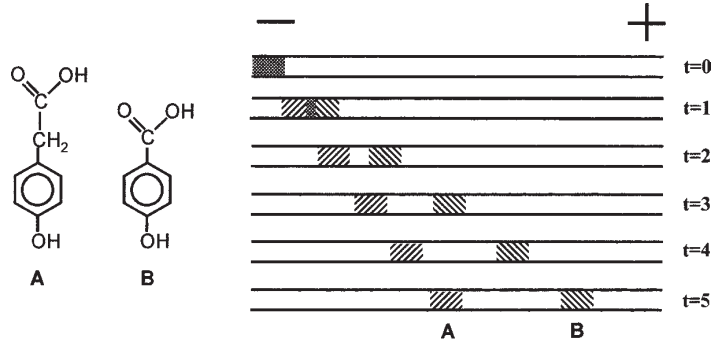

Fig. 2. Capillary electrophoresis.

1.1.2. Electrophoresis in a Capillary

The electrophoretic process in a capillary has all of the features previously described. In addition, the small diameter of the capillary and its large aspect ratio (length/width) contribute additional factors. What actually defines a capillary? For this chapter the discussion will be limited to capillaries formed from fused silica and having an inner diameter (i.d.) of 100 μm or less. The usual range of inner diameters is from 20–100 μm. Typically the capillaries that are used in CE are circular in cross-section. However, capillaries with square cross-sections have been produced. These may offer some advantages in terms of thermal regulation and detection sensitivity *(2)*, but to date they have not been widely used.

1.1.2.1. Advantages of the Capillary

Capillaries were introduced into electrophoresis as an anti-convective and heat controlling innovation. In wide tubes thermal gradients cause band mixing and loss of resolution. The use of glass capillaries of 200–500-μm i.d. was reported by Virtanen *(3)* in 1969. Jorgensen's *(4)* introduction of 75 μm capillary tubes was the start of modern "high-performance" CE.

Figure 2 illustrates the separation of a mixture of two components (both negatively charged) in a capillary. A small plug of sample is introduced into one end. When an electrical field is applied, the components begin to move in the field as previously described. The narrow capillary reduces lateral diffusion and insures that temperature differences between the center of the capillary and the wall are quite small. Because the two components in this example move at different velocities, they can be separated. The geometry and other properties of the capillary electrophoretic separation also lead to a condition known as plug flow. Under ideal plug flow conditions, the only

factor leading to sample dispersion is diffusion. This contributes to the high efficiency of CE separations.

1.1.2.2. Electroosmosis

The small diameter of the capillary contributes to another aspect of the separation process. This is the phenomenon known as electroosmosis, electroendoosmotic flow, or simply EOF. EOF exists in any electrophoretic system. It will occur whenever the liquid near a charged surface is placed in an electrical field resulting in the bulk movement of fluid near that surface. Because the surface to volume ratio is very high inside a capillary, EOF becomes a significant factor in CE.

The velocity of the electroosmotic flow through a capillary is given by the Smoluchowski equation *(5)* (Eq. 1),

$$v_{eof} = -(\varepsilon\zeta/4\pi\eta)E \quad (1)$$

where ε is the dielectric constant of the electrolyte, ζ is the zeta potential (Volts), η is the viscosity (Poise), and *E* is the potential applied (Volts/cm). In CE the zeta potential (ζ) is a measure of the charge on the wall of the capillary. This charge arises from both the nature of the material that composes the capillary and the composition of the electrolyte (buffer). The most commonly employed capillary material is fused silica. The surface of a fused silica capillary can be hydrolyzed to yield a negatively charged surface as described in Section 1.2.1. The negatively charged wall attracts cations that are hydrated from the electrolyte solution, creating an electrical double layer. In an electrical field they migrate toward the cathode, pulling water along and creating a pumping action. The zeta potential increases with the density of the charge on the surface. For fused silica and many other materials charge density will vary with pH *(6)*. Bare fused silica behaves much like a weak acid with a pKa of 6.25. The relationship between EOF and pH is shown in Fig. 3. EOF also decreases with the square root of the concentration of the electrolyte, i.e., increasing buffer concentration decreases the velocity of EOF. Although the aforementioned discussion has focused on bare fused silica capillaries, other types of surfaces can be chemically created from fused silica. The new surface may be positive, negative, or neutral. The direction of the electroosmotic flow will therefore depend on the sign of the charge on the wall of the capillary. Flow is always toward the electrode that has the same charge as the capillary wall. Thus an uncharged wall will have, in theory, no EOF. In reality this is difficult to accomplish.

In the narrow confines of the capillary the velocity of liquid is nearly uniform across the i.d. of the capillary resulting in what has been termed "plug flow" *(4)*. This is in contrast to the laminar flow exhibited by pumped

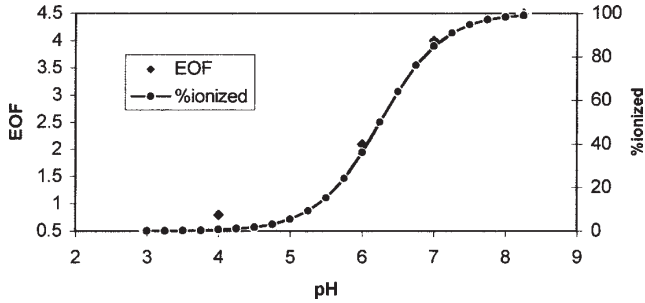

Fig. 3. EOF as a function of pH.

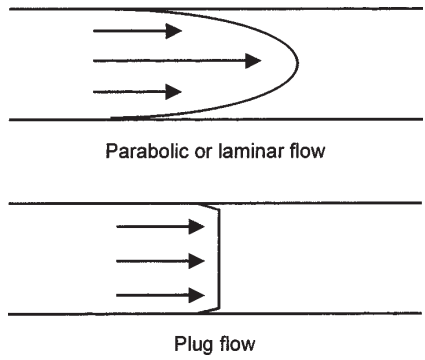

Fig. 4. Flow profiles.

systems, which creates a velocity profile across the diameter of the tube. On the other hand, plug flow greatly reduces the band broadening seen in systems (such as high-performance liquid chromatography [HPLC]) that are pumped by a pressure differential. A comparison of laminar flow and plug flow is shown in Fig. 4.

In the example shown in Fig. 2 the EOF is assumed to be zero. In practice it is very difficult to completely eliminate EOF, although it can be reduced to a value near zero. In many separations it is a significant factor, and it may affect the net movement of the analyte molecules more than does the electrophoretic force. The movement (vectors) due to the electrophoretic and electroosmotic forces may even be in opposite directions. Figure 5A shows a separation of two negatively charged species in a fused silica capillary at pH 8.3. Both analytes have a charge of –1 under these conditions. The EOF

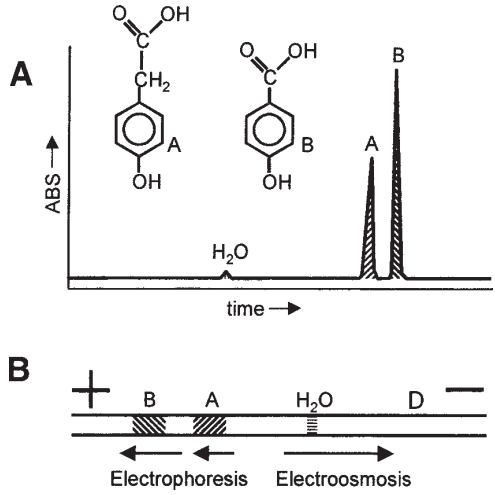

Fig. 5. Separation with EOF.

in this case is toward the cathode, while the analytes are moved electrophoretically toward the anode. The EOF contribution is larger than the electrophoretic, so that the net movement is toward the cathode. The small peak labeled H_2O represents the water in which the sample was dissolved. This water plug does not have any electrophoretic mobility; the movement of this plug is due solely to electroosmotic flow, and it is the first peak to pass the detector. The next peak to emerge, A, has the higher mass/charge ratio of the two analytes. This is in contrast to the case of Fig. 1 (no EOF) where the first peak to emerge had the lower mass/charge ratio. Note also that the polarity of the electrodes has been reversed compared to Fig. 1. Figure 5B shows a snapshot of the inside of the capillary during the separation but prior to any peak reaching the detector (D). Because A and B are influenced by both the EOF and the electrophoretic force, their net movement is the sum of the electroosmotic and electrophoretic vectors.

1.1.2.3. Capillaries

Many materials have been suggested and tested for the construction of capillaries for CE. These include fused silica, borosilicate glass, and polytetrafluoroethylene (Teflon*). Fused silica is now the preferred material for the construction of capillaries. The silica used is of very high purity,

*Teflon is a registered trademark of DuPont.

similar to that used to produce silicon chips for electronic components. Tubes of silica are heated to over 1000°C, then stretched to produce the final dimensions. Fused silica capillaries are extremely brittle. To facilitate handling they are coated with a layer of polyimide 10–25 μm thick, rendering the capillary very flexible. It can easily be wound on a spool 2.5 cm in diameter without breaking. The coating, however**,** has the disadvantage of being virtually opaque to UV light. It must be removed to create a "window" to allow on-capillary detection. This makes an area that is very fragile, warranting careful handling to prevent breakage.

The thickness of the wall of the capillary and the polyimide coating is generally much greater than the i.d. of the capillary *(7)*. A typical capillary will have an external diameter of 350 μm. If the internal diameter is 50 μm, this leaves a wall thickness of 150 μm. The thickness of the wall is critical, because the heat that is produced when an electrical current passes through the electrolyte must be removed through this wall. Reducing the wall thickness increases the fragility of the capillary at the same time as it increases the heat transfer capacity. External diameter is thus a trade-off between strength and heat transfer. Heat transfer will be discussed in more detail in Subheading 1.2.3.

1.2. Variables in CE

CE offers many directions from which to approach an analytical problem. This can be advantageous because as the number of options available increases, the likelihood that a solution to the analytical problem can be found also increases. This can also be a disadvantage, particularly to the newcomer, because the wide choice of options can appear daunting. This is compounded by the interactive nature of some of these factors. For example**,** a change in pH can affect the current; a change in current can affect temperature; a change in temperature can affect pH, the factor we were trying to vary in our experiment. Understanding these key factors will contribute to successful analysis.

1.2.1. The Capillary Surface

The inner surface of a capillary is an extremely important factor in CE. The inner wall is in contact with the separation chemistry and the samples. As noted earlier, the capillary wall is the site of the mechanism by which EOF is created. In order to understand how to control the effect of the surface on the separation, it important to understand the behavior of the capillary surface. The inner surface of a CE capillary is more analogous to the surface of the particles packed inside an HPLC column than it is to the HPLC column wall. Unlike the wall of an HPLC column, the capillary wall partici-

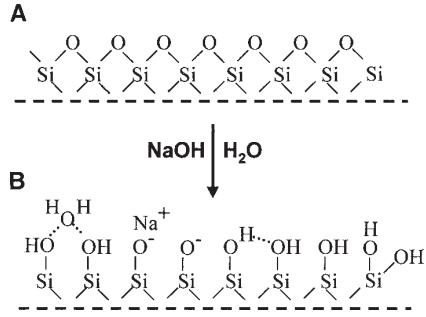

Fig. 6. Silica surface chemistry. Surface of fused silica before **(A)** and after **(B)** NaOH hydrolysis.

pates in the separation process. In CE the goal is usually to prevent interaction between the analytes and the inner wall of the capillary while simultaneously controlling EOF. In order to have control over the separation process it is necessary to have control over this surface.

1.2.1.1. Surface Modifications

The nature of the surface of a bare fused silica capillary depends somewhat on the treatment it has experienced *(8)*. A freshly manufactured silica capillary has a surface similar to that shown in Fig. 6A. Most of the surface is in a siloxane form; there are few groups present that can create a charged surface. Treatment with base (NaOH or KOH) **c**auses the hydrolysis of the siloxane group, creating the surface shown in Fig. 6B. Sometimes referred to as an etched surface, this layer is rich in silanol groups, which are the weak acids referred to in the discussion of EOF. To convert completely the surface of the capillary into this form may require long periods of exposure with 0.1–1.0 *M* base at elevated temperatures. Incomplete conversion may lead to poor reproducibility in peak migration times.

Not all separations can be optimized using bare silica. However, the silica surface is chemically reactive and can be derivatized in a variety of ways. Silane derivatization reactions are commonly employed to link other groups to silica. This can result in a linkage through a Si-O-Si-C bond or through a Si-C bond depending on the chemistry employed. The Si-C bond is said to be the more stable of the two at high pH values *(9)*. Using a molecule that has a silane at one end and a second functional group at the other allows a second layer to bond. This approach allows more complex coatings to be applied in layers. In this way many types of coatings have been created including polyacrylamide *(9,10)*, polyvinyl alcohol, polyethylene glycol *(11)*, and polyvinylpyrolidone *(12)*. Each of these coatings will have differ-

ent properties of charge and adsorptivity. Most were designed to reduce protein binding to the capillary wall. Basic proteins**,** being positively charged, are particularly difficult to analyze in untreated capillaries because they bind tightly to the negatively charged wall *(13)*. Coatings are also employed to modify the charge of the wall such that EOF is drastically altered or reversed.

Presently, the analyst seldom prepares covalently coated capillaries, as many types are commercially available. With commercially available fixed coatings, it is possible to obtain charges ranging from acidic to neutral to basic and from hydrophilic to hydrophobic. The analyst may also wish to incorporate noncovalent coatings into a separation method using a bare silica capillary. These transient or dynamic coatings rely on ionic interactions to hold them on the capillary wall. Polyamines will stick to the negatively charged silica, effectively shielding the negatively charged wall from the solution and creating a positively charged surface *(14)*. In such a capillary, the EOF will be toward the anode. Coatings of this type may be stable for several runs. Cationic detergents such as tetradecyltrimethylammonium bromide (TTAB) can be used to form a positively charged surface as well *(15)*. Such coatings are actually bilayers. The first layer has the positively charged head of the detergent attached to the negatively charged silanol surface. The hydrophobic tails of the molecules extend radially inward, forming a hydrophobic surface. A second layer of detergent forms with the hydrophobic tails in contact with the tails of the first layer and the positively charged heads forming the inner surface of the capillary. Again, EOF will be toward the anode. These detergent bilayers are easy to form but lack stability. They are easily disrupted by organic solvents and usually require that the detergent be added to the run buffer (where it may interact with analytes).

Whenever a capillary is used, the buffer being used modifies the surface. Most workers have come to recognize that capillaries perform best when they are "dedicated" to a specific type of buffer species. For example, if a capillary that has been equilibrated with a phosphate buffer is used with a borate buffer of similar concentration and pH the migration times will drift from run to run until a new equilibrium condition has been established. Although neither borate nor phosphate would be expected to form a coating on the capillary wall, it is apparent that the capillary does "remember" the buffer it has held. This dedication of a capillary to one type of system is a relatively inexpensive way to improve results.

1.2.1.2. Capillary Regeneration

Regeneration is the process of creating the same surface on the inner wall of the capillary prior to the start of every analytical run with a given method. Although it is only necessary to recreate the same surface condition every

time, it is in fact easier in the long run to fully regenerate the surface. There are two goals to regeneration: 1) to clean the capillary of any residual buffer and sample from the previous analysis; and 2) to create the same surface from run to run, thereby enhancing reproducibility. If the method has been properly designed these steps should not be necessary. If sample components are sticking to the capillary wall in sufficient quantity to affect subsequent runs, it is advisable to change the method to prevent the sample from sticking (perhaps by using a coating or by increasing the buffer concentration) rather than incorporating a cleaning procedure. In practice this can be difficult to accomplish, particularly with samples containing protein (such as serum) that is inherently troublesome.

This chapter will focus on the regeneration of bare silica capillaries. The special handling of coated surfaces will be discussed in Chapter 3. However, when using commercially coated capillaries, it is important to follow the manufacturer's instructions for cleaning and regeneration. This is because coated capillaries are easily converted into bare silica capillaries.

Freshly purchased fused silica capillaries, even those sold as "ready to use," may not be clean when first received. Therefore, some variation of the following procedure should be used whenever a new piece of bare silica capillary is used.

1. Flush the capillary with methanol. This will remove any organic residues (such as oil) and will aid in the subsequent wetting of the surface.
2. Flush with water.
3. Flush with 1 *N* hydrochloric acid. This removes any residual cations.
4. Flush with water.
5. Flush with 1 *N* NaOH. This step hydrolyzes the siloxanes to create the charged silanol groups that provide EOF.
6. Flush with water.
7. Flush with electrolyte.

Many variations on this procedure exist. For example, a second hydrochloric acid wash can be added after the NaOH if the removal of residual sodium is desired (as prior to a sodium analysis). The time and temperature of each step is important. A minimum of three capillary volumes should be used over at least 5 min (Section 1.3.1. will describe how to calculate fluid delivery). Static soaking should be avoided in favor of a continuous flow. As expected, the use of elevated temperature (35–40°C) will enhance the cleaning rate. The final flush with electrolyte, although critical to obtain assay reproducibility, is often neglected. The procedure described above creates a virgin surface that may change as analysis proceeds. There is usually an equilibration between the capillary wall and even simple buffers, such as sodium phosphate. Applying voltage to the capillary facilitates the exchange of ions at the surface after filling with the electrolyte.

The regeneration process between sample runs can be milder than the initial cleaning, particularly if the method is not detrimental to the surface and the samples are not sticky. In the best of cases it is only necessary to flush with fresh run buffer. At other times exposure to acid or base, or even an organic solvent, may be required to restore the surface to the original condition. The regeneration procedure, however**,** should be matched to the separation method. For separations using sodium borate buffer, pH 8.3, regeneration with NaOH is appropriate. For separations using phosphate buffer, pH 2.5, the use of NaOH or other base rinses should be avoided. At low pH, EOF is suppressed because the silanol groups are poorly ionized. Using NaOH to regenerate the capillary will increase the time needed to re-equilibrate the surface with the low pH electrolyte (phosphate), thus rinsing with a higher concentration of low pH buffer or even with 1 *N* acid (i.e., phosphoric) will generally give better results.

Rinsing coated capillaries adds a further complication, in that the coating itself may be labile in the presence of the cleaning solution. Exposure to NaOH will strip many covalently bonded phases from the capillary surface. It is critical to follow the manufacturer's recommendations for cleaning and regenerating coated capillaries. Dynamic coatings such as TTAB should be reconstructed by rinsing with a more concentrated solution of the coating material than is used in the run buffer. For example, if the running buffer contains 0.5 m*M* of detergent, a pre-rinse with 20 m*M* detergent followed by the actual running buffer would be appropriate.

1.2.2. Separation Buffers

The significance of the capillary wall in controlling the process of separation in CE cannot be overstated. The separation, however, takes place in the separation buffer. It is here that the conditions are such that the differences in mobility can exist. Buffers can either be made or purchased. Buffer quality is independent of the instrument system used. However, even the best instrument system will not perform properly with a poorly prepared buffer.

1.2.2.1. Significance of pH

The most common measurement in analytical chemistry is probably the measurement of pH. It is also the measurement that is most often made improperly. In CE it is extremely important to properly control pH since it affects analyte charge, electroosmotic flow, and, by affecting current, heat production. Thus small changes in pH tend to have greater impact in CE than do comparable pH variations in HPLC.

Figure 3 shows the titration curve of the silica surface, which behaves as a typical weak acid (e.g., acetic acid). The ionization of acetic acid (pK_a = 4.76) is shown in Eq. 2. At the pK_a, the analyte will be 50% charged. This

does not mean that each molecule carries one-half of a charge, but rather that at any point in time one half of the molecules are charged and the other half are uncharged. A titration curve like that shown in Fig. 3 represents the probability than any given molecule will be charged at a given pH.

$$H_3C\text{-}COOH \rightleftarrows H_3C\text{-}COO^{(-)} + H^{(+)} \quad (2)$$

When the molecule is uncharged it is under the influence of the electroosmotic flow but not the electrical field. When charged the molecule responds to both forces. As the fraction of the time the molecule spends in each state varies with pH, the net migration velocity will change. As discussed earlier, the EOF also changes with pH. Failure to properly control pH is one of the major causes of poor reproducibility in CE.

1.2.2.2. Buffer Species

Buffers are compounds that are used to control the pH of a solution. They are generally weak acid or bases that can accept or donate protons. Buffers reduce the change in pH that is caused by the introduction of additional acid or base; CE buffers are selected on the basis of the pH range that is to be maintained, as well as other factors.

A wide range of buffers has been employed in CE systems. The most commonly employed are phosphate, borate, citrate, acetate, and Tris (trishydroxymethylamino methane). The zwitterionic buffers that have been recommended by several authors have the advantage of carrying less current than do mono-functional buffer molecules. However, the selection of a buffer for CE should be based on several factors, including: 1) pH value desired; 2) operating temperature; 3) charge of the buffer relative to the analytes and the capillary wall; and 4) effects on detection. These factors are further described as follows:

1. For any buffer the effective buffering range is defined as within one pH unit of the buffers pK_a. Thus a buffer with a pK_a of 5 would be usable from pH 4.0 to pH 6.0. For CE it is much better to have the pH as close to the pK_a of the buffer as possible, certainly within ± 0.5 pH units, to reduce the buffer's contribution to variability.
2. Buffer molecules exhibit a temperature coefficient. The pH of a buffered system will tend to change with temperature. In addition, buffers tend to differ in their sensitivity to temperature. For example, phosphate has a low temperature coefficient compared to Tris. The temperature coefficient of the buffer is also important because during electrophoresis the analyst has only limited control of the temperature inside the capillary. This is discussed in more detail in Subheading 1.2.3.
3. Interaction between the buffer and the analytes can change the effective charge on the analyte molecules, changing their migration velocity. Ion pairing can also occur between the buffer molecules and the analytes affecting the separation.

4. Buffers differ widely in their absorbance spectra. This is much less of a problem in CE than in HPLC because of the short path-length in CE detection. However, for high-sensitivity work the background absorbance may become an issue.

Once the buffer composition has been selected, it is essential that it be prepared properly. In order to make a buffer consistent it is critical to make the buffer the same way every time. For example, preparing a 50 m*M* sodium phosphate buffer by starting with 50 m*M* sodium phosphate and adjusting the pH with phosphoric acid will result in a buffer with a phosphate concentration higher than 50 m*M*. Such a buffer is best prepared by titration of phosphoric acid with NaOH.

A buffer that has found wide application in CE is sodium borate. However, the preparation of borate buffers can be confusing because boric acid combines with sodium hydroxide according to Eq. 3.

$$4\ H_3BO_3 + 2\ NaOH \rightleftarrows Na_2B_4O_7 + 7\ H_20 \quad (3)$$

Four moles of boric acid react with sodium hydroxide to form one mole of sodium tetraborate. A borate buffer will contain both boric acid and tetraborate. In discussing these systems it is customary to refer to the concentration of Boron. Thus, 4 *M* boric acid and 1 *M* sodium tetraborate have the same concentration of boron.

1.2.2.3. Buffer Concentration

Increasing the concentration of a buffer will increase the buffering capacity of the system, making it less likely to change pH with the addition of acid or base. In addition, there are other factors in CE that can be affected by the concentration of the buffer *(16,17)*. These factors can be critical when trying to achieve optimum resolution and reproducibility.

If the composition of the sample plug is different from the composition of the buffer in the capillary, the phenomenon of stacking may occur *(18,19)*. Properly exploited stacking can result in sharper peaks and greater sensitivity. One way to achieve stacking is to keep the conductivity of the sample lower than the conductivity of the running buffer by reducing the concentration of the sample buffer relative to the run buffer. To understand this phenomenon, consider the buffer filled capillary as a wire. If the buffer concentration is uniform from one end of the capillary to the other end, the voltage drop will be linear. Inserting a plug of dilute sample is like inserting a resistor into the wire. The voltage drop now becomes steeper in the region of the plug than it is elsewhere. Since velocity is proportional to the voltage gradient, the velocity of the analytes will be faster in this region. Analytes will move rapidly until they reach the boundary between the dilute sample plug and the more concentrated running buffer. On reaching this point they

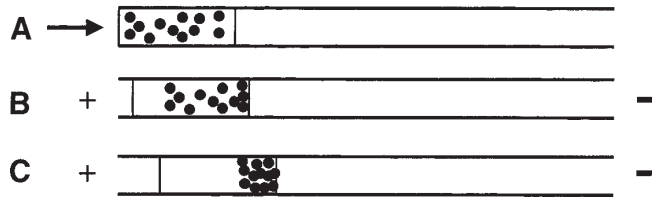

Fig. 7. Sample stacking. **(A)** In an injected sample plug the analyte molecules are distributed randomly. **(B)** When the voltage is applied the entire sample plug begins to move under EOF. The analytes in the sample move more rapidly than the bulk flow due to the difference in ionic strength between the sample plug and the run buffer. **(C)** Before the analytes begin to migrate out of the sample plug they have been concentrated. Note that the length of the sample plug is unchanged.

will stack, increasing the local concentration of analyte and creating a zone that is narrower than the original injection plug. This is illustrated in Fig. 7.

Because stacking is caused by a difference in conductivity between the buffer and the sample, it might be assumed that dissolving the sample in pure water would create the highest peak efficiencies. In practice, however, this is not usually the case. The resistance across a plug of nearly pure water can be so high that localized heating results. This heating causes convective mixing and de-stacking. There should be always be some buffer ions in the sample plug; keeping samples at about 1/10 the concentration of the running buffer is a good rule of thumb *(20)*.

One way to maximize stacking would be to increase the concentration of the buffer, particularly if the samples as received are high in salt. As stated in Section 1.1.2.2., EOF decreases with the square root of the buffer concentration. If EOF is to remain constant it will be necessary to increase it in some other way, such as a change in pH, temperature, or voltage. If desired, EOF can be reduced simply by increasing the buffer concentration. However, by increasing the buffer concentration the current that passes through the capillary will also increase. This in turn will increase the heat generated inside the capillary and, if not adequately dissipated, a variety of problems can result. Without adequate cooling the ability to exploit higher buffer concentrations is limited.

When trying to separate basic compounds or proteins that tend to interact with the capillary wall using bare silica capillaries, problems are frequently encountered. In extreme situations they may be totally adsorbed on the capillary wall and never pass the detector. In other cases the wall may provide a drag force that results in peak tailing, loss of resolution, and decreased signal-to-noise ratio. Although coated capillaries are one solution to this prob-

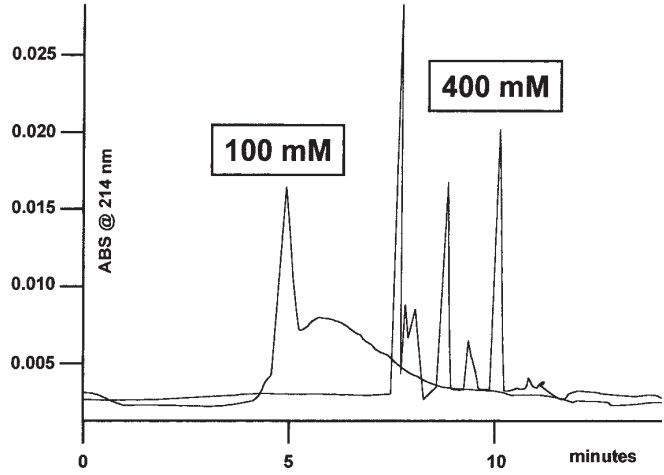

Fig. 8. Effect of buffer concentration on peak shape. The proteins in this sample interact strongly with the wall of the capillary. Increasing the buffer concentration can reduce this effect.

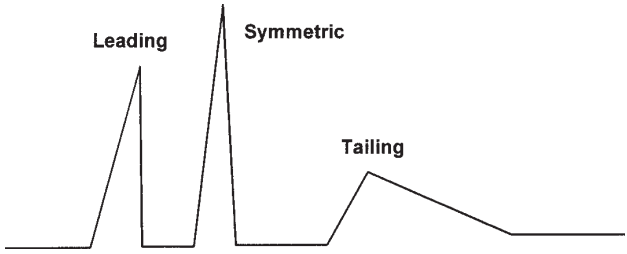

Fig. 9. Peak shape in CZE mode. The peaks shown here are indicative of variations between the mobility of the analytes and the mobility of the buffer ions. Peak shapes such as these do not necessarily indicate adsorption phenomena.

lem, merely increasing the concentration of the electrolyte can often reduce these interactions *(15,21)*. Figure 8 shows the effect on a protein separation of increasing the phosphate concentration.

Peak shape in many modes of CE differs from the bell shape that is commonly seen in HPLC and other chromatographic separations. In open capillary modes (such as capillary zone electrophoresis [CZE]), it is not uncommon to see peak shapes like those in Fig. 9. In CE the electrical field influences the buffer ions just as it does the analyte ions. The peak shape in

these separations is due to the relative migration rates of the buffer ions and the analyte ions. The degree of asymmetry will increase with increased difference in mobility. Buffer mismatch can so distort the peak shape that analytes may be difficult to quantitate or may even be lost.

Because the buffer ions migrate in the electrical field, the concentration of the buffer ions in the inlet and outlet vials will change over time. Using buffer vials that are too small (i.e., do not contain an adequate volume of buffer) or running too many samples without replacing the buffer can result in ion depletion. This process can result in a change in the sample migration times or resolution.

1.2.2.4. Additives

Other reagents are frequently added to the buffer systems used in CE. The most common are detergents, such as sodium dodecyl sulfate (SDS) *(22)*, viscosity modifiers, such as linear polyacrylamide *(23)*, organic solvents, such as acetonitrile *(24)*, denaturants, such as urea *(25)*, or combinations of these additives.

The addition of detergent to a buffer used in CE can change dramatically the separation properties of the system. Detergents can aid in solubilizing analytes and in reducing analyte-wall interactions. They may also bind to the capillary wall, affecting the EOF. If the detergents are above their critical micellar concentrations (CMC), they can create a pseudostationary phase. Under these conditions the separation mechanism is referred to as micellar electrokinetic chromatography (MEKC), which is discussed in Section 2.5. Detergents for CE may be anionic, cationic, or nonionic. Protein separations can also be achieved with detergents. By titrating the concentration of SDS in the run buffer it is sometimes possible to achieve protein separations based on differential binding of the detergent to different proteins. This is not a separation by MEKC because the protein molecules are too large to be included in the micelle *(26)*.

Viscosity modifiers provide a physical retardation to the movement of molecules. Suitable viscosity modifiers include polymers, such as linear polyacrylamide or soluble cellulose derivatives, as well as small molecules like urea. By retarding large molecules more than they do small molecules the dimension of size separation is added to the electrophoretic separation. This is exploited fully in capillary gel electrophoresis (CGE) which is discussed in Section 2.2. At lower concentrations, viscosity modifiers can aid in the separation of very similar analytes. Viscosity modifiers can also have significant effects on EOF, both by the change in viscosity and by interacting with the capillary wall *(27)*.

Organic additives routinely used include acetonitrile, methanol, formamide, and dimethylformamide. These solvents improve the solubility

of organic molecules that would otherwise be poorly soluble in an aqueous system. At sufficiently high concentrations, they can also reduce the degree of ionization of charged analytes. In MEKC they can alter the relative hydrophobicity of the micellar and non-micellar phases. Organic additives can also affect viscosity and wall charge (and hence EOF). The effect of solvents on viscosity can be complex. Acetonitrile has a lower viscosity than water, and any combination of water and acetonitrile will have a viscosity between that of water and acetonitrile. Methanol also has a lower viscosity than water, but combinations of water and methanol can be substantially more viscous than either solvent alone. In nonaqueous CE (described in Section 2.7.) organic solvent completely replaces water in the separation solution.

1.2.3. Temperature

Temperature control is crucial to reproducible separations in CE. However, temperature regulation is complicated by several factors. First is that the passage of electrical current through the buffer-filled capillary results in the production of heat. This self-heating effect is inherent in electrophoretic separations and is called Joule heating. Thus temperature control in CE is as much a task of removing heat as it is maintaining a constant temperature environment. Second is that the temperature of the contents inside the capillary is difficult to measure. A layer of silica and a layer of polyimide separate the small fluid volume inside the capillary from the thermostatted medium. Any heat must be transferred to and from the separation buffer through these layers.

Although the temperature inside the capillary cannot easily be measured it can be estimated. Equation 4 is used to estimate the Joule heating (Q). In this equation

$$Q = E^2 \Lambda c \tag{4}$$

Q is the Joule heat generated (Ω/cm^3), E is the voltage gradient (V/cm), Λ is the molar conductivity of the electrolyte ($cm^2\,mol^{-1}\,W^{-1}$) and c is the concentration of the electrolyte (mol/L). Since the conductivity of the electrolyte will vary with temperature, a positive feedback mechanism occurs. In other words, as the temperature increases, the current will also increase. This in turn generates more heat thus generating more current. This feedback loop is moderated by the ability of the cooling system to remove heat and thus limit current. Because the cooling system must act at the surface of the capillary and not internally, a temperature gradient exists from the center of the capillary to the outside, which is illustrated in Fig. 10.

The temperature gradient within the capillary is parabolic. The difference in temperature between the center of the capillary and the wall will increase

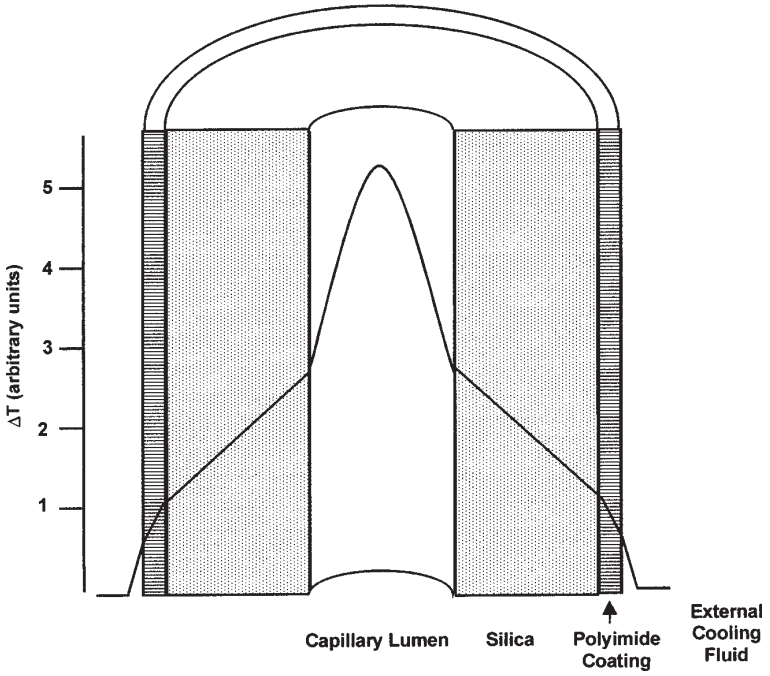

Fig. 10. Theoretical radial temperature profile. Joule heating occurs when current passes through the buffer-filled capillary. The temperature can be expected to be highest in the center of the capillary. Each layer through which the heat must pass will have its own thermal conductivity leading to a complex pattern such as that illustrated here. Adapted from Knox *(28)*.

with increasing capillary internal diameter. Current will also increase as the diameter increases (current α radius^2). The combination of these two factors can lead to the conclusion that heating will cause band broadening and that the band broadening will increase as the internal capillary diameter increases. In systems with adequate temperature control, this thermal band broadening does not become a problem unless the capillary diameter is larger than about 100 μm. A more detailed discussion of heat transfer in CE can be found in an article by Knox *(28)*.

A second temperature gradient can also exist in poorly designed systems if the entire length of the capillary is not maintained in the same thermal environment. The regions of the capillary that are in buffer vials, passing through cartridge walls, or in detector cells are frequently much less efficiently cooled than are other regions of the capillary. Depending on the sys-

tem and the capillary length, this uncooled region can account for as much as 25% of the capillary's effective length and can account for much of the variation in performance seen between instruments of different design.

Dynamically cooled CE systems are conveniently divided into two groups: those employing gas and those employing liquid as a heat exchange medium. Liquid cooling is generally considered to be more efficient than gas cooling simply because the heat capacity of liquids exceed that of air. The most effective liquid coolant would be water because of its enormous heat capacity. However, when dealing with electrical equipment, the use of water as a heat exchange medium is not advisable. The requirements of safety has led to the use of perfluorinated organic molecules, having the general formula (F_3C-$[CF_2]_n$-CF_3)**,** because of their very low electrical conductivity. Because of their greater heat capacity, liquids tend to be slower to adjust to changes in temperature than gases. Liquid cooling also necessitates sealed recirculating systems, adding to the expense and complexity of the instrument. Whatever the cooling medium, most modern instrument systems use computer controlled Peltier devices to control the temperature of the cooling fluid. Peltier devices are semiconductor mechanisms that can be made to heat or cool by reversing the direction of current flow through them.

Changes in temperature can also impact other factors inside the capillary. For most liquids, an increase in temperature causes a decrease in viscosity. For example, water has a viscosity of 1.002 centipoise at 20°C. This drops to 0.798 centipoise at 30°C. In the absence of any other changes this change in viscosity will result in a substantial increase in EOF. In addition, the pH of a buffered solution may also change with temperature. A 10°C change in temperature will cause a 0.3 pH unit change in the pK_a of Tris. This may be sufficient to change both EOF and the charge on the analyte molecules.

A simple way to determine if a CE system is capable of coping with the heat that will be produced during separation is by creating an Ohm's Law plot. Ohm's Law states that V = IR. If we vary voltage (V), the current (I) should change in a linear manner so long as resistance (R) remains constant. An increase in temperature within the capillary generally results in a decrease in resistance (and hence an increase in current at constant voltage). By measuring the current at a variety of voltages a graph of current vs voltage can be created. Regardless of the type of cooling system the current will become nonlinear at some voltage. For the buffer system being considered the point at which the line deviates from linearity indicates the maximum voltage possible for that buffer system/capillary combination on that instrument. In practice it is possible to run at somewhat higher voltage if it is understood that the temperature within the capillary is higher than the thermostatted temperature under these conditions. It is good practice to

monitor current during a run because an increasing current during a run may indicate that the heat dissipation capacity of the instrument is being exceeded. In the worst case the heat in the capillary may become sufficient to boil the buffer locally, usually at some poorly cooled location. This usually breaks the electrical circuit (and sometimes the capillary).

1.3. Volume Relationships

The dimensions employed in CE are much smaller than those with which most chemical analysts are accustomed to work. Capillary diameters typically are measured in microns and as such the entire volume of a capillary is usually a few microliters. Injected sample volumes are in nanoliters (10^{-9} L). At these scales the tolerances required of instrument systems are extremely small and difference of 1 μm in the diameter of a capillary can create very large differences in the results of an analysis.

1.3.1. Flow Dynamics

Most often the sample being analyzed by CE is injected into the capillary by pressure. To calculate the volume of liquid injected it is necessary to use the Poiseuille equation (Eq. 5), which estimates the flow of liquid through a cylinder.

$$V = (\Delta P d^4 \pi t)/(128 \eta L) \quad (5)$$

In this equation, ΔP is the pressure drop down the length of the cylinder (Pascals), d is the cylinder's inside diameter (m), t is time the pressure is applied (s), η is the fluid viscosity (Pascal-seconds), and L is the total length of the cylinder (m). This equation has wide applicability in fluid dynamics and can be used to calculate fluid flow in CE capillaries, blood vessels, or a water main. It shows that the delivery of fluid into a capillary by pressure is directly proportional to the pressure that is applied and the length of time the pressure is applied. Fluid delivery is inversely proportional to the liquid viscosity and the length of the capillary, and proportional to the fourth power of the diameter. Temperature is indirectly involved in this relationship because the viscosities of most fluids change with temperature.

Table 1 exemplifies the volumes typically encountered in CE. This example assumes the viscosity of water at 25°C (1.000 centipoise). Typically injection and rinse pressures are 0.5 and 20 psi, respectively.

As predicted by Eq. 5, the volume of fluid delivered at a given pressure increases dramatically as the diameter of the capillary increases. Doubling the diameter of the capillary increases the delivered volume 16-fold. The implications of this are significant. As shown in Table 2, the volume injected under constant condition changes dramatically when the capillary diameter changes over a narrow range. Because tolerances of 1–2 μm are not uncom-

Table 1
Typical Volumes Encountered in Capillary Electrophoresis[a]

Diameter (μm)	Length (cm)	Capillary volume (nL)	Injected nL/s @ 0.5 psi	Injection plug (mm)	Rinse nL/s @ 20 psi
20	25	79	0.060	0.19	2.43
20	50	157	0.030	0.09	1.21
25	25	123	0.148	0.30	5.93
25	50	245	0.074	0.15	2.96
50	25	491	2.37	1.2	94.92
50	50	982	1.18	0.6	47.46
75	25	1104	12.0	2.71	480
75	50	2209	6.00	1.35	240
100	50	3927	18.9	2.41	759

[a]These values were calculated using Eq. 5.

Table 2
Injection Volume Variability with Capillary Internal Diameter[a]

Diameter (μm)	18	19	20	21	22
nL/s @0.5 psi	1.99	2.47	3.03	3.69	4.44

[a]Capillary for CE is typically manufactured to tolerances of +/- 2 μm.

mon in commercially available capillary, selecting different pieces of capillary nominally 20 μm in diameter could give peak areas that vary substantially. Cutting pieces from a large spool of capillary is no guarantee of uniformity because the diameter may vary along the length of the spool.

The injection plug length in Table 1 is the linear distance within the capillary that is occupied by the injected sample volume. An injection plug that is too long may result in wide bands and loss of resolution, particularly if the analytes cannot focus. Increasing the capillary diameter allows the injection of substantially larger volumes of sample without increasing the plug length. Because the volume of a cylinder increases with the square of the radius a doubling of the capillary diameter will allow the injection of four times as much sample without changing the plug length.

The viscosity of the fluid being delivered is the most difficult parameter to know with accuracy. The aforementioned examples have assumed the viscosity of water at 25°C. Within the range of temperatures typically used in CE separations (15–60°), the viscosity of water varies in a nonlinear manner from 1.138 to 0.467 centipoise. Almost anything added to the water will alter both the viscosity and the temperature-viscosity relationship. The addi-

tions of macromolecules, such as cellulose derivatives or acrylamide polymers, are extreme cases. These molecules display remarkable viscosity behavior with changes in flow rates. Linear polymers, for example, can extend and align themselves when forced through a capillary. They can show a reduction in viscosity with increased flow. Other systems may increase in viscosity with flow because of polymer entanglement. Even systems as simple as methanol-water can show complex behavior. For example, a 1:1 mix of methanol and water has a higher viscosity than either pure solvent. It is also important to remember that when calculating volumes injected into fluid-filled capillaries, the viscosity of the fluid in the capillary is usually more significant than the viscosity of the sample (unless one is analyzing highly viscous samples).

Entering the Poiseuille equation into a spreadsheet program simplifies fluid delivery calculations such as these. There is also a Windows*-compatible computer program called "CE Expert" that can perform these calculations (this program is currently available at no cost directly from Beckman Coulter, Inc.).

1.3.2. Sample Handling

Four basic strategies are used to deliver these fluid volumes into capillaries: Positive pressure, vacuum, gravity, and electrophoresis. Positive pressure and vacuum have been the most common methods of filling and rinsing capillaries. Positive pressure up to 100 psi (7 bar) has been used for rinsing. This pressure is delivered either from a source of compressed gas, such as nitrogen, or from an on-board air pump that applies pressure to the headspace of a buffer reservoir. Vacuum delivery is limited to 10 psi or less but can be useful for drawing fluid from containers that cannot be made pressure tight.

Gravity and electrophoresis are not practical for filling and rinsing capillaries, but they are used, along with pressure and vacuum, for injecting samples into capillaries. Gravity injection (sometimes called hydrostatic injection) is accomplished by inserting the inlet end of the capillary into the sample vial and raising the vial and capillary relative to the outlet end. Reproducible gravity injection requires that the vial be raised to the same height for the same duration of time at each injection (gravity itself being a fairly reliable source of motive power). Pressure and vacuum injections are more complicated. Regardless of how the pressure differential is created, a finite time is required for the pressure to reach a steady state. Changes in pressure on the order of 0.05 psi can be significant at the low pressures commonly employed (0.1–1 psi). Systems that rely on pressure or vacuum

*Windows is a trademark of Microsoft.

must have some sort of feedback mechanism to compensate for these variations. These systems either adjust the delivered pressure or the delivery time to maintain the desired product of pressure and time. In well-engineered systems a 3-s injection at 1 psi and a 10-s injection at 0.3 psi should give identical results, because both are 3 psi-second injections. In practice the longer, lower pressure injection usually gives better performance because it allows a longer time for the system to respond to variances.

Electrophoretic or electrokinetic injections do not conform to the Poiseuille equation. In this method of sample introduction, the inlet of the capillary is inserted into the sample and the outlet into a buffer vial. Voltage is briefly applied. Through a combination of electrophoresis and electroosmotic flow sample is drawn into the capillary. This technique is valuable when delivering sample to a gel-filled capillary or when pressure delivery is not possible. There is a possibility of bias when using this technique *(29)*. Components that migrate more rapidly in the electrical field will be over-represented in the sample compared to slower moving components. To maximize the volume injected, the sample should be at a considerably lower ionic strength than the run buffer. Subsequent injections will show reduced peak areas because each injection delivers salts from the capillary buffer into the sample vial, raising the ionic strength of the sample. This effect can be minimized (and injected quantity increased) by pre-injecting from pure water immediately prior to the sample injection.

Most commercial systems employ some sort of carousel or X-Y-Z robotic system to move the buffer and sample vials to the capillary. These systems may also provide refrigerated storage for labile samples. In this case the sample storage temperature should be regulated independently of the capillary thermostatting system.

1.4. Detection

In order to gain useful information from the separation technique, it is necessary to detect and measure the analytes. Detection may be qualitative and/or quantitative. Most CE detection is done on-capillary; that is, a section of the capillary is linked to the detection device and the capillary itself is the detection cell. It is also possible to couple to detectors that are outside of the separation capillary although this does require a specialized interface.

1.4.1. Geometry and Path-Length

The most frequently used method of detection involves absorbance of energy as the analytes move through a focused beam of light. The scale of the detection apparatus and of the signal produced has created some unique challenges for the instrument developer.

On-capillary detection eliminates the problems of coupling the capillary and its power supply to flow cells or other devices. However, detection through the capillary is complicated by the curvature of the capillary itself. The capillary and the fluid it contains make up a complex cylindrical lens. The curvature of this lens must be accounted for in order to gather the maximum amount of light and thereby maximize signal-to-noise ratio. The effective length of the light path through the capillary is actually about 63.5% the stated i.d. of the capillary. Thus a 50-μm capillary has an effective path length of only 32 μm. This can be compared to typical HPLC detectors that have detectors in the 5–10 mm range. Because of this very small light path, the absorbance signal obtained from a CE system is also correspondingly small. Therefore, a peak with an absorbance of 0.002 AU is a significant peak. Noise levels are correspondingly small and are usually measured in microabsorbance units. The maximum absorbance of typical CE detectors is 0.2 AU. The capillary lumen occupies only a small part of the diameter of the capillary; the remainder is transparent silica. This geometry allows for large amounts of stray light to enter the detector so that a fully opaque sample would not reduce the light passing through the capillary to zero. This factor must be considered in the design of effective CE detector hardware and software.

Several novel approaches have been taken to increase the path length (and the sensitivity) of CE detectors. One approach has been to use a specially constructed low-volume flow cell. These cells carry the analytes through two right-angle bends *(30)*. The segment between the bends, which may be over 1000 μm long, is thus at right angles to the direction of the capillary and parallel to the direction of the light beam. Properly designed, these cells can dramatically increase sensitivity but at the risk of some loss of resolution. Another approach has been to create a wide zone or "bubble" in the capillary at the window *(31)*. A 50-μm i.d. capillary may have a window that is 150 μm in diameter, giving a sensitivity increase of approximately threefold.

1.4.2. Absorbance

Absorbance detectors are the most commonly encountered types of detector in CE instrument systems. They rely on the absorbance of light energy by the analytes. This absorbance creates a shadow as the analytes pass between the light source and the light detector. The intensity of the shadow is proportional to amount of material present.

The simplest absorbance detector, shown in Fig. 11A uses only portion of the available energy. The broad-spectrum light from a source lamp is passed through a filter or diffracted by a grating so that a narrow range of the spectrum is used. In some cases lamps (such as hollow cathode types) are used, that produce light at only a few discrete wavelengths. Monochromatic

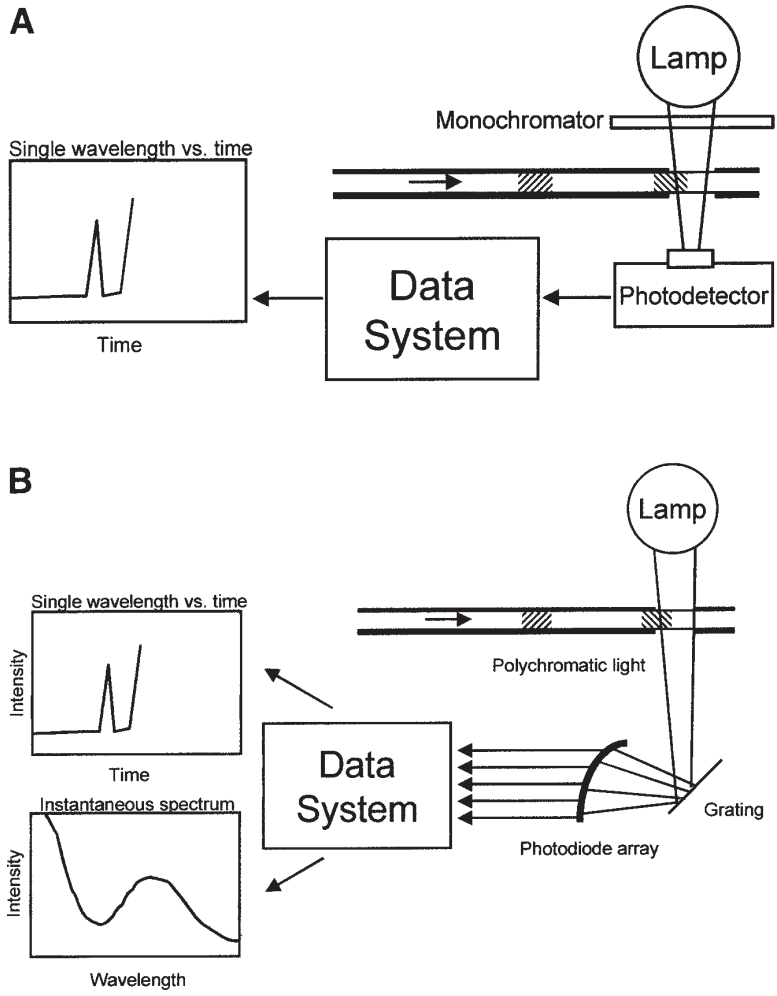

Fig. 11. Comparison of single wavelength **(A)** and photodiode array **(B)** absorbance detectors.

absorbance detectors are relatively inexpensive and rugged. However, because only a part of the spectrum is used, information may be lost from complex samples that have components with differing absorbance maxima.

Another type of absorbance detector looks at changes over a wide range of wavelengths simultaneously. The photodiode array detector (PDA) shown in Fig. 11B delivers the entire spectrum of light available from the source

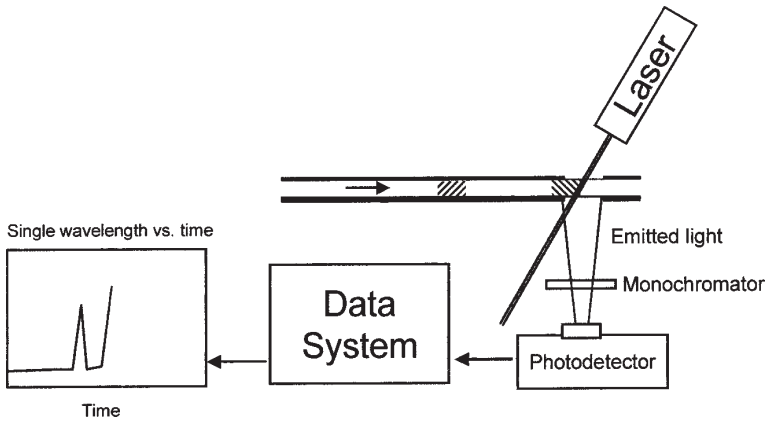

Fig. 12. Laser-induced fluorescence detector.

lamp to the capillary window. The light passing through the capillary is diffracted into a spectrum that is projected on a linear array of photodiodes. In this manner it is possible to record the entire absorbance spectrum of analytes as they pass by the detector window. PDA-type detectors are more expensive and less rugged than are monochromatic detectors. Because the spectrum can be divided into as many as 512 channels the amount of data acquired in a single run can be very large. The PDA type detector, however, is not suitable for all applications because nearly all the energy of the source lamp is focused onto a very small region of the capillary. Some capillary coatings and buffers will decompose under this onslaught of energy unless some of the energy is filtered out. Despite these limitations the information provided by the PDA detector can be valuable for confirming the identity of analytes. By comparing the change in spectral signature across a peak it is possible to estimate peak purity *(32)*.

1.4.3. Fluorescence

Fluorescence detectors do not rely on the measurement of shadows. These systems use an external energy source to excite the analyte molecules to a higher energy state. When these excited molecules return to the normal state they emit energy of a lower wavelength, which can be detected and recorded as evidence of the passage of the analytes. Fluorescent detectors in CE systems often use lasers as the source of the excitation energy (LIF detection, Fig. 12). Lasers have the advantage of producing intense light at a single wavelength. The intensity of the light contributes to good excitation efficiency. In addition, the monochromatic nature of the laser beam makes it

easy to filter out any stray laser light to keep it from interfering with the detection of analytes. Analytes will vary in their excitation and absorbance wavelengths so that a fluorescent detector will not see all the components that may be in a sample. For analytes that are fluorescent or can be made fluorescent by a chemical reaction, the sensitivity of this type of detector can be 10–1000 times better than an absorbance detector *(33)*.

1.4.4. Amperometry and Conductivity

These two detection techniques potentially can offer a high degree of sensitivity and be applied to a wide variety of analytes, including those without appreciable UV absorption. Amperometry and conductivity are difficult to do in practice, and to date these devices are not commercially available. Both of these detection schemes require the use of sensing electrodes that are scaled to the dimensions of the electrophoresis capillary. In addition, these detectors place some limitations on the type of separation buffer employed.

In amperometric detection, an electroactive analyte undergoes an electrochemical reaction inside a detector cell *(34)*. The CE separation is generally carried out at microampere currents and kilovolt potentials, whereas the detection cell must operate at picoampere currents and millivolt potentials. The two circuits must therefore be isolated, usually by connecting the capillary to the high voltage at a point prior to the end of the capillary where the detection cell is located. This system depends on EOF to carry the analyte past the high voltage electrode to the detection cell. Amperometric detectors have been used successfully for the detection of biogenic amines at levels as low as 10^{-8} m *(35)*. With continued advances in microfabrication this type of detector should become routinely available.

There are two types of conductivity detector *(36,37)*. Both require that two electrodes be placed within the separation capillary. In the first type a short distance down the length of the capillary, separates the electrodes. Because there is a voltage gradient down the length of the capillary a portion of that voltage gradient can be measured between the two sensing electrodes. The presence of analyte zones will change the potential drop in the area of the zone. This change in potential will be sensed as the zone passes the electrodes. In the other arrangement the electrodes are placed opposite one another across the diameter of the capillary. In this case there is no voltage drop between the two electrodes. A circuit is constructed that passes a sensing voltage across the capillary diameter. If the conductivity of the system changes as analytes pass between the electrodes, there will be a change in the current in the sensing circuit. Conductivity detectors work best when there is a substantial difference between the conductivity of the

analyte zones and the background buffer *(38)*. These conditions are not optimized for peak shape and lead to unacceptable peak asymmetry. Various schemes have been described for avoiding this problem. Conductivity detectors have been used for the measurement of inorganic ions and in isotachophoretic separations (Section 2.4.).

1.4.5. Capillary Electrophoresis-Mass Spectrometry

Hybrid systems such as CE-mass spectrometry (CE-MS) offer an additional dimension of analysis in addition to detection. CE data consists of migration time, quantity, and (using a PDA detector) spectral signature. MS adds the additional data of molecular weight and, using collision dissociation and MS-MS systems, structural information as well. This combination of techniques provides an orthogonal approach to analysis in a single analytical run.

The predominant form of MS that has been coupled to CE has been electrospray MS *(39)*. A more in-depth discussion of CE-MS can be found in Chapter 15. This section will focus on that technique. The outlet end of the CE capillary is inserted into the electrospray interface. Because the volume of liquid emerging from the capillary is very small, a make-up liquid is pumped through an axial needle. This sheath flow also provides a return connection to the high-voltage power supply of the CE system. The liquid is mixed with a flowing gas stream and nebulized into a spray. The spray vaporizes and the ionized analyte particles are carried into the MS detector. The MS system is usually set to scan across an expected range of mass values. Because the width of peaks in CE can be very small, the MS instrument must be able to scan across the desired mass range very rapidly or peaks may be missed.

Most mass spectroscopists prefer to use buffer systems that are volatile, such as ammonium formate, in order to reduce the accumulation of buffer salts inside the MS instrument. These buffers may not be optimized for the separation of the analyte mixture, although an incomplete separation can be acceptable in CE-MS because the MS systems provide an additional dimension of separation.

Many of the existing commercial CE systems have been interfaced to commercial MS systems. The design of these systems allows the use of UV or other detectors prior to the MS interface, but usually require quite long and awkward reaches of capillary to connect the two systems. Unlocking the true potential of this method will require the development of a CE system that is fully integrated with the MS system.

1.4.6. Indirect Detection

In the previous discussions it has been assumed that detection will be direct, that is, the presence of the sample in the detector cell or window will

cause an increase in the output signal. This is not always the case. There are certain applications where the decrease of the background signal provides evidence of the passage of analytes through a detector. In some cases a detector that is useful for indirect detection would not detect the analytes in direct mode. An example of this is the detection of inorganic ions such as sodium or sulfate with a UV detector.

To detect such analytes, the capillary is filled with a buffer that has mobility close to that of the analytes and also has a significant UV absorbency. The buffer/chromophore must carry the same charge as the analyte. For the analysis of sulfate, a buffer containing sodium chromate is used *(40)*. In the area of the capillary occupied by the sulfate band the chromate is displaced, creating a chromate depleted region. Because chromate absorbs light at 254 nm, by monitoring background electrolyte absorbance at this wavelength the presence of sulfate will be indicated by a negative peak, with an area corresponding to the amount of chromate displaced and hence to the amount of sulfate present. Most CE data analysis packages can invert these negative peaks, producing a normal-appearing electropherogram.

1.4.7. Data Analysis

The collection and analysis of CE data has many characteristics in common with other chromatography-like analyses. Many analysts have utilized data systems developed for HPLC and GC systems. These data systems may not be optimal for CE for three reasons:

1. The signals obtained from CE are usually very small. A typical HPLC peak may have a maximum absorbance of 0.2 AU, whereas a CE detector may have a full range of 0.2 AU. A typical peak in CE may have a maximum absorbance of 0.002 AU.
2. CE peaks can be quite narrow with a width of only a few seconds. Some older data systems (particularly HPLC systems) cannot respond sufficiently fast to deal with this data. If data is to be collected digitally, it must be collected at a high data rate to define adequately the peak shape.
3. CE peaks are often non-Gaussian. Because of the low band broad spreading in CE and the effects of electrofocusing CE peaks tend to be more triangular and less bell-shaped than peaks in HPLC and GC. At times CE peaks may have one edge that is nearly vertical. Some data systems have difficulty locating the peak start and stop times for these shapes.

Two types of peak-detection methods dominate analytical data analysis: slope sensitive algorithm and the moving median filter algorithm. Slope sensitive algorithms look at the slope of the baseline over some interval of time. When the slope exceeds a pre-determined value, a peak is said to have begun. The point at which the slope goes to zero identifies the peak apex, and the point at which the slope returns to the starting value defines the peak end.

The slope-sensitive method looks for peaks independently of the baseline shape. Most commercial software packages use this method.

Moving median filter algorithms take a different approach. Peaks are relatively high frequency (impulse) events when compared to baseline drift. These algorithms seek to define how the baseline would look in the absence of peaks by filtering out all impulse events. Whatever differs from the baseline is defined as a peak. In practice, the algorithm makes several passes through the data set (iterations) to determine the best fit. This type of algorithm, commercially available as "Caesar," is more successful at dealing with the abrupt slope changes in CE data than are slope-sensitive algorithms *(41)*.

A data system for CE needs to include some calculations that can be deemed "CE specific." These include the calculation of mobility (a measure of the velocity of an analyte through the capillary) and corrected peak area. Corrected area is necessary because the peaks passing through a CE detector do not all pass through at the same velocity. Early eluting peaks move through more rapidly than do later eluting peaks. This is unlike the situation in HPLC where the velocity through the detector cell is dependent only on the flow rate and not on the retention time. Because later eluting peaks are moving more slowly, they appear to be larger relative to earlier eluting peaks. Corrected peak area normalizes peak area to unit migration time and allows accurate comparison of the components in a mixture.

1.4.8. Fraction Collection

Fraction collection is placed under the heading of detection under the assumption that fractions are being collected for analysis outside the CE instrument. At first glance, fraction collection in CE appears to be analogous to fraction collection in HPLC, but there are significant differences *(42)*.

The velocity of peaks through the HPLC detector is constant if the flow rate is constant. This is not true in CE where earlier eluting peaks are moving more rapidly than later eluting peaks. The delay time between peak detection and elution (the time for transit from the window to the capillary outlet) will therefore vary, becoming longer with each successive peak. This time can be quite long. Consider a capillary 60-cm long with the window located 10-cm from the outlet end. If a peak passes the detector at $t = 5$ min it will not emerge from the end of the capillary until $t = 6$ min. A peak detected at $t = 10$ min will emerge at $t = 12$ min. Peaks that pass the detector between 5 and 10 min will all be in the outlet segment of the capillary between $t = 6$ and $t = 12$ min. A further complication is that collection requires that the outlet of the capillary be moved to a new collection vial for each peak that is to be collected. At each move the circuit will be broken. The time needed for the power supply to ramp down and ramp up at each

move, and the diminished peak velocities during the ramping segments cannot be disregarded. Complex algorithms are needed to do this process smoothly.

A more serious limitation to the collection of fractions from CE is the very small amount of sample that the fraction will contain. Consider a capillary 100 μm in diameter and 50 cm long, with the window located 10 cm from the end. A 5 psi-sec injection into this capillary will inject about 189 nL of sample (*see* Section 1.3.1.). If the analyte concentration is 1 mg/mL in the starting sample and the peak is collected with 100% efficiency, the recovered sample will be 189 ng. Repeating this run 10 times would yield less than a two micrograms of product. Although fraction collection in CE is possible, one must question whether it is worth the effort.

2. MODES OF SEPARATION IN CE

A CE system can be operated in several different modes. These modes offer the analyst a variety of ways to approach an analytical problem. The choice of mode will be based on the analytical problem under consideration. This section will describe the major modes of capillary electrophoretic separation that are currently in use and provide some applications for each.

2.1. Capillary Zone Electrophoresis (CZE)

2.1.1. Mechanism

Section 1 of this chapter dealt almost entirely with this form of CE. CZE is characterized by the use of open capillaries and relatively low viscosity buffer systems. Analyte molecules move from one end of the capillary to the other according to the vector sum of electrophoresis and electroosmotic mobility.

2.1.2. Applications

CZE is the most widespread mode of CE. It has been used for analytes as diverse as sodium ions, drugs, and protein molecules. Analyte species can be separated by CZE if they migrate at different velocities in the electrical field.

2.2. Capillary Gel Electrophoresis (CGE)

2.2.1. Mechanism

Capillary gel electrophoresis (CGE) is separation based on viscous drag. In this mode of CE the capillary is filled with a gel or viscous solution. EOF is often suppressed so that the migration of the analytes is solely by electrophoresis. Larger molecules tend to be retarded more by the viscous separation medium than are smaller molecules, so that the separation is effectively based on the molecular size.

2.2.2. Applications

This is the method of choice for molecules that differ in size but not in mass/charge ratio. DNA molecules, for example, can vary greatly in length, but the charge per unit length is quite constant. In a pure CZE separation, all the molecules move at very nearly the same velocity and no separation results. In a viscous medium, the longer molecules are retarded more than shorter molecules. Thus shorter pieces of DNA pass the detector sooner than do larger pieces of DNA. See Chapters 13 and 14 for a more detailed discussion of DNA separation by CE.

Protein molecules are composed of more complex subunits than DNA molecules. Even though proteins vary widely in their size, the wide range of charge states possible in a protein sequence complicates size separation. In order to separate proteins by size, it is necessary to mask the native charge and create a more nearly uniform charge-mass ratio. This is done by treating the proteins with the detergent SDS. Although there are exceptions, typically proteins bind to a constant number of SDS molecules per unit length. Because the SDS molecules are highly negatively charged the amino acid subunit charge contributes little to the mobility of the molecules and a separation based on chain length is possible. This technique of SDS-CGE is exactly analogous to SDS-polyacrylamide gel electrophoresis (SDS-PAGE).

2.3. Capillary Isoelectric Focusing (cIEF)

2.3.1. Mechanism

Molecules that carry both positively and negatively charged groups exhibit, at a specific pH, an equal number of positive and negative charges. At this pH, known as the isoelectric pH or pI, the molecule, although charged, behaves as if it is neutral because its positive and negative charges cancel each other. The molecule, therefore, has no tendency to migrate in an electrical field. In isoelectric focusing, special reagents called ampholytes are used to create a pH gradient within the capillary. These ampholytes are mixtures of buffers with a range of pK_a values. In an electrical field, ampholytes will arrange themselves in order of pK_a; this gradient is trapped between a strong acid and a strong base. Analytes introduced into this gradient will migrate to the point where the pH of the gradient equals their pI. At this point the analyte, having no net charge, ceases to migrate. It will remain at that position so long as the pH gradient is stable, typically as long as the voltage is applied.

2.3.2. Applications

Capillary isoelectric focusing (cIEF) is used almost exclusively for the separation of closely related protein species. Hemoglobin can be separated

into several bands by this technique, whereas separation by SDS-CGE usually results in a single form being identified. In this application, the protein sample is mixed with the ampholyte solution and the mixture is pumped into the capillary. When voltage is applied, the proteins and the ampholytes migrate to their appropriate positions in the gradient. When focusing is complete, the proteins in the mixture are distributed throughout the length of the capillary. In order to detect the proteins it is necessary to mobilize them so that they pass by the detector in turn. There are two ways to accomplish this mobilization. Pressure mobilization utilizes positive pressure applied to one end of the capillary to drive the entire fluid column through the window. In order to prevent band distortion, this pressure must be applied carefully, preferably while the voltage is still being applied. Chemical mobilization requires that one end of the capillary be transferred to a salt solution after focusing has taken place. Upon application of voltage, salt will migrate into the capillary, disrupting the pH gradient and allowing the proteins to migrate past the detector by electrophoresis.

cIEF is also widely used for examining the distribution of carbohydrate isoforms of glycoproteins. In other techniques, such as SDS-CGE or CZE, these proteins tend to move as diffuse bands and to generate broad peaks. cIEF can often resolve these bands into peaks that differ by as little as one charged sugar group.

2.4. Capillary Isotachophoresis (cITP)

2.4.1. Mechanism

In this technique the sample plug is introduced between two different buffers. One of these, the leading electrolyte, has the highest mobility in the separation. The second, trailing electrolyte, has a mobility lower than anything else does. The sign of the charge on the analytes and the buffers must be the same. When the voltage is applied the ions in the sample form discrete zones that are not separated into peaks; one zone is adjacent to the next. The concentration of the analyte within a zone is constant within that zone and the length of the zone is proportional to the concentration within the zone. Because the voltage drop is uniform within a zone, isotachophoretic methods are quite compatible with conductance detectors (*see* Section 1.4.4.).

2.4.2. Applications

Capillary isotachophoresis (cITP) of peptides and proteins has been used with MS detection *(43)*. Since this mode of separation delivers zones of uniform concentration to the MS, it may be an ideal separation mechanism for this detection technique.

2.5. Micellar Electrophoresis

2.5.1. Mechanism

Electrophoresis is not possible for analytes that are not charged. In order to analyze such analytes, it is necessary to employ some agent in the separation buffer that will transport them through the capillary. The most commonly used mode of CE for these analytes is MEKC. In this technique a suitable charged detergent, such as SDS, is added to the separation buffer in a concentration sufficiently high to allow the formation of micelles. These micelles are arrangements of detergent molecules that have a hydrophobic inner core and a hydrophilic outer surface. Micelles are dynamic and constantly form and break apart. For any given analyte, there is a probability that the molecules of that analyte will associate within the micelle at any given time. This probability is the same as the partition coefficient in classical chromatography. When associated with the micelle, the analyte molecule will migrate at the velocity of the micelle. When not in the micelle, the analyte molecule will migrate with the EOF (if any). Differences in the time that analytes spend in the micellar phase will determine the separation.

2.5.2. Applications

MEKC is useful for a wide range of small molecules such as drugs, pesticides, and food additives that are not charged and are sufficiently hydrophobic to associate with the micelle. While SDS is probably the most widely used detergent for this purpose, cationic detergents such as TTAB can also be employed. Nonionic detergents by themselves do not provide mobility to uncharged analytes, but in combination with charged detergents they will modify the separation. Some detergents are useful in specific applications. For example, sodium cholate is useful in the separation and analysis of a variety of steroids. The micelles formed in this case are not the classical spherical shape but are probably sodium cholate molecules arranged on each other like a stack of coins. Different uncharged steroids differ in their tendencies to participate in these stacks.

2.6. Chiral Electrophoresis

2.6.1. Mechanism

Chiral molecules are molecules that can exist in two stereo-specific forms. These chiral forms or enantiomers are identical in molecular weight and chemical formula but differ in the arrangement of the atoms in space. Separation of these enatiomeric forms depends on the tendency to associate differentially with other chiral molecules known as chiral selectors. By incorporating a chiral selector into the CE buffer, it is often possible to sepa-

rate enantiomers of a chiral molecule. This is analogous to MEKC described previously. The complex of the analyte and the selector will migrate at a different rate than will the analyte alone. Because one of the two enantiomers associates more strongly with the selector than does the other form a separation can be achieved.

2.6.2. Applications

The most commonly employed chiral selector in CE is cyclodextrin, ring shaped carbohydrates made up of 6, 7, or 8 D-glucose subunits. Cyclodextrins may be chemically modified to alter their hydrophobicity or charge. Uncharged cyclodextrins are not suitable for the analysis of uncharged analytes since the complex will move with the EOF. However, cyclodextrins modified to carry a charge by addition of sulfate groups, can serve both as chiral selectors and as carrier molecules (similar to the detergent in MEKC). Other molecules, such as the antibiotic vancomycin, have also been employed as chiral selectors.

Chiral CE can be used to separate the enantiomeric forms of pharmaceuticals as well as natural substances, such as amino acids. Impurities as small as 0.1% are easily detected by this method.

2.7. Nonaqueous Electrophoresis

2.7.1. Mechanism

Electrophoresis usually is considered to occur only in aqueous solutions. However, CE can be performed using nonaqueous systems based on such solvents as acetonitrile, methanol, formamide, and dimethylformamide, to which are added small amounts of anhydrous acid or buffer salts. The separation is by simple electrophoresis as EOF is very low under these conditions.

2.7.2. Applications

There are times when two analytes have the same charge to mass ratio and are not easily separated. In some cases this same analyte pair can be separated in a nonaqueous environment where they may have different pK_a values than those expressed in water. The degree of solvation, and hence the radius of the solvated species, may also differ in aqueous and nonaqueous environments. Hence, nonaqueous CE offers an alternative for analytes that are difficult to separate under aqueous conditions. In addition, some analytes are difficult to solubilize in aqueous systems but dissolve readily in organic solvents, thus nonaqueous CE offers an alternative to MEKC for these analytes. Separations by nonaqueous CE have been reported for drugs, dyes, preservatives, surfactants, and inorganic ions.

2.8. Capillary Electrochromatography

2.8.1. Mechanism

Capillary electrochromatography (CEC) is a hybrid technique between liquid chromatography and electrophoresis. It is a partitioning technique in which molecules distribute between a stationary and a moving phase. As described for MEKC, different analytes will tend to associate to a greater or lesser extent with the stationary phase, effecting a separation.

CEC capillaries are packed with particles like those used in HPLC columns. Unlike conventional LC techniques, CEC uses electroosmotic flow to drive the mobile phase down the column. The resulting plug flow improves the separation efficiency over that of the laminar flow of pressure driven systems.

2.8.2. Applications

As of this writing, most of the work done on CEC has used model systems, such as polyaromatic hydrocarbons. A great deal of effort is underway to identify applications where this technique can be the method of choice. Because the buffers used are typically high in organic content and hence volatile, CEC may be useful when coupled to mass spectroscopy (CEC-MS).

REFERENCES

1. Janini, G. M., and Issaq, H. J. (1993) The buffer in capillary zone electrophoresis, in Capillary Electrophoresis Technology, (Guzman, N.A., ed.), Marcel Dekker, Inc., New York, pp. 119–160.
2. Cifuentes, A. and Poppe, H. (1994) Rectangular capillary electrophoresis: some theoretical considerations. Chromatographia 39, 391–404.
3. Virtanen, R. and Kivalo, P. (1969) Quantitative high-voltage zone electrophoresis method. Suomen Kemistilehti B 42, 182.
4. Jorgensen, J. W. and Lukacs, K. D. (1981) Zone electrophoresis is open-tubular glass capillaries. Anal. Chem. 53, 1298–1302.
5. Smoluchowski, M. V. (1905) Elektrosche kataphorese. Physik. Z. 6, 529.
6. Lukacs, K. D. and Jorgensen, J. W. (1985) Capillary zone electrophoresis: effect of physical parameters on separation efficiency and quantitation J. High Resolut. Chromatogr. Chromatogr. Commun. 8, 407.
7. Kuhn, R. and Hoffstetter-Kuhn, S. (1993) Capillary Electrophoresis Principle and Practice. Springer Verlag, Berlin, p. 153.
8. Lambert, W. J. and Middleton, D. L. (1990) pH hysteresis effect with silica capillaries in capillary zone electrophoresis. Anal. Chem. 62, 1585–1587.
9. Cobb, K. A., Dolnik, V., and Novotny, M. (1990) Electrophoretic separations of proteins in capillaries with hydrodynamically stable surface structures. Anal. Chem. 61, 2478–2483.
10. Hjerten, S. (1988) High performance electrophoresis: elimination of electroendosmosis and solute absorption. J. Chromatogr. 347, 191–198.

11. Bruin, G. M., Chang, J. P., Kuhlmann, R. H., Zegers, K., Kraak, J. C., and Poppe, H. (1992) Capillary zone electrophoretic separations of proteins in polyethylene glycol-modified capillaries. J. Chromatogr. 471, 429–436.
12. McCormick, R. M. (1988) Capillary zone electrophoretic separation of peptides and proteins using low pH buffers in modified silica capillary. Anal. Chem. 60, 2322–2328.
13. Novotny, M. V., Cobb, K. A., and Liu, J. (1990) Recent advances in capillary electrophoresis of proteins, peptides, and amino acids. Electrophoresis 11, 735–749.
14. Wiktorowicz, J. E. and Colburn, J. C. (1990) Separation of proteins via charge reversal in capillary electrophoresis. Electrophoresis 11, 769–773.
15. Emmer, A, Jansson, M., and Roeraade, J. (1991) Improved capillary zone electrophoretic separation of basic proteins, using a flurosurfactant buffer additive. J. Chromatogr. 547, 544–550.
16. VanOrman, B. B., Liversidge, G. G., and McIntire, G. L. (1991) Effects of buffer composition on electroosmotic flow in capillary electrophoresis. J. Microcol. Septo. 2, 176.
17. Ferguson, N. J., Braunschweiger, K. I., Braunschweiger, W. R., Smith, J. R., McCormick, J. J., Wasmann, C. C., et al. (1980) Hydrogen ion buffers for biological research. Anal. Biochem. 104, 300–310.
18. Vinther, A. and Soeberg, H. (1991) Temperature elevation of the sample zone in free solution capillary electrophoresis under stacking conditions. J. Chromatogr. 559, 3–26.
19. Vinther, A. and Soeberg, H. (1991) Mathematical model describing dispersion in free solution capillary electrophoresis under stacking conditions. J. Chromatogr. 559, 27–47.
20. Burgi, D. S. and Chien, R-L. (1991) Optimization in sample stacking for high-performance capillary electrophoresis. Anal. Chem. 63, 2042–2047.
21. Bullock, J. A. and Yuan, L. C. (1991) Free solution capillary electrophoresis of basic proteins in untreated fused silica capillary tubing. J. Microcol. Sep. 3, 241.
22. Terabe, S. (1989) Electrokinetic chromatography: an interface between electrophoresis and chromatography. Trends Anal. Chem. 8, 129.
23. Heiger, D. N., Cohen, A. S., and Karger, B. L. (1990) Separation of DNA restriction fragments by high performance capillary electrophoresis with low and zero crosslinked polyacrylamide using continuous and pulsed electric fields. J. Chromatogr. 516, 33–48.
24. Fujiwara, S. and Honda, S. (1987) Effect of addition of organic solvents on the separation of positional isomers in high-performance capillary electrophoresis. Anal. Chem. 59, 487–490.
25. Josic, D., Zeilinger, K., Reutter, W., Bottcher, A., and Schmitz, G. (1990) High performance capillary electrophoresis of hydrophobic membrane proteins. J. Chromatogr. 516, 89–98.
26. Harrington, S. J., Varro, R., and Li, T. M. (1992) High-performance capillary electrophoresis as a fast in-process control method for enzyme-labeled monoclonal antibodies. J. Chromatogr. 559, 385–390.
27. Reijenga, J. C., Aben, G. V. A., Verheggen, Th. P. E. M., and Everaerts, F. M. (1992) Effect of electroosmosis on detection in isotachophoresis. J. Chromatogr. 594, 317–324.

28. Knox, J. H. (1988) Thermal effects and band spreading in capillary electroseparation. Chromatographia 26, 329.
29. Jorgensen, J. W. and Lukacs, K. D. (1983) Capillary zone electrophoresis. Science 222, 266–272.
30. Chervet, J. P., van Soest, R. E. J., and Ursem, M. (1991) Z-shaped flow cell for UV detection in capillary electrophoresis. J. Chromatogr. 543, 439–449.
31. Gordon, G. B. (1991) Capillary zone electrophoresis cell system. United States Patent 5,061,361, October 29.
32. Kobayashi, S., Ueda, T., and Kikumoto, J. (1989) Photodiode array detection in high-performance capillary electrophoresis. J. Chromatogr. 480, 179–184.
33. Wu, S. and Dovichi, N. J. (1992) Capillary zone electrophoresis separation and laser-induced fluorescence detection of zeptomole quantities of fluorescein thiohydantoin derivatives of amino acids. Talanta 39, 173.
34. Wallingford, R. A. and Ewing, A. G. (1987) Characterization of a microinjector for capillary zone electrophoresis. Anal. Chem. 59, 678–681.
35. Wallingford, R. W. and Ewing, E. M. (1990) Separation of serotonin from catechols by capillary zone electrophoresis with electrochemical detection. Anal. Chem. 61, 98–100.
36. Huang, X., Pang, T. K., Gordon, M. J., and Zare, R. N. (1987) On column conductivity detector for capillary zone electrophoresis. Anal. Chem. 59, 2747–2749.
37. Huang, X., Zare, R. N., Sloss, S., and Ewing, A. G. (1991) End column detection for capillary zone electrophoresis. Anal. Chem. 63, 189–192.
38. Huang, X., Luckey, J. A., Gordon, M. J., and Zare, R. N. (1989) Quantitative analysis of low molecular weight carboxylic acids by capillary zone electrophoresis/conductivity detection. Anal. Chem. 61, 766–770.
39. Banks, J. F. (1997) Recent advances in capillary electrophoresis/electrospray/mass spectrometry. Electrophoresis 18, 2255–2266.
40. Wildman, B. J., Jackson, P. E., Jones, W. R., and Alden, P. G. (1991) Analysis of anion constituents of urine by inorganic capillary electrophoresis. J. Chromatogr. 546, 459–466.
41. Wanders, B. J. (1997) Data analysis in CE, in Handbook of Capillary Electrophoresis, 2nd ed. (Landers, J. P., ed.), CRC, Boca Raton, FL.
42. Kuhn, R. and Hoffstetter-Kuhn, S. (1993) Capillary Electrophoresis Principle and Practice. Springer Verlag, Berlin, p. 156.
43. Smith, R. D., Leo, J. A., Edmonds, C. G., Barinaga, C. J., and Usdeth, H. R. (1990) Sensitivity considerations for large molecule detection by capillary electrophoresis-electrospray ionization mass spectrometry. J. Chromatogr. 516, 157–165.